

Stress-Induced Cytokinin Synthesis Increases Drought Tolerance through the Coordinated Regulation of Carbon and Nitrogen Assimilation in Rice¹[C][W][OPEN]

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The effects of water deficit on carbon and nitrogen metabolism were investigated in flag leaves of wild-type and transgenic rice (*Oryza sativa japonica* 'Kitaake') plants expressing *ISOPENTENYLTRANSFERASE* (*IPT*; encoding the enzyme that mediates the rate-limiting step in cytokinin synthesis) under the control of P_{SARK} , a maturation- and stress-induced promoter. While the wild-type plants displayed inhibition of photosynthesis and nitrogen assimilation during water stress, neither carbon nor nitrogen assimilation was affected by stress in the transgenic $P_{SARK}::IPT$ plants. In the transgenic plants, photosynthesis was maintained at control levels during stress and the flag leaf showed increased sucrose (Suc) phosphate synthase activity and reduced Suc synthase and invertase activities, leading to increased Suc contents. The sustained carbon assimilation in the transgenic $P_{SARK}::IPT$ plants was well correlated with enhanced nitrate content, higher nitrate reductase activity, and sustained ammonium contents, indicating that the stress-induced cytokinin synthesis in the transgenic plants played a role in maintaining nitrate acquisition. Protein contents decreased and free amino acids increased in wild-type plants during stress, while protein content was preserved in the transgenic plants. Our results indicate that the stress-induced cytokinin synthesis in the transgenic plants promoted sink strengthening through a cytokinin-dependent coordinated regulation of carbon and nitrogen metabolism that facilitates an enhanced tolerance of the transgenic plants to water deficit.

Plant hormones control many aspects of plant growth and development and the responses of plants to abiotic and biotic stresses. Cytokinins (CKs) have been shown to regulate plant cell differentiation, leaf senescence, and other key developmental processes (Sakakibara, 2006). It has also been shown that CKs regulate assimilate partitioning (Ronzhina and Mokronosov, 1994), sink strength (Kuiper, 1993), and source/sink relationships (Roitsch, 1999). The localized expression in tobacco (*Nicotiana tabacum*) of a promoterless *ISOPENTENYLTRANSFERASE* (*IPT*), a gene encoding the enzyme that catalyzes the rate-limiting step in CK synthesis, enhanced the local sink strength and

quickly mobilized nutrients to the tissues with elevated CK (Guivarc'h et al., 2002). Changes in sink/source relationships were also observed in CK-deficient tobacco shoots and roots (Werner et al., 2008). Elevated CK levels enhanced the survival of plants under water-stress conditions (Rivero et al., 2007). The overexpression of *IPT* under the control of *SENESCENCE-ASSOCIATED RECEPTOR KINASE* (*SARK*; a maturation- and stress-induced promoter) improved the drought tolerance of both eudicots (Rivero et al., 2007; Qin et al., 2011) and monocots (Peleg et al., 2011). After a water-stress episode during the reproductive stages (pre and post anthesis), transgenic $P_{SARK}::IPT$ rice (*Oryza sativa*) plants displayed higher grain yield than the wild type (Peleg et al., 2011). The transgenic $P_{SARK}::IPT$ rice exhibited a differential expression of genes encoding enzymes associated with hormone synthesis and hormone-regulated pathways. These results suggested that changes in hormone homeostasis induced the modification of source/sink relationships in the transgenic plants, resulting in higher grain yields under stress conditions (Peleg et al., 2011).

The maintenance of carbon (C) and nitrogen (N) assimilation is of paramount importance to ensure sink strength and improve stress tolerance without yield penalties. The interactions between C and N metabolism are vital for plant growth and development, and complex mechanisms operate in the plant to coordinate C assimilation with N metabolism (Nunes-Nesi et al., 2010). Thus, plants respond to changes in C and N metabolites through the regulation of translation and

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posttranslational modification mechanisms. C and N metabolites activate signaling pathways that regulate enzyme and transporter activities that control C and N fluxes, optimizing the plant response to developmental and environmental cues changing source/sink relationships (Coruzzi and Zhou, 2001).

Plant hormones affect, either directly or indirectly, these pathways and can act antagonistically or synergistically when responding to environmental stress (Wilkinson et al., 2012). The exposure of plants to water-limiting conditions results in abscisic acid (ABA) synthesis that induces ABA-dependent gene expression (Yamaguchi-Shinozaki and Shinozaki, 2006), triggering the closure of stomata and reducing water loss during drought (Wilkinson and Davies, 2010). Other hormones, in particular CK, salicylic acid, ethylene, and jasmonic acid, also play direct or indirect roles in the plant responses to abiotic stress (Peleg and Blumwald, 2011). Under drought stress, plant CK content decreases, and the reduction in CK increases the plant responses to increasing ABA (Davies and Zhang, 1991), inducing stomata closure and inhibiting photosynthesis (Rivero et al., 2010). Our previous results suggested that the stress-induced CK synthesis, driven by a stress-induced promoter, protected against the deleterious effects of water deficit on the photosynthetic apparatus, allowing higher photosynthetic rates and higher yields after water deficit in tobacco (Rivero et al., 2009) and cotton (*Gossypium hirsutum*; Kuppu et al., 2013) plants grown in the greenhouse and peanut (*Arachis hypogaea*) plants grown under field conditions (Qin et al., 2011).

Here, we analyzed gene expression profiles, metabolites, and enzymatic and photosynthetic activities of flag leaves of wild-type and transgenic rice expressing $P_{SARK}::IPT$ exposed to water deficit during the reproductive stage and identified metabolic processes associated with the enhanced tolerance of the transgenic plants to water deficit. Our results indicate that the stress-induced CK synthesis in the transgenic plants promoted sink strengthening through the maintenance and coordination of N and C assimilation during water stress.

RESULTS

Effects of Water Stress on C Assimilation in Wild-Type and Transgenic $P_{SARK}::IPT$ Rice Plants

Compared with wild-type plants, which exhibited severe drought stress and reduced growth after 3 d of water stress, transgenic $P_{SARK}::IPT$ rice plants displayed enhanced growth and tolerance to drought stress (Fig. 1A). In addition, $P_{SARK}::IPT$ rice recovered from drought upon rewatering, while wild-type plants did not (Fig. 1A, right panel). Under water-stress conditions, the transgenic plants were able to maintain higher photosynthetic rates, as compared with wild-type plants (Fig. 1B). The relative water content (RWC) of wild-type plants was drastically reduced during water stress, while the decrease in RWC of $P_{SARK}::IPT$ plants was

attenuated and remained above 80% (Fig. 1C). The maximum rate of carboxylation (V_{max}), maximum rate of electron transport (J_{max}), and triose phosphate utilization (TPU) were calculated in order to evaluate Rubisco activity, the regeneration of ribulose biphosphate, and phosphate limitations, respectively (Sharkey, 1985; Fig. 1, D–F). V_{max} , J_{max} , and TPU remained unchanged in the transgenic plants, while a 75% reduction was noted in the wild-type plants. Transgenic $P_{SARK}::IPT$ plants were able to regulate stomata conductance (Fig. 1G) and displayed a lesser increase in ABA content during water stress (Fig. 1H), which allowed gas exchange and the maintenance of photosynthetic activity during the stress episode (Fig. 1B). In wild-type plants, the stomatal closing after 3 d of water stress correlated well with the decrease in carboxylation efficiency. The maintenance of a constant ratio of variable to maximal chlorophyll fluorescence (F_v/F_m), an indicator of the maximum quantum efficiency of PSII and stress-induced photo-inhibition (Krause et al., 1989), further supported the ability of the transgenic plants to maintain C assimilation during the stress episode (Fig. 1I).

Impact of IPT Expression on the Regulation of C Metabolism Under Water Stress

Changes in C metabolism in $P_{SARK}::IPT$ rice plants were associated with the maintenance of photosynthetic activity during water stress. Although both wild-type and $P_{SARK}::IPT$ plants showed reductions in starch content under stress (Fig. 2) correlated with the increase in amylolytic activity with the stress (Table I), $P_{SARK}::IPT$ rice plants displayed higher starch content than wild-type plants grown under both well-watered and water-stress conditions (Fig. 2B; Peleg et al., 2011). Despite the increase in amylolytic activity found in wild-type plants with the stress, no significant differences were found in amylolytic activity during stress between transgenic and wild-type plants (Table I). Suc, Glc, Fru, and Glc-6-P contents were similar in wild-type and $P_{SARK}::IPT$ plants grown under well-watered conditions (Fig. 2). During water stress, Suc contents increased in $P_{SARK}::IPT$ plants (Fig. 2), and this increase was correlated with higher Suc synthesis (sucrose phosphate synthase [SPS] activity) and lower cytosolic Suc degradation (sucrose synthase [SuSy] and cytosolic invertase activities; Table I). The reduction in Suc synthesis seen in wild-type plants during water stress was accompanied by increased Suc degradation (Table I) and the concomitant increase in Glc and Fru (Fig. 2). The flow of C from starch and/or Suc to Glc and Fru indicated a typical osmotic adjustment response of the wild-type plants to water stress (Merewitz et al., 2011; Nio et al., 2011). This osmotic stress response was not seen in the transgenic $P_{SARK}::IPT$ plants. The increase in cytosolic invertase and SuSy activities in wild-type plants during water stress was paralleled by the increase in transcripts encoding for vacuolar and cytosolic invertases (Fig. 3) that resulted in

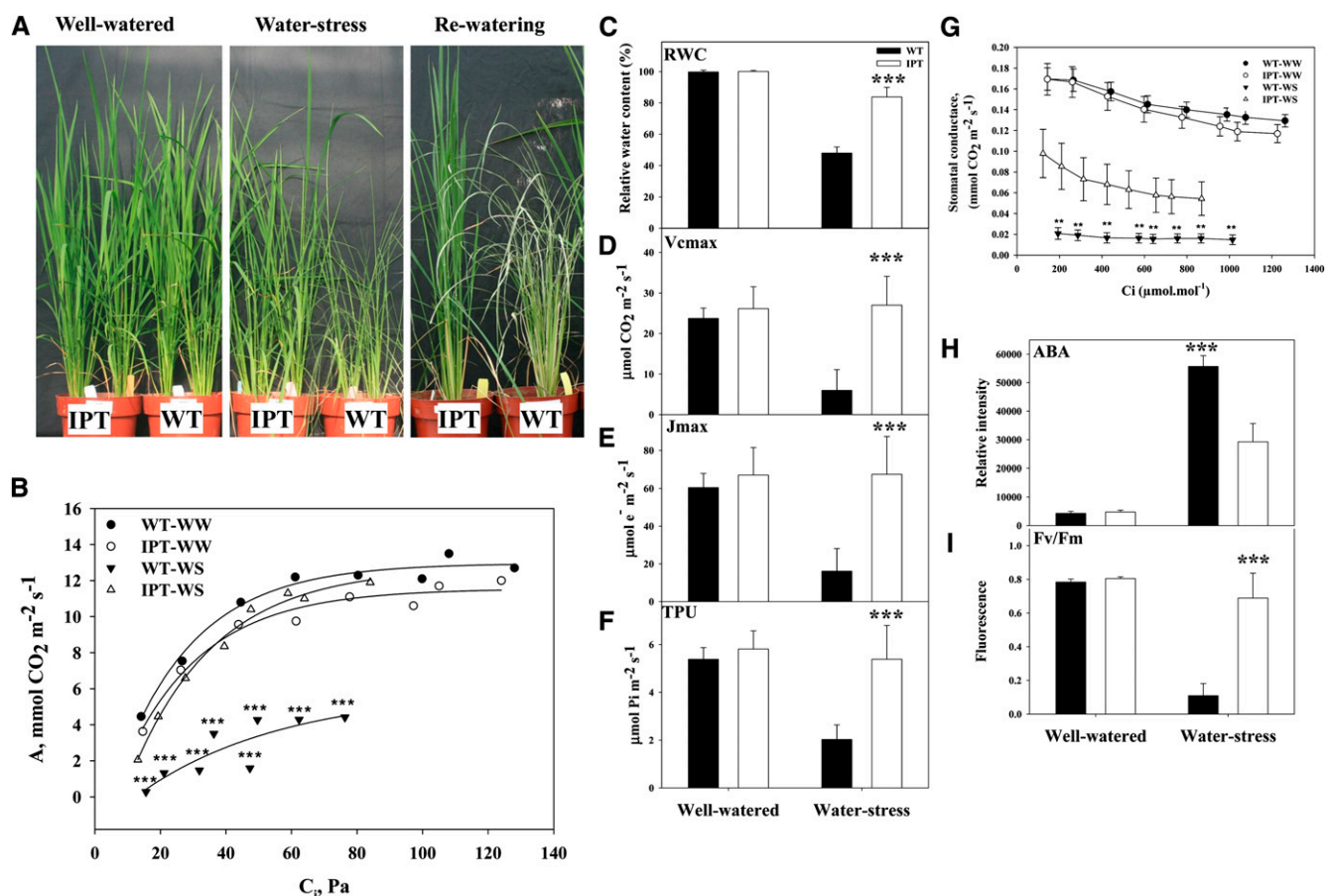


Figure 1. Analysis of carbon assimilation in flag leaves of wild-type (WT) and transgenic $P_{SARK}::IPT$ rice plants subjected to 3 d of water stress. A, Plants grown under well-watered conditions, 3 d of water stress, and 3 d after rewatering at pre anthesis (booting) stage. B, A/C_i curves of wild-type well-watered (WW; black circles), wild-type stressed (WS; black triangles), $P_{SARK}::IPT$ well-watered (white circles), and $P_{SARK}::IPT$ stressed (white triangles) plants. C, RWC. D, V_{cmax} . E, J_{max} . F, TPU. G, Stomatal conductance ($\text{mmol water m}^{-2} \text{s}^{-1}$). H, ABA content. I, F_v/F_m . Values are means \pm SD ($n = 7$). Seven individual plants per genotype/treatment were used in each of the measurements, and the data were analyzed using one-way ANOVA followed by Student's t test. Asterisks indicate significant differences between genotypes per treatment (** $P \leq 0.01$, *** $P \leq 0.001$).

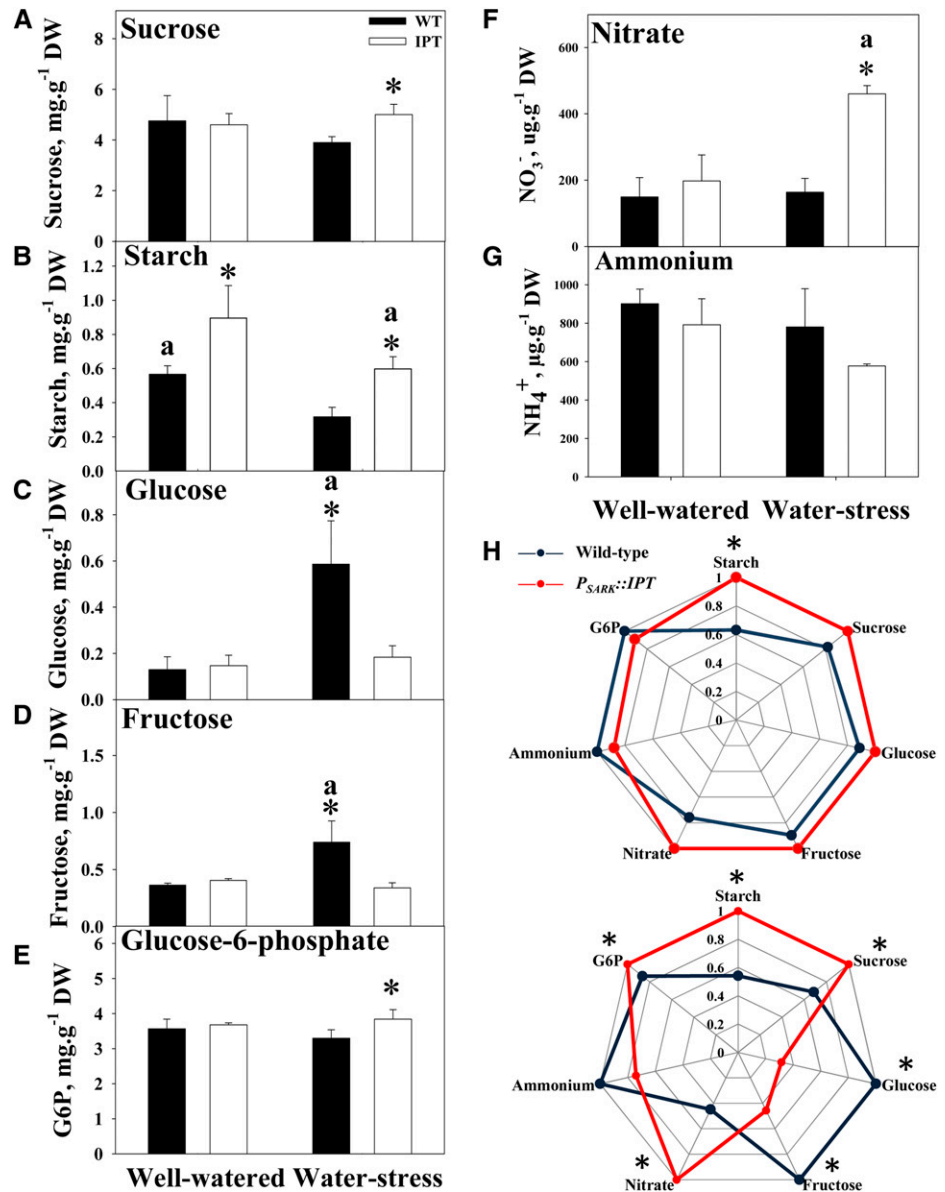
the increase of Glc and Fru contents. Also, the increase in SPS activity in the transgenic $P_{SARK}::IPT$ plants was matched by an increase in transcripts encoding SPS (Fig. 3). Trehalase activity displayed similar patterns in response to stress between wild-type and transgenic plants under both conditions (Table I). Interestingly, $P_{SARK}::IPT$ plants displayed higher hexokinase (HK) activity than wild-type plants under both experimental conditions, and the high HK activity correlated well with the Glc-6-P contents of the transgenic plants (Fig. 2). These results appear to be consistent with the role of Glc-6-P as a substrate for Suc synthesis via SPS, in contrast to the wild-type plants, where Suc was degraded. Our results show that increased HK activity in $P_{SARK}::IPT$ plants is not associated with detrimental effects on photosynthesis and growth or in accelerating senescence, indicating that the differences between the regulation driven by changes in gene expression and

protein activity might involve different signaling pathways, as suggested previously (Cho et al., 2009).

Effects of Water Stress on the Interaction between C and N Metabolism

No difference in NH_4^+ contents were seen in wild-type and $P_{SARK}::IPT$ transgenic plants under well-watered and water-stress conditions (Fig. 2). However, equal NH_4^+ contents could result from different NH_4^+ generation processes. Under drought stress, wild-type plants displayed increased glutamate dehydrogenase (GDH) deamination activity (Table II). This process has been shown to be active under stress conditions and serves to direct amino acid synthesis in conditions where N assimilation is decreased (Masclaux-Daubresse et al., 2010), as reflected by the decrease in nitrate reductase (NR) activity in wild-type plants under stress (Table II).

Figure 2. Sugar and N-related metabolite contents in flag leaf tissue at the pre anthesis stage of wild-type (WT) and transgenic *P_{SARK}::IPT* plants. A, Suc. B, Starch. C, Glc. D, Fru. E, Glc-6-P. F, Nitrate. G, Ammonium. H, Radar charts comparing the metabolic profile of wild-type (blue) and transgenic *P_{SARK}::IPT* (red) plants under control (well-watered) conditions (top) and water-stress conditions (bottom). Values are means \pm SD ($n = 6$). Six individual plants per genotype/treatment were used in each of the measurements, and the data were analyzed using one-way ANOVA followed by Student's *t* test. Asterisks indicate significant differences by genotypes ($*P \leq 0.05$); a indicates significant differences between treatments ($P \leq 0.05$). DW, Dry weight.



P_{SARK}::IPT plants were able to maintain primary N assimilation without the activation of reassimilation processes, increasing NO₃⁻ uptake together with higher NR and nitrite reductase (NiR) activities (Table II).

The water stress-induced inhibition of photosynthesis (Fig. 1) and the reduction in C and N assimilation in the wild-type plants were accompanied by a dramatic accumulation of amino acids (Table III). The increase in amino acid contents, together with the accumulation of Glc and Fru (Fig. 2), are characteristic hallmarks of plant osmotic adjustments to water deficit, providing protection against structural and functional damage caused by dehydration (Chen and Jiang, 2010).

The accumulation of pipercolate and 4-guanidine butanoate during stress in the *P_{SARK}::IPT* plants is noteworthy (Table III). Pipercolate and 4-guanidine

butanoate are intermediate components of Lys and Arg metabolism, respectively. In plants, these compounds act as N reservoirs and control the catabolism of Lys and Arg when stress conditions recede (Moulin et al., 2006). These results are consistent with the N remobilization patterns shown by *P_{SARK}::IPT* plants, which were able to maintain N metabolism levels similar to those under control growth conditions (Fig. 4) and recovered after rewatering.

The total activities of glutamate synthase (GOGAT), glutamine synthetase (GS), and amino acid transferases (Ala and Asp transferases) remained unchanged during water stress in both wild-type and *P_{SARK}::IPT* plants, suggesting that amino acid synthesis was not affected by water stress (Fig. 4B; Table II). However, differences between wild-type and transgenic *P_{SARK}::IPT*

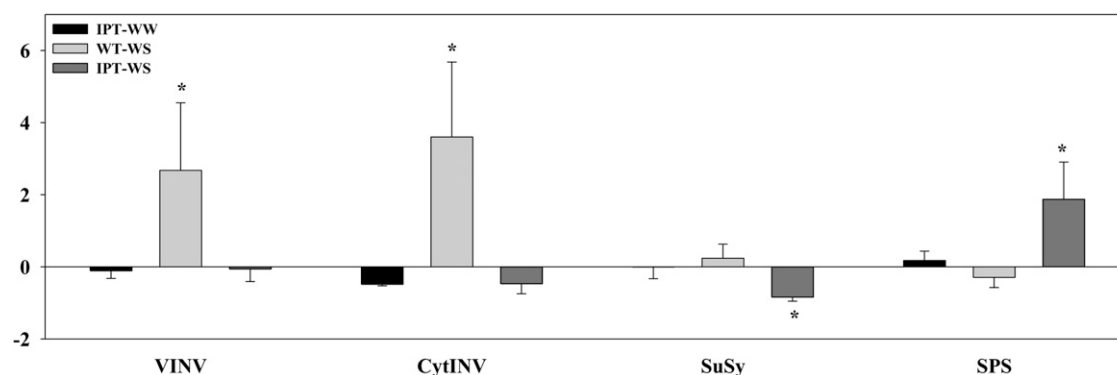


Figure 3. Relative expression of C-related genes in flag leaves of wild-type (WT) and transgenic $P_{SARK}::IPT$ plants grown under well-watered (WW) and water-stress (WS) conditions. Values were calculated and normalized using the transcription elongation factor (LOC_Os03g08060) as an internal control. Expression values were calculated relative to the expression of wild-type plants grown under well-watered conditions ($2^{-\Delta\Delta Ct} = 0$). Values represent means \pm SD ($n = 6$). C-related genes are as follows: VINV, vacuolar invertase (LOC_Os02g01590); CytINV, cytosolic neutral invertase (LOC_Os01g22900); SuSy (LOC_Os07g42490); SPS (LOC_Os06g43630). Asterisks indicate significant differences between genotypes ($*P \leq 0.05$).

plants were seen in N assimilation processes under water-stress conditions. The increase in NR and the maintenance of NiR activities in the $P_{SARK}::IPT$ plants during water stress (Table II; Fig. 4B) indicated the positive effects of CK synthesis on N assimilation in transgenic plants. Wild-type plants displayed increased GDH deamination activity and decreased N assimilation (NR and NiR) activities (Table II). The increase in the GDH deamination activity was consistent with the amounts of NH_4^+ found in wild-type stressed plants (Supplemental Fig. S2). GDH deamination activity fulfills a dual role: providing 2-oxoglutarate to the tricarboxylic acid (TCA) cycle (limited by the reduction of C assimilation in wild-type plants) and providing the required NH_4^+ for the maintenance of amino acid synthesis (Fig. 4).

Regardless of the 2-oxoglutarate input driven by GDH, the wild-type plants subjected to water stress

displayed a reduction of glycolysis- and TCA-related metabolites, such as citrate, cis-aconitate, 2-oxoglutarate, succinate, 3-Phosphoglyceric acid, or pyruvate (Table III). The decrease in these metabolites was well correlated with the decreased expression of genes encoding enzymes involved in these pathways, including isocitrate dehydrogenase (LOC_Os02g38200 and LOC_Os01g46610), citrate synthase (LOC_Os02g10070), citrate transporter (LOC_Os03g05390), methylisocitrate lyase 2 (LOC_Os04g31700), enolase (LOC_Os10g08550), and aconitate hydratase (LOC_Os03g04410; Supplemental Fig. S1).

The increased ammonia reassimilation activities in the wild-type plants were paralleled by the increased expression of *GS1* (cytosolic Gln synthetase1), *GDH*, and *AMT* (NH_4^+ transporter; Fig. 5). The significant decrease in Gln and Glu contents in the transgenic plants during water stress (Table III) compared with

Table I. C-related enzyme activities during pre anthesis in flag leaves

C-related enzyme activities were determined in flag leaves of wild-type and transgenic $P_{SARK}::IPT$ plants subjected to 3 d of water stress. Enzyme activities are expressed as moles of metabolite generated/consumed per milligram of protein per unit of time. Additional details are provided in "Materials and Methods." Values are presented as means \pm SD of six individual plants per genotype/treatment. The data were analyzed using one-way ANOVA followed by Student's *t* test. Boldface values indicate significant differences by genotype comparisons ($P \leq 0.05$); asterisks indicate significant differences by treatment comparisons ($P \leq 0.05$); CWInv, Cell wall invertase.

Enzyme Activities	Flag Leaf			
	Well Watered		Water-Stress	
	Wild Type	$P_{SARK}::IPT$	Wild Type	$P_{SARK}::IPT$
SPS ($\mu\text{mol Suc mg}^{-1} \text{ protein h}^{-1}$)	24.9 \pm 6.5*	18.5 \pm 2.7	16.3 \pm 3.4	24 \pm 2.6*
SuSy (synthesis) [$\mu\text{mol Suc mg}^{-1} \text{ protein h}^{-1}$]	5.9 \pm 1.3	5.8 \pm 2.6	5 \pm 2.4	5.8 \pm 2.72
SuSy (degradation) [$\mu\text{mol Fru mg}^{-1} \text{ protein h}^{-1}$]	4.7 \pm 0.5	3.3 \pm 0.7	5.7 \pm 0.4	4. \pm 0.8
CytINV ($\mu\text{mol Glc mg}^{-1} \text{ protein h}^{-1}$)	2.9 \pm 0.6	2.9 \pm 0.2	4.4 \pm 0.5	2.3 \pm 0.6
VINV ($\mu\text{mol Glc mg}^{-1} \text{ protein h}^{-1}$)	7.7 \pm 1.7	9.1 \pm 1.2	11.6 \pm 1.7*	9.5 \pm 0.6
CWInv ($\mu\text{mol Glc mg}^{-1} \text{ protein h}^{-1}$)	9 \pm 3.2	11 \pm 3.4	11.8 \pm 3	9.5 \pm 3.9
Amylolytic activity ($\mu\text{mol Glc mg}^{-1} \text{ protein h}^{-1}$)	6 \pm 0.8	5.8 \pm 0.8	7.9 \pm 1.6*	6.7 \pm 0.7
Trehalase ($\mu\text{mol Glc mg}^{-1} \text{ protein h}^{-1}$)	0.6 \pm 0.1	0.5 \pm 0.2	1 \pm 0.06*	1.2 \pm 0.1*
HK (nmol NADH $\text{mg}^{-1} \text{ protein min}^{-1}$)	6.1 \pm 0.6	9.4 \pm 0.4	8.8 \pm 0.9*	12 \pm 1.3*

Table II. N-related enzyme activities during pre anthesis in flag leaves

N-related enzyme activities were determined in flag leaves of wild-type and transgenic $P_{SARK}::IPT$ plants subjected to 3 d of water stress. Enzyme activities are expressed as moles of metabolite generated/consumed per milligram of protein per unit of time. Additional details are provided in "Materials and Methods." Values are presented as means \pm SD of six individual plants per genotype/treatment. The data were analyzed using one-way ANOVA followed by Student's t test. Boldface values indicate significant differences by genotype comparisons ($P \leq 0.05$); asterisks indicate significant differences by treatment comparisons ($P \leq 0.05$).

Enzyme Activities	Flag Leaf			
	Well Watered		Water-Stress	
	Wild Type	$P_{SARK}::IPT$	Wild Type	$P_{SARK}::IPT$
NR (nmol NO ₂ mg ⁻¹ protein min ⁻¹)	3.2 \pm 0.4*	3.4 \pm 0.3	1.7 \pm 0.5	5.1 \pm 0.2*
NiR (nmol NO ₂ mg ⁻¹ protein min ⁻¹)	98.9 \pm 26.1*	138.1 \pm 38.4	14.7 \pm 8.3	118 \pm 8
NADH-GOGAT (nmol NADH mg ⁻¹ protein min ⁻¹)	7.7 \pm 1.2	7.3 \pm 1.2	8.5 \pm 1	6.9 \pm 0.1
GS (μ mol Gln mg ⁻¹ protein h ⁻¹)	198.3 \pm 53.5	191.6 \pm 23.1	215.4 \pm 51.7	215.4 \pm 26.1
GDH (amination) [nmol NADH mg protein ⁻¹ min ⁻¹]	8.1 \pm 1.5	7.1 \pm 1.1	6.6 \pm 0.6	6.2 \pm 1
GDH (deamination) [nmol NADH mg ⁻¹ protein min ⁻¹]	3.5 \pm 0.3	5.3 \pm 0.7	8.4 \pm 2.4*	4.7 \pm 1.5
Ala aminotransferase (nmol NADH mg ⁻¹ protein min ⁻¹)	45 \pm 6.5	41.5 \pm 3	35.9 \pm 5.6	43.5 \pm 11.6
Asp aminotransferase (nmol NADH mg ⁻¹ protein min ⁻¹)	13.4 \pm 0.9	12.9 \pm 1.1	13.13 \pm 1.6	13.9 \pm 1.5

the opposite trend seen in the wild-type plants indicated the capacity of the transgenic plants to maintain protein synthesis during the stress episode. A comparison between wild-type and $P_{SARK}::IPT$ plants revealed a differential expression of genes encoding proteins associated with ribosomal assembly and translation (Supplemental Fig. S2). These results indicated that amino acid accumulation in wild-type plants during stress was a consequence of a decrease in protein synthesis (Supplemental Fig. S2) together with the increase in protein degradation, as supported by the increase in protease activities (Fig. 6B).

As a consequence of protein oxidation, carbonyl groups are added into the side chains of proteins. Protein oxidation is a well-accepted mechanism to target proteins for degradation (Xiong et al., 2007). Wild-type stressed plants displayed an increase in protein oxidation (Fig. 6A) determined by the immunodetection of derivatized carbonyl groups to 2,4-dinitrophenylhydrazine (DNPH) in oxidized proteins. Consistent with the increase in protein oxidation seen in wild-type plants during stress, the levels of protease activities showed an increment (Fig. 6B) with a concomitant decrease in flag leaf protein contents (Fig. 6C).

DISCUSSION

We have shown previously that the synthesis of CK, mediated by the expression of *IPT* under the control of a maturation- and stress-induced promoter (P_{SARK}), led to enhanced drought tolerance of transgenic $P_{SARK}::IPT$ rice (Peleg et al., 2011) and tobacco (Rivero et al., 2007) plants subjected to severe and long-term stress, respectively. Here, we studied the responses of C and N metabolism to drought stress, aiming to characterize physiological responses and metabolic pathways associated with the CK-induced stress tolerance in $P_{SARK}::IPT$ rice plants. Wild-type rice plants under water stress displayed a reduction in stomatal conductance and an

inhibition of photosynthetic activity and C assimilation. Lauer and Boyer (1992) demonstrated that, during drought stress, the limitation in C assimilation was due to the reduction of the biochemical capacity of photosynthesis. The authors showed that although a simultaneous reduction of stomatal opening occurred during water deficit, calculated substomatal CO₂ concentration (C_i) was maintained or even increased. In the initial stages of water stress, a decrease in stomatal conductance mediated by ABA can limit the internal CO₂ concentration used in C assimilation (Zhang and Davies, 1990; Tardieu and Davies, 1993). As the stress progresses, the decrease in photosynthetic activity and the continuation of respiration both contribute to the maintenance (or increase) of internal CO₂ in plants subjected to stress (Lawlor and Tezara, 2009). Our results support this concept. The inhibition of photosynthetic activity in wild-type rice plants during stress was accompanied by a decline in stomatal conductance without altering C_i values until the external CO₂ concentration reached 1,200 μ L L⁻¹ (Fig. 1G), indicating that the impairment of C assimilation was predominantly associated with the biochemical inhibition of photosynthesis. In contrast, and in spite of a reduction in C_i (associated with a reduction in stomatal conductance during water stress), transgenic $P_{SARK}::IPT$ rice maintained photosynthesis at levels similar to those of well-watered plants. Similar results were obtained previously with transgenic tobacco plants expressing $P_{SARK}::IPT$, where the stress-induced increase in CK resulted in the protection of biochemical processes associated with photosynthesis during water stress (Rivero et al., 2009).

A reciprocal control between C and N assimilation coordinating the production of sugars and amino acids, according to the plant requirements, has been postulated (Kaiser and Brendle-Behnisch, 1991; Foyer et al., 1998, 2004). During water stress, the wild-type plants displayed an increase in vacuolar invertase, cytosolic invertase, and *SuSy* transcripts and increased

Table III. Free amino acid content and relative TCA- and glycolysis-related metabolite contents in flag leaf of wild-type and transgenic $P_{SARK}::IPT$ plants grown under control and water-stress conditions

Values are means \pm SD for each compound and were calculated from semiquantitative data that were scaled with respect to the median response observed for each metabolite and in which null values were imputed with the minimum observed value. Six individual plants were used per genotype/treatment. Data were analyzed using one-way ANOVA followed by Student's t test. Boldface values indicate significant differences by genotype ($P \leq 0.05$); asterisks indicate significant differences by treatment ($P \leq 0.05$). BDL, Below detection limits; DW, dry weight.

Amino Acid/Metabolite	Well Watered		Water-Stress	
	Wild Type	$P_{SARK}::IPT$	Wild Type	$P_{SARK}::IPT$
Amino acid composition (nmol g ⁻¹ DW)				
Ala	1.4 \pm 0.1	0.8 \pm 0.2	2.1 \pm 0.2*	0.8 \pm 0.2
Arg	0.2 \pm 0.07	0.2 \pm 0.01*	0.8 \pm 0.1*	0.07 \pm 0.02
4-Guanidinobutanoate	0.06 \pm 0.01*	0.06 \pm 0.01	0.03 \pm 0.003	0.05 \pm 0.01
Asn	2.8 \pm 0.4	3.3 \pm 0.4*	3.9 \pm 0.03*	2.4 \pm 0.2
Asp	1.5 \pm 0.2	1.8 \pm 0.3	1.6 \pm 0.21	1.5 \pm 0.1
Creatine	0.2 \pm 0.04	0.2 \pm 0.04	0.3 \pm 0.2	0.1 \pm 0.05
Ethanolamine	0.1 \pm 0.01	0.1 \pm 0.02	0.2 \pm 0.02	0.13 \pm 0.02
γ -Aminobutyric acid	0.2 \pm 0.01	0.1 \pm 0.01	0.2 \pm 0.07	0.1 \pm 0.02
Glu	3.8 \pm 0.5	4.5 \pm 0.01*	5.4 \pm 0.03*	3.3 \pm 0.3
Gln	2.6 \pm 0.27	2.1 \pm 0.3*	5.8 \pm 1.2*	1.5 \pm 0.04
Gly	0.1 \pm 0.01	0.07 \pm 0.01*	0.2 \pm 0.05	0.04 \pm 0.01
His	0.06 \pm 0.01	0.05 \pm 0.01	0.6 \pm 0.1*	0.04 \pm 0.01
Ile	0.04 \pm 0.002	0.04 \pm 0.01	1.2 \pm 0.1*	0.03 \pm 0.01
Leu	0.04 \pm 0.001	0.03 \pm 0.01	1.7 \pm 0.2*	0.03 \pm 0.01
Lys	0.05 \pm 0.004	0.05 \pm 0.01	0.5 \pm 0.06*	0.03 \pm 0.01
Pipecolate	0.04 \pm 0.01	0.06 \pm 0.02	0.04 \pm 0.01	0.1 \pm 0.03*
Met	0.04 \pm 0.02	0.03 \pm 0.003	0.2 \pm 0.01*	0.03 \pm 0.01
Pro	BDL	0.04 \pm 0.01	1.1 \pm 0.1*	BDL
Phe	0.05 \pm 0.01	0.05 \pm 0.01	1.2 \pm 0.2*	0.04 \pm 0.01
Sarcosine	1.4 \pm 0.5	1.2 \pm 0.2	1.1 \pm 0.3	1.4 \pm 0.2
Ser	1 \pm 0.07	0.96 \pm 0.1*	1.2 \pm 0.1	0.6 \pm 0.1
Thr	0.4 \pm 0.07	0.47 \pm 0.03*	1.6 \pm 0.1	0.3 \pm 0.04
Trp	BDL	BDL	0.6 \pm 0.2	BDL
Tyr	0.05 \pm 0.01	0.04 \pm 0.003	0.7 \pm 0.1	0.04 \pm 0.003
Val	0.07 \pm 0.005	0.08 \pm 0.02	1.8 \pm 0.2	0.05 \pm 0.01
TCA cycle metabolites (relative intensity)				
2-Oxoglutarate	1 \pm 0.2*	1.2 \pm 0.2	0.8 \pm 0.3	1 \pm 0.3
cis-Aconitate	1.1 \pm 0.2*	1.1 \pm 0.2	0.8 \pm 0.1	1 \pm 0.07
Citrate	1.2 \pm 0.2*	1.4 \pm 0.2*	0.8 \pm 0.08	0.9 \pm 0.08
Fumarate	1 \pm 0.06	1.1 \pm 0.1	1 \pm 0.1	1.1 \pm 0.3
Malate	1 \pm 0.08	1.1 \pm 0.2	1 \pm 0.2	0.9 \pm 0.08
Succinate	1.2 \pm 0.1	1.1 \pm 0.2	0.8 \pm 0.05	1 \pm 0.2
Glycolysis (relative intensity)				
1,3-Dihydroxyacetone	0.9 \pm 0.05	1 \pm 0.1	1 \pm 0.1	1.1 \pm 0.07
Fru-6-P	1.1 \pm 0.1	1.1 \pm 0.3	0.8 \pm 0.26	0.8 \pm 0.4
Glc	0.07 \pm 0.01	0.07 \pm 0.02	0.2 \pm 0.06*	0.1 \pm 0.02
Glc-6-P	1 \pm 0.08	1 \pm 0.02	0.9 \pm 0.07	1.1 \pm 0.06
Glycerate	1 \pm 0.08	1.1 \pm 0.2	0.8 \pm 0.1	1.1 \pm 0.3
Pyruvate	1.2 \pm 0.1*	1.3 \pm 0.3*	0.5 \pm 0.1	0.7 \pm 0.2
Fru	0.1 \pm 0.05	0.1 \pm 0.01	0.2 \pm 0.06*	0.07 \pm 0.03
Lipid metabolism (relative intensity)				
Glycerol	0.9 \pm 0.1	1 \pm 0.3	1.5 \pm 0.3*	1 \pm 0.2
Glycerol 3-phosphate	0.9 \pm 0.08	1 \pm 0.1	1.2 \pm 0.2*	1 \pm 0.1

invertases and SuSy activities that resulted in higher Glc and Fru contents. The increase in Glc and Fru was not correlated with increases in glycolytic metabolites, indicating that the sugar accumulation together with the increase in free amino acid contents (see below) constitute part of the osmotic adjustment response of wild-type plants to dehydration brought about by water stress (Kameli and Lösel, 1993; Wang et al., 1995;

Kim et al., 2000; Merewitz et al., 2011; Nio et al., 2011). On the other hand, the maintenance of photosynthesis in the transgenic $P_{SARK}::IPT$ plants during water stress was well correlated with increased SPS transcription, increased SPS activity, and reduced SuSy and cytosolic invertase activities compared with wild-type plants, leading to increased Suc contents in the flag leaves. This sustained C assimilation in transgenic $P_{SARK}::IPT$

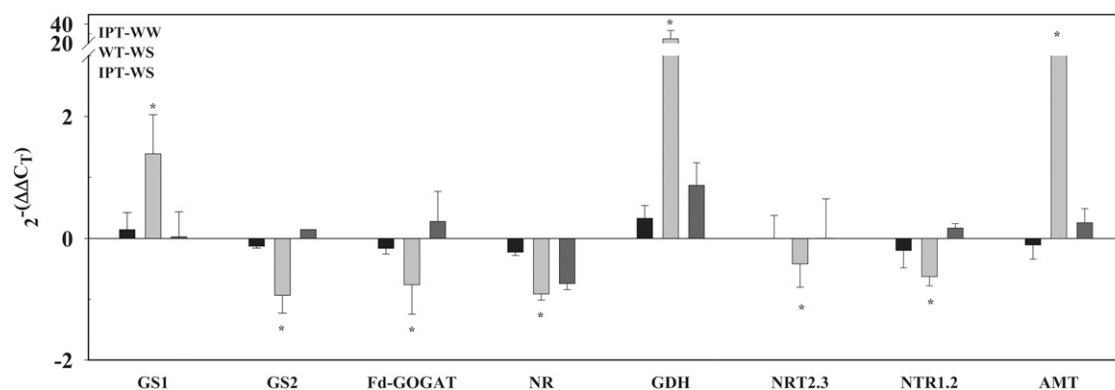


Figure 5. Relative expression of N-related genes in flag leaves of wild-type (WT) and $P_{SARK}::IPT$ transgenic plants grown under well-watered (WW) and water-stress (WS) conditions. Values were calculated and normalized using the transcription elongation factor (LOC_Os03g08060) as an internal control. Expression values were calculated relative to the expression of wild-type plants grown under well-watered conditions ($2^{-\Delta\Delta C_t} = 0$). Values are means \pm SD ($n = 6$). N-related genes are as follows: GS1 (LOC_Os02g50240); GS2 (LOC_Os04g56400); ferredoxin (Fd)-GOGAT (LOC_Os07g46460); NR (LOC_Os08g36480); GDH (LOC_Os04g45970); NTR2.3, high-affinity nitrate transporter2.3 (LOC_Os01g50820); NTR1.2, nitrate transporter1.2 (LOC_Os06g38294); AMT, ammonium transporter (LOC_Os03g62200). Asterisks indicate significant differences between genotypes ($*P \leq 0.05$).

(hormones, light, or sugar availability) controlling signaling pathways in plants. Interestingly, HK roles in sugar sensing and signaling seem to be independent of the enzyme activity, as demonstrated using catalytically inactive OsHXX alleles of OsHXX5 and OsHXX6 in rice plants (Cho et al., 2009). This notion was further supported by the higher HK activity found in $P_{SARK}::IPT$ plants that correlated well with an increase in Glc-6-P without detrimental effects on photosynthesis, growth, or senescence. On the other hand, higher Glc-6-P contents would enhance the ribulose biphosphate regeneration capacity during water-stress conditions and maintain C assimilation. Also, high Glc-6-P would sustain enhanced Suc synthesis mediated by SPS activity. Interestingly, a gene encoding the cytosolic form of the enzyme Glc-6-P dehydrogenase (G6PDH; LOC_Os2g38840) was highly up-regulated in the transgenic plants during stress. Since there is a good correlation between *G6PDH* mRNA levels, protein abundance, and enzyme activities (Hauschild and von Schaewen, 2003), we can assume that levels of G6PDH were also elevated in $P_{SARK}::IPT$ plants. G6PDH supplies the NADPH required for assimilatory processes via the pentose phosphate pathway (Kruger and von Schaewen, 2003). NADPH participates in a number of key reactions, such as ATP production (Liu et al., 2008) and the regeneration of reduced glutathione (Noctor et al., 1998). NADPH is also a limiting factor in NR-mediated N assimilation (Kaiser et al., 2000; Dutilleul et al., 2005). NR showed a dramatic increase under stress conditions in the transgenic plants, indicating that CK might mediate the production of NADPH in the $P_{SARK}::IPT$ plants in response to water stress.

Plants possess complex regulatory machinery that coordinates N assimilation with C metabolism (Nunes-

Nesi et al., 2010). This metabolic coordination varies during the day and is controlled by changes in light and/or sink demand (Ainsworth and Bush, 2011). This coordination involves sensing mechanisms for the availability of sugars (Rolland et al., 2002), nitrate and amino acids (Wang et al., 2004), C-N balance (Coruzzi and Zhou, 2001), as well as hormones. CK can affect C and N partitioning (Sakakibara et al., 2006), although the specific mechanisms underlying this regulation remain unknown. One of the most relevant routes proposed for the interaction between CKs and N is the role of CK in the regulation of N uptake and signaling, controlling the N status of the plant (Sakakibara et al., 2006; Kiba et al., 2011). C-N interactions are based on the ability of N to regulate CK biosynthesis in response to N availability (Sakakibara et al., 2006). In $P_{SARK}::IPT$ plants, we found an increased N assimilation capacity that was mediated by the increase in endogenous CK in a C-N-dependent manner.

It has been proposed that CKs play a negative role in the regulation of N uptake-related genes (Kiba et al., 2011). The exogenous application of CK to *Arabidopsis thaliana* seedlings resulted in the reduced expression of *AtNRT* genes (encoding nitrate transporters) as well as some ammonium and amino acid transporters (Kiba et al., 2011). A similar CK-induced down-regulation of genes associated with nutrient acquisition was reported in 2-week-old rice seedlings grown hydroponically and treated with exogenously applied CK (Hirose et al., 2007). Since the CK-induced repression of *AtNRT* genes was observed under both low and high N, it was suggested that the CK effects were independent of the N status of the plant (Kiba et al., 2011). The notion that CKs negatively regulate the expression of genes encoding nitrate transporters should be considered with caution, because in the

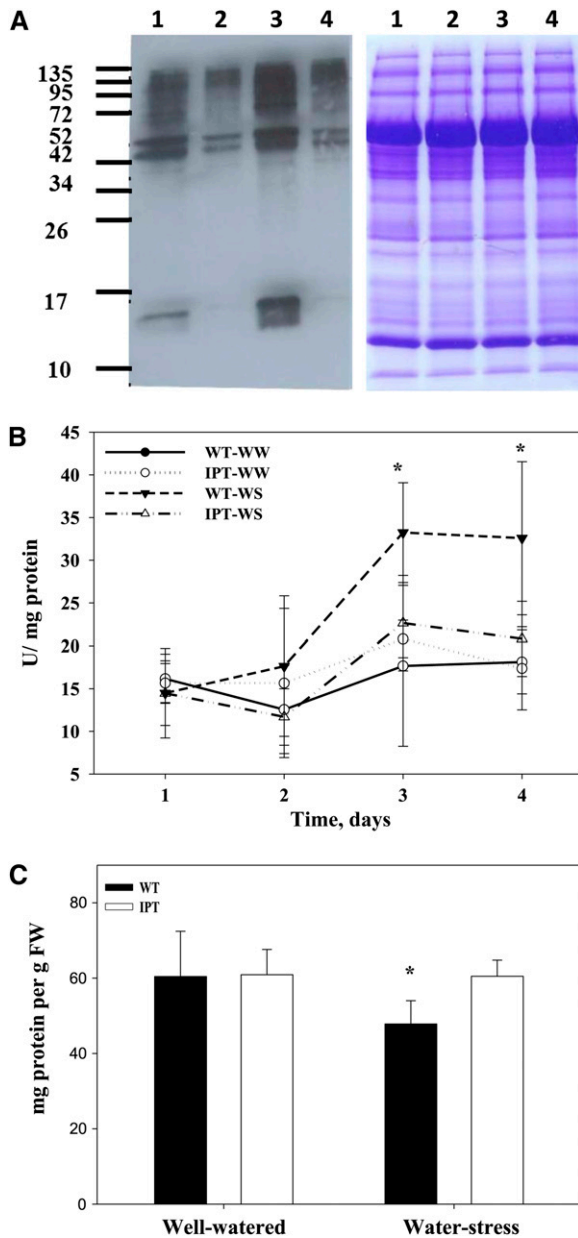


Figure 6. Protein stability and oxidation of wild-type (WT) and transgenic $P_{SARK}::IPT$ rice plants. A, Immunodetection of derivatized protein carbonyl groups using anti-DNP-hydrazone antibody to evaluate oxidative damage of proteins (blot on the left) at well-watered (WW) and water-stress (WS) conditions: wild-type well watered (lane 1), wild-type stressed (lane 2), $P_{SARK}::IPT$ well watered (lane 3), and $P_{SARK}::IPT$ stressed (lane 4). A loading control using SDS-PAGE gels followed by staining with Coomassie Brilliant Blue R-250 is shown at right. B, Protease trypsin-like activity in wild-type well-watered (black circles), wild-type stressed (black triangles), $P_{SARK}::IPT$ well-watered (white circles), and $P_{SARK}::IPT$ stressed (white triangles) rice. C, Protein content of wild-type (black columns) and transgenic $P_{SARK}::IPT$ (white columns) plants grown under well-watered and water-stress conditions. FW, Fresh weight. Values are means \pm SD ($n = 8$). Asterisks indicate significant differences between genotypes ($*P \leq 0.05$). [See online article for color version of this figure.]

reports cited above, only some of the *NRT* genes were down-regulated while others were up-regulated, and most *NRTs* expressed in the shoots were up-regulated (Kiba et al., 2011). The seedlings used in these studies were very young (3-d-old *Arabidopsis* seedlings and 14-d-old rice seedlings) and grown under non-physiological conditions (*Arabidopsis* seedlings were grown on agar plates containing a growth medium supplemented with Suc; rice seedlings were grown hydroponically). In addition, CK was applied exogenously, and it was probably catabolized via CK oxidase (Paces et al., 1971). Our results showed that, during water stress, the transgenic $P_{SARK}::IPT$ plants displayed enhanced flag leaf nitrate contents, higher NR activity, and sustained ammonium contents. These results were correlated with the higher expression of *OsNRT2.3* (LOC_Os01g50820) in the $P_{SARK}::IPT$ transgenic plants, which has been shown previously in rice to be implicated in the long-distance transport of NO_3^- to shoots (Tang et al., 2012) and the rice ortholog of the *Arabidopsis NRT1.2* (LOC_Os06g38294.1), described as a NO_3^- and ABA transporter able to regulate stomatal opening in *Arabidopsis* plants (Kanno et al., 2012). The expression of other rice nitrate transporters (LOC_Os08g05910 [ortholog of *AtNRT1.1*], LOC_Os01g68510 [ortholog of *AtNRT1.7*], LOC_Os02g47090, LOC_Os02g37040, LOC_Os04g50940, and LOC_Os02g02190) either did not show significant differences between genotypes under both conditions tested or their expression was not detectable in the leaf tissue (i.e. LOC_Os04g50940; data not shown). Our results indicate that the stress-induced CK synthesis, driven by the stress-induced *SARK* promoter, played a role in maintaining nitrate acquisition in the transgenic plants.

While N assimilation in $P_{SARK}::IPT$ plants was not affected by water stress, wild-type plants displayed a marked inhibition of NR and NiR activities. The inhibition of NR and NiR activities by water stress has been reported previously, and the decrease in activities was dependent on the severity of the stress (Ramanjulu and Sudhakar, 1997). In spite of the inhibition of N assimilation in the wild-type plants, the ammonium content in wild-type and $P_{SARK}::IPT$ plants was similar. Wild-type plants compensated for the inhibition of NiR by increasing the activity of GDH that mediates the deamination of Glu. Increased GDH deamination activity has been associated with catabolic processes during starvation (Krapp et al., 2011) and during the response of rice plants to salt stress (Nguyen et al., 2005). The increase in GDH deamination activity seen in wild-type plants during water stress would increase the amounts of 2-oxoglutarate, contributing to the maintenance of the TCA cycle and providing ammonia needed for amino acid synthesis (Miyashita and Good, 2008).

Sink capacity is the ability of tissues to import and use photosynthates for later growth and biomass production (Ainsworth et al., 2004). The ability of CKs to increase sink capacity has been associated with their role in growth, through the control of cell division (Kuiper, 1993; Werner et al., 2008), photosynthesis (Paul

and Foyer, 2001), and N metabolism (Sakakibara et al., 2006). Previously, we showed that the induction of CK synthesis in transgenic $P_{SARK}::IPT$ rice influenced changes in source-sink interactions that resulted in the improvement of yield and grain quality (Peleg et al., 2011). Here, we show that the ability of $P_{SARK}::IPT$ plants to yield during water stress is associated with the maintenance of photosynthetic activity and N assimilation in the flag leaves, supporting the role of CK in improving sink capacity (Bartrina et al., 2011). In rice, flag leaves are the main source for the growing panicles (Mae, 1997), and C and N remobilization from the flag leaves are the main contributors to grain yield (Chen and Wang, 2008). The CK-dependent increase in SPS, NR, and NiR activities in the $P_{SARK}::IPT$ plants during water stress could have contributed to the regulation of C and N supply to the growing sinks. The improvement of the flag leaf sink capacity ensured grain development during post anthesis stages, resulting in increased grain yields in the transgenic plants subjected to water stress during the pre anthesis stage (Peleg et al., 2011).

Under water stress, wild-type plants displayed enhanced protease activities, decreased protein contents, down-regulation of protein synthesis-related genes, and increased free amino acids. The inhibition of protein synthesis in plants growing under water stress is well documented (Hsiao, 1973; Dhindsa and Cleland, 1975), and the stress-induced decline in protein contents is accompanied by the accumulation of free amino acids in plant tissues (Good and Zaplachinski, 1994; Ramanjulu and Sudhakar, 1997). Protein degradation is an important mechanism for N remobilization, and chloroplasts are the major source of cellular proteins (Hörtensteiner and Feller, 2002). During stress, nutrient mobilization driven by the degradation of chloroplast proteins influences the abundance and activity of enzymes that control N and C metabolism (Ishida et al., 2008). Protein degradation in the chloroplast results in an increased amino acid pool (i.e. Glu that serves as GDH substrate) and in the loss of function of plastid enzymes such as GS2 (Kamachi et al., 1992). Thus, under stress, the cytosolic form of GS (GS1) plays an important role in Gln synthesis (Masclaux-Daubresse et al., 2006; Martinelli et al., 2007). These responses were seen in wild-type rice with a reduced expression of GS2 and *Fd-GOGAT*, both encoding chloroplast enzymes and the up-regulation of *GS1* expression, together with the loss of chloroplast function (and the concomitant inhibition of photosynthesis) and the inhibition of primary N assimilation. In the transgenic $P_{SARK}::IPT$ plants, the stress-induced CK synthesis maintained the availability of C and N needed for protein synthesis.

In conclusion, stress-induced CK synthesis in the $P_{SARK}::IPT$ transgenic plants enhanced the sink capacity of the flag leaf through a CK-dependent coordinated regulation of C and N metabolism that maintained photosynthetic activity and nitrate assimilation during the water deficit episode.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of wild-type rice (*Oryza sativa japonica* 'Kitaake') and transgenic plants expressing $P_{SARK}::IPT$ were germinated on moist germination paper for 4 d at 28°C in the dark. Seedlings were then transplanted into 8-L pots filled with a mix of 80% sand and 20% peat. Two plants were planted per pot and placed in water trays. Greenhouse conditions were kept at 12-h/12-h day/night under an illumination of 1,200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 30°C/20°C. Plants were fertilized with a solution 50% N:phosphorus:potassium (20:10:20) and 50% ammonium sulfate (total of 0.5 g of N) every 10 d until panicle initiation. Water stress treatments were applied at pre anthesis (end of booting stage toward panicle emerging) by withholding water. Plants were rewatered when visual stress symptoms (i.e. leaf rolling) appeared in the transgenic plants.

Physiological Analysis

For photosynthesis and chlorophyll fluorescence measurements, rates of CO_2 assimilation were determined in flag leaves of wild-type and $P_{SARK}::IPT$ plants after 3 d of stress using a LI-COR 6400-40 with an integrated fluorescence chamber head (Li-COR). The leaf cuvette was set at photosynthetic photon flux density of 1,200 $\mu\text{mol m}^{-2} \text{s}^{-1}$, relative humidity of 70%, and 30°C. For measurements of net CO_2 assimilation rate (A) versus Ci (A/Ci curves), the CO_2 concentration in the leaf cuvette was set at 11 levels (50, 100, 200, 300, 370, 400, 600, 800, 1,000, 1,200, and 1,500 $\mu\text{mol m}^{-2} \text{s}^{-1}$). A/Ci curves were fitted in SigmaPlot version 11 (Systat Software) using a nonlinear exponential rise to maximum model based on the equations calculated by Farquhar et al. (1980) and subsequently modified by Sharkey (1985). J_{max} , V_{cmax} , and TPU were calculated using the Photosyn Assistant software (Dundee Scientific) based on the model developed by Farquhar et al. (1980). Chlorophyll fluorescence measurements of F_v/F_m (Butler, 1978) were used to assess the maximum quantum efficiency of PSII in dark-adapted plants for 20 min, following manufacturer settings (Li-COR). Stomatal conductance was measured using a LI-COR 6400-40. Measurements of flag leaf RWC were taken from wild-type and $P_{SARK}::IPT$ plants grown under well-watered and water-stress conditions. Flag leaves were sampled before dawn and placed in a preweighed air-tight vial. Vials were weighted to obtain leaf fresh weight (FW), after which the samples were immediately hydrated to full turgidity (6 h). The leaf samples were weighted again to obtain turgid weight (TW). Samples were then oven dried at 80°C for 24 h, and dry weight (DW) was determined. RWC was calculated according to the equation:

$$\text{RWC}(\%) = \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \times 100$$

Soluble Sugar Quantification

Leaves were collected in the early morning (7–8 AM) and immediately frozen in liquid N. The frozen samples were freeze dried, and 0.1 g was used for the soluble sugar extraction as described previously (Peleg et al., 2011). The samples were dissolved in distilled, deionized water, filtered (0.45 μm), and subsequently analyzed by high-performance anion-exchange chromatography on a HPLC apparatus using a Carbohydrate Analysis column (Aminex HPX-87P 300X7.8 mm). Starch quantification was determined as described by Smith and Zeeman (2006). The release of Glc was determined at 340 nm using a Glc assay (GAHK20; Sigma-Aldrich) with a spectrophotometer (DU-640; Beckman Coulter). Starch content was calculated as 90% of Glc content.

NO_3^- and NH_4^+ Contents

Leaf freeze-dried tissue (0.01 g) already ground was used for NH_4^+ and NO_3^- quantification. NH_4^+ was determined according to Forster (1995). Absorbance was measured at 660 nm in a spectrophotometer (DU-640; Beckman Coulter). NO_3^- was determined according to Doane and Horwath (2003) by reducing nitrate to nitrite using vanadium (III) chloride followed by the addition of sulfanilamide and *N*-(1-naphthyl)-ethylenediaminedihydrochloride. Subsequent colorimetric nitrite analysis was done at 540 nm in a spectrophotometer (DU-640; Beckman Coulter). Nitrite analysis was done without the use of vanadium (III) chloride and then was subtracted from the nitrite content determined for NO_3^- analysis. Standard curves were obtained using KNO_3 as a standard.

Amino Acid Quantification

Leaf frozen tissue was ground and freeze dried. Free amino acids were extracted as described previously by Hacham et al. (2002). Tissue (0.1 g) was ground in the presence of 400 μL of water:chloroform:methanol (3:5:12, v/v) and centrifuged for 2 min at 12,000g. This step was repeated twice, the supernatants were combined, and a mixture of 200 μL of chloroform and 300 μL of water was added. After 2 min of centrifugation at 14,000g, the supernatant was collected and dried. Determination of free amino acids was carried out on a Hitachi L-8900 amino acid analyzer.

Metabolite Profiling

Metabolites were analyzed in flag leaves of wild-type and *P_{SARK::IPT}* plants grown under well-watered and water-stress conditions. Samples ($n = 6$) were submitted to Metabolon. The sample preparation and analysis process was carried out as described previously (Lawton et al., 2008; Evans et al., 2009; Oliver et al., 2011). Compounds were identified by comparison with library entries of purified standards or recurrent unknown entities using Metabolon's proprietary software. The identification of known chemical entities was based on comparison with metabolomics library entries of purified standards (DeHaven et al., 2010).

Enzyme Assays

Enzyme activities were determined in flag leaves of wild-type and transgenic *P_{SARK::IPT}* plants subjected to 3 d of water stress and collected in the early morning (7–8 AM). Flag leaf homogenates were obtained from leaf tissue collected 2 to 10 cm from the apical meristem. Enzyme activities are expressed as moles of metabolite generated/consumed per milligram of protein per unit of time.

SPS activity measurements were based on Klann et al. (1993). Sugars were quantified using Anthrone (Van Handel, 1968), and absorbance was measured using a microplate reader (Synergy MxMonochromator-Based Multi-Mode Microplate Reader; Biotek) at 620 nm.

SuSy activity was determined in the direction of Suc synthesis as well as Suc degradation. Suc synthesis mediated by Suc synthase was determined according to Klann et al. (1993). Suc cleavage was assayed as described by Zrenner et al. (1995). Absorbance was measured using a microplate reader (Synergy MxMonochromator-Based Multi-Mode Microplate Reader; Biotek) at 540 nm using Fru as a standard.

Cell wall invertase, cytosolic invertase, and vacuolar invertase were assayed according to Roitsch et al. (1995). Controls were boiled before incubation with the reaction buffer. Reaction was stopped using 100 μL of 1 M NaOH, and reducing sugars were determined after reaction time (Miller, 1959).

HK, trehalase, total amylolytic, and α -amylase activities were measured according to Wiese et al. (1999), Müller et al. (1995), and Lee et al. (2009), respectively.

For NR and NiR, leaf frozen tissue (0.1 g) was ground in the presence of 1 mL of buffer containing 50 mM KH_2PO_4 -KOH buffer, pH 7.5, 2 mM EDTA, 2 mM dithiothreitol, and 1% polyvinylpyrrolidone. Extracts were centrifuged at 20,000g for 20 min at 4°C. NR activity was measured according to Kaiser and Lewis (1984) with some modifications. The reaction was initiated by the addition of 700 μL of reaction buffer (50 mM KH_2PO_4 -KOH buffer, pH 7.5, 10 mM KNO_3 , and 0.1 mM NADH) to 100 μL of total soluble proteins. Samples were incubated at 28°C for 15 min, and controls were boiled before incubation with the reaction buffer. The reactions were stopped, and NO_2^- was determined following the method established by Hageman (1971). NiR activity measurements were performed according to Lillo (1984).

NADH-GOGAT and GS were determined according to Matoh and Takahashi (1982) and O'Neal and Joy (1973), respectively.

Both amination and deamination activities of GDH were assessed according to protocols described previously by Loyola-Vargas and de Jimenez (1984). Asp and aminotransferase activities were assayed according to Lillo (1984).

Protein Quantification

The Bradford assay (Bradford, 1976) was used for protein quantification using bovine serum albumin as a standard.

Oxidative Modification of Proteins

Protein oxidation was evaluated using the OxyBlot Kit (Millipore). Immunodetection of derivatized carbonyl groups to DNP-hydrazone was performed following kit protocol.

Proteinase Activity Assay

Trypsin-like activity was determined using the QuantiCleave Protease Assay Kit (Pierce Chemical). Leaf homogenates were prepared using 50 mM HEPES-KOH, pH 7.5, 2 mM EDTA, 5 mM MgCl_2 , 0.05% Triton X-100, 5 mM β -mercaptoethanol, and 1% polyvinylpyrrolidone (w/v). Fifty microliters of the extract was used for the assays following the manufacturer's instructions. Absorbance was measured at 450 nm. Blanks were prepared without the addition of substrate (succinyl casein solution).

Quantitative PCR Analysis

RNA was extracted from flag leaves of wild-type and transgenic *P_{SARK::IPT}* rice plants under well-watered and water-stress conditions. First-strand complementary DNA synthesis, primer design, and quantitative PCR were performed as described before (Peleg et al., 2011). The different sets of primers used for the amplification of the different target genes are listed in Supplemental Table S1. Analysis of the relative gene expression was performed according to the Comparative Cycle Threshold Method as described by Livak and Schmittge (2001).

Processing Microarray Data

Normalization and statistical analyses were performed using the JMP Genomics (version 5.1) statistical package (SAS Institute) as described before (Peleg et al., 2011). The CELL data files of the 12 Affymetrix GeneChip results of this study were deposited in the public microarray database National Center for Biotechnology Information Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi>) with accession number GSE 23211.

Statistical Analysis

The JMP (version 8.0) statistical package (SAS Institute) was used for statistical analyses. ANOVA was employed to test the effect of genotype on trait. The LSMean t test (Least Square Means) was used to compare means by genotype/treatment at a probability level of 5%. Levels of significance are represented by asterisks as follows: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$; n.s. indicates not significant. The experiments were based on a randomized complete block design using six replicates.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Heat map of the list of genes involved in C and N metabolism

Supplemental Figure S2. Heat map of the list of genes encoding enzymes involved in protein synthesis.

Supplemental Table S1. List of Primers Used in qPCR (Real Time PCR).

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