Enhanced Cytokinin Synthesis in Tobacco Plants Expressing $P_{SARK}$::IPT Prevents the Degradation of Photosynthetic Protein Complexes During Drought

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To identify genes associated with the cytokinin-induced enhanced drought tolerance, we analyzed the transcriptome of wild-type and transgenic tobacco (Nicotiana tabacum ‘SR1’) plants expressing $P_{SARK}$::IPT (for senescence-associated receptor kinase::isopentenyltransferase) grown under well-watered and prolonged water deficit conditions using the tomato GeneChip. During water deficit, the expression of genes encoding components of the carotenoid pathway leading to ABA biosynthesis was enhanced in the wild-type plants, but repressed in the transgenic plants. On the other hand, transgenic plants displayed higher transcript abundance of genes involved in the brassinosteroid biosynthetic pathways. Several genes coding for proteins associated with Chl synthesis, light reactions, the Calvin–Benson cycle and photosynthesis were induced in the transgenic plants. Notably, increased transcript abundance of genes associated with PSII, the cytochrome $b_6/f$ complex, PSI, NADH oxidoreductase and the ATP complex was found in the $P_{SARK}$::IPT plants. The increased transcript abundance was assessed by quantitative PCR and the increased protein levels were confirmed by Western blots. Our results indicated that while the photosynthetic apparatus in the wild-type plants was degraded, photosynthesis in the transgenic plants was not affected and photosynthetic proteins were not degraded. During water deficit, wild-type plants displayed a significant reduction in electron transfer and photochemical quenching, with a marked increase in non-photochemical quenching, suggesting a decrease in energy transfer to the PSII core complexes and an increase in cyclic electron transfer reactions.

Keywords: Cytokinins • Drought • IPT • Nicotiana tabacum ‘SR1’ • Photosynthesis • Water deficit.

Abbreviations: BR, brassinosteroid; CET, cyclic electron transport; CK, cytokinin; cyt, cytochrome; ETR, electron transport rate; Fd, ferredoxin; FDR, false discovery rate; IPT, isopentenyltransferase; JA, jasmonic acid; MgCh, magnesium chelatase; NPQ, non-photochemical quenching; OEC, oxygen-evolving complex; PGR5, PROTON GRADIENT REGULATION 5; PQ, plastoquinone; qP, photochemical quenching; ROS, reactive oxygen species; RuBP, ribulose-1,5-bisphosphate; SAM, statistical analysis of microarray; SARK, senescence-associated receptor kinase; WT, wild type.

Introduction

Water deficit imposes severe physiological and biochemical limitations on plant growth and productivity. During drought stress, a series of hormonal changes have been reported [such as a decrease in cytokinins (CKs) or an increase in ABA] (Davies and Zhang 1991). These changes in hormone homeostasis due to water deficit typically lead to the inhibition of photosynthesis (Chaves 1991). The inhibition of photosynthetic activity under stress is due to a decrease in CO$_2$ availability caused by the limitation of CO$_2$ diffusion (Cornic 2002, Flexas et al. 2007) and/or changes in the biochemical control of photosynthesis where ribulose-1,5-bisphosphate (RuBP) synthesis, ATP synthesis, electron transfer, etc. are inhibited (Lawlor 2002, Rivero et al. 2009). Although during relatively mild water stress stomatal limitations account for most of the decrease in photosynthesis, during more severe or prolonged drought stress the breakdown of the photosynthetic machinery is a major factor in the reduction of CO$_2$ assimilation (Tambussi et al. 2000). Of the several energy-transducing complexes of the photosynthetic machinery, PSII is a critical multisubunit complex composed of a core, the light-harvesting chlorophyll antenna and the oxygen-evolving complex (OEC). The core is comprised of the reaction center proteins D1 and D2, the internal antenna proteins CP43 and CP47, cytochrome (cyt) $b_559$ (Giardi et al. 1997) and the phosphoprotein TSP0 (Hansson et al. 2007). The PSII proteins D1 and D2 form a heterodimer. This heterodimer is essential for initiating the transport of electrons liberated from the breakdown of H$_2$O and to produce energy (ATP) and
Reduced equivalents (NADPH) for CO₂ assimilation. The electrons are transferred from the D1–D2 heterodimer to the PSI through the cyt b₆/f complex and eventually regenerate NADPH. The resulting proton gradient across the thylakoid membrane is used to generate ATP (Shikanai 2007). This process, called linear electron transport, is coupled to cyclic electron transport (CET) which comprises PSI, ferredoxin (Fd) and the plastoquinone (PQ) pool. During CET, the electrons are recycled from NAD(P)H or Fd to PQ to generate a proton gradient without NADPH accumulation (Shikanai 2007). Although CET plays important roles in cyanobacteria, algae and C₄ plants, it is thought to have minimal influence in C₃ plants under steady-state photosynthesis (Long et al. 2008). However, CET plays a significant role under environmental stress (Munekage et al. 2002, Nandha et al. 2007, Okegawa et al. 2008). During prolonged water deficit, the steady-state levels of major PSII proteins decline, possibly because of the increased protein degradation and inhibition of protein synthesis (Aro et al. 1993, He et al. 1995). In addition, reactive oxygen species (ROS) accumulate in the chloroplast, inducing the damage to the thylakoid membranes and photoinhibition (Tambussi et al. 2000).

Hormones mediate the adaptation of plant growth and development to changing environmental conditions (Wolters and Jurgens 2009), and extensive overlaps exist between drought-associated genes in plants in response to drought and gene expression in plants in response to hormones including ABA, auxins, CKs, gibberellic acid, jasmonic acid (JA) and brassinosteroids (BRs) (Huang et al. 2009). However, while CKs and ABA have been clearly associated with senescence and drought responses, respectively, the roles of other plant hormones such as gibberellic acid, auxin, ethylene and BRs in the response of plants to water deficit are relatively less well characterized. Nevertheless, a dynamic interaction among the various hormones, changing with development and tissue types at the same developmental stage, can be expected (Achard et al. 2006).

We have shown previously that stress-induced leaf senescence could be delayed in transgenic plants expressing isopentenyltransferase (IPT), an enzyme that catalyzes the rate-limiting step in CK synthesis under the control of senescence-associated receptor kinase (SARK), a maturation- and stress-inducible promoter (Rivero et al. 2007). Transgenic plants expressing Pₛ₅₃₉::IPT grown under water deficit conditions displayed enhanced levels of CKs but did not show a consistent pattern for the steady-state levels of ABA (Rivero et al. 2007). Increased CK production resulted in enhanced drought tolerance of the transgenic Pₛ₅₃₉::IPT plants, with minimal yield loss (Rivero et al. 2007). Following a severe drought treatment, the production of CKs in transgenic plants expressing Pₛ₅₃₉::IPT led to enhanced photosynthesis and improved water use efficiency (Rivero et al. 2007). When wild-type (WT) and transgenic Pₛ₅₃₉::IPT tobacco (Nicotiana tabacum ‘SR1’) were grown under optimal or restricted (30% of optimal) watering regimes, there was no significant difference in stomatal conductance between leaves from WT or transgenic plants, but the WT

Results and Discussion

Drought stress-responsive genes in WT and Pₛ₅₃₉::IPT plants

We have shown previously that transgenic plants expressing the IPT gene under the control of SARK, a stress- and senescence-induced promoter, displayed a remarkable tolerance when grown under reduced water regimes that inhibited the growth and yield of WT plants (Rivero et al. 2007, Rivero et al. 2009). (Supplementary Fig. S2) To identify genes associated with the CK-induced enhanced drought tolerance, we assayed and compared the transcriptomes of WT and Pₛ₅₉::IPT plants growing under well-watered (1,000 ml d⁻¹) and restricted watering conditions (300 ml d⁻¹) (Fig. 1A). Because a microarray platform for N. tabacum was not available within these experiments were initiated, we used the expressed sequence tag (EST)-based tomato GeneChip from Affymetrix, representing ~9200 transcripts, for heterologous hybridization. Roughly 41% of the probe sets on the tomato array had a present call when processed RNA from Nicotiana was hybridized. The data generated were examined for quality, corrected for background and normalized as described in the Materials and Methods. The differential expression analysis was performed using statistical analysis of microarray (SAM) software. We used an arbitrary cut-off of a 1% false discovery rate (FDR) for identifying differentially expressed genes among samples. Most of the genes that fulfilled the stringent differential expression criteria were up- or down-regulated by at least a 2-fold change. Using the analysis described above we first sought to identify the differentially expressed genes in the two genotypes in response to 45 d of growth under water deficit conditions. A comparison between WT plants growing under well-watered and water deficit conditions identified 121 probe sets up-regulated and 322 probe sets down-regulated in response to water stress (Figure 1B; comparison 3, Supplementary Tables S1, S2). A similar stress triggered fewer transcript changes in Pₛ₅₉::IPT
In summary, the drought stress-responsive gene sets were markedly diverged in the transgenic plants expressing P_{SARK::IPT}. If the number of transcript changes in response to drought is considered a proxy for relative tolerance, this analysis indicated a less perturbed transcriptome of highly tolerant P_{SARK::IPT} plants.

**Contrasting responses between the two genotypes during drought stress**

We extended our analysis of the drought-responsive genes by attempting to identify genes that displayed a contrasting response to water deficit between the two genotypes. To this end, we searched for genes which were induced in P_{SARK::IPT} plants but repressed in WT plants during water deficit, and found seven genes (Supplementary Table S5). Notable among these was an auxin-responsive gene (LesAffx.71035.1.S1_at) that was induced in transgenic plants (1.8-fold, FDR 0%) but repressed in WT plants (2.3-fold, FDR 0%). The Arabidopsis Response data set (www.geneinvestigator.com; www.tair.org) indicated that the Arabidopsis ortholog of this gene (At3g03840) was strongly repressed by drought, ABA and high light but was activated by the phytohormones auxin, zeatin (a CK) and brassinolide treatments. The induction of Les Affx.71035.1.S1_at expression in the transgenic plants would suggest an effect of increased CK possibly overriding an ABA effect (Supplementary Fig. S1). In addition, genes coding for a chloroplast ATP synthase and a cytochrome oxidase also displayed expression trajectories parallel to the auxin-responsive gene. A converse comparison, i.e. for genes that were induced in the WT during drought, but repressed in transgenic plants, yielded a null set.

**Differential transcriptome regulation by increased CK levels**

Our objective is to understand the shift in transcriptome dynamics associated with activation of the P_{SARK::IPT} gene, the production of CKs and the concomitant improved plant survival and growth during water deficit. To address this fundamental question on the role of CKs, we first compared the transcriptome of WT and transgenic P_{SARK::IPT} plants grown under well-watered conditions (Fig. 1B; comparison 1, Supplementary Tables S6, S7). Using the defined statistical criteria described above, we identified 322 probe sets that were up-regulated and 393 that were down-regulated in P_{SARK::IPT} plants as compared with WT plants. A parallel comparison for stressed plants (Fig. 1B; comparison 2, Supplementary Tables S8, S9) indicated that 515 and 475 probe sets were up- and downregulated respectively, in both genotypes. The comparison (between 3 and 4, Fig. 1B) clearly indicated a diverged transcriptome response resulting from a direct or a downstream effect(s) by P_{SARK::IPT} expression. To ensure that the minimal overlap between the two genotypes was not a result of our stringent statistical cut-off, we performed the same comparison using a relatively relaxed threshold of a 5% FDR. Although the number of differentially expressed genes increased greatly, the overlap between comparisons 3 and 4 (Fig. 1B) reflecting the conservation of transcript level responses was even lower. In summary, the drought stress-responsive gene sets were

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**Fig. 1** Experimental design and differential expression comparisons. (A) Scheme of the experimental design and time points at which the samples were collected for expression analysis (indicated by green ovals). The green broken line indicates daily watering (1,000 ml d^{-1}) of WT and P_{SARK::IPT} transgenic plants. The blue line represents both genotypes under limited water (300 ml d^{-1}) conditions harvested after 45 d. The orange line represents a severe drought treatment (no water for 1 week) for both genotypes. (B) The pair-wise differential expression comparisons (1–5) performed between plants at the 45 d time point. The genotypic comparisons are represented by blue bars. The number of genes differentially expressed (induced or repressed) at a 1% FDR are indicated in green for each of the comparisons. Complete lists as well as the 1 week comparisons are provided as Supplementary data.
These results are surprising because under well-watered conditions, the WT and \( P_{\text{SARK}}\)\(\cdot\)\(\text{IPT} \) plants did not display any phenotypic differences, and physiological and biochemical characterization indicated similar levels of ROS detoxification and photosynthesis (Rivero et al. 2007; Rivero et al. 2009).

To elucidate the role of CKs in the tolerance to water deficit, we first examined the gene sets generated from the genotypic differential expression analyses. Several biological features emerged from the gene sets. Some of the salient features observed included a shift in certain hormone-associated pathways, and activation of the tetrapyrrole biosynthesis pathways, starch metabolism-associated genes and genes encoding photosynthetic electron transport components. While these features are notable and could explain the observed contrasting phenotypic differences between the \( P_{\text{SARK}}\)\(\cdot\)\(\text{IPT} \) and WT plants, they are not an exhaustive coverage of the gene lists provided as supplementary data (Supplementary Tables S1–S9).

Distinct plant hormonal regulation in \( P_{\text{SARK}}\)\(\cdot\)\(\text{IPT} \) plants

Plant hormones play important roles in almost all aspects of plant growth, development and their response to a multitude of environmental cues. Some of the well-studied hormones include auxins, CKs, gibberellins, ethylene, ABA, JA and BRs. The hormonal homeostasis that defines the physiological and developmental status of a plant is probably determined by the cross-talk among the different hormones. For instance, \( JAZ1 \) is a protein that represses JA signaling pathways that was recently reported to be auxin inducible (Grunewald et al. 2009). Another instance of the antagonistic relationship among plant hormones was reported in Arabidopsis, where CKs and IAA inhibit ABA-induced stomatal closure by enhancing ethylene production (Tanaka et al. 2006). During drought stress the endogenous plant hormone levels change and these changes appear to impact grain yield and quality in wheat (Xie et al. 2003). Xie et al. (2003) demonstrated that changes in wheat yield and grain starch and protein content under drought were associated with reduced IAA, zeatin riboside and gibberellic acid levels and elevated ABA levels in plants, especially in grains. These and other reports highlight a shift in hormone levels in response to changing environment. In the \( P_{\text{SARK}}\)\(\cdot\)\(\text{IPT} \) plants a gene coding for gibberellin 20-oxidase1 (GA20ox1) was up-regulated by \( >8\)-fold (FDR \( 0\% \)) relative to the WT plants (Fig. 1B; comparisons 1 and 2). GA20ox1 is involved in gibberelin synthesis, indicating that the increased CK levels could promote increased transcription of gibberelin synthetic pathways. The expression of the Arabidopsis ortholog of GA20ox1 is strongly suppressed by water stress and to some degree by ABA treatments (www.geneinvestigator.com; www.tair.org). Interactions between CK and gibberellin levels vary during plant development; high CK and low gibberellin levels are required for shoot apical meristem development, the site of active cell development (Jasinski et al. 2005). On the other hand, low CK and high gibberellin signals are essential in mature and elongating cells (Greenboim-Wainberg et al. 2005, Yanai et al. 2005).

Repression of ABA-associated genes by increased CKs

A gene encoding carotenoid cleavage dioxygenase (NCED1) was induced in the WT plants but not in \( P_{\text{SARK}}\)\(\cdot\)\(\text{IPT} \) plants (Rivero et al. 2009; Supplementary Fig. S1). The oxidative cleavage by NCED1 is considered to be the first committed and probably the rate-regulating step in ABA biosynthesis downstream of the \( C_{\text{40}} \) carotenoid pathway (Schwartz et al. 2003). Interestingly, the gene encoding the upstream enzyme \( \beta \)-carotene hydroxylase, which converts \( \beta \)-carotene to zeaxanthin, was strongly repressed in the transgenic plants (Supplementary Fig. S1; comparisons 1, 2). Further, the transcript abundance of violaxanthin de-epoxidase (NPQ1) was highly increased (7.8-fold, FDR \( 0\% \)) in the \( P_{\text{SARK}}\)\(\cdot\)\(\text{IPT} \) plants relative to the WT (Supplementary Fig. S1). NPQ1 operates in the reverse direction (away from ABA biosynthesis), converting violaxanthin to antheraxanthin and zeaxanthin (Havaux and Nigoyi 1999).

In addition to genes associated with ABA synthesis, we also found several genes coding for ABA signaling components that were differentially regulated between the two genotypes (Supplementary Fig. S1). Among these, a protein phosphatase 2C (HAB2; homolog to ABI2) was repressed in \( P_{\text{SARK}}\)\(\cdot\)\(\text{IPT} \) plants. Also, the expression of \( \text{RCAR1} \) was repressed in the \( P_{\text{SARK}}\)\(\cdot\)\(\text{IPT} \) plants relative to the WT during stress. \( \text{RCAR1} \) was shown recently to mediate the interaction between ABA and ABI2, the negative regulator of ABA responses (Ma et al. 2009). In general, the expression data indicated that ABA biosynthesis and ABA-responsive genes were expressed at higher levels in the WT plants compared with the \( P_{\text{SARK}}\)\(\cdot\)\(\text{IPT} \) plants (Supplementary Fig. S1).

\( P_{\text{SARK}}\)\(\cdot\)\(\text{IPT} \) positively regulates the transcription of genes associated with BR biosynthesis

BRs are hormones involved in plant growth and development, and mediate plant responses to the environment such as light and pathogen challenge (Bishop and Yokota 2001). A comparison between WT and \( P_{\text{SARK}}\)\(\cdot\)\(\text{IPT} \) plants showed a strong differential expression regulation in the early sterol biosynthetic pathway and its derivate hormone BR (Fig. 3). \( \text{FACKEL} \), \( \text{SMT2} \), \( \text{DWARF1} \) (\( \text{DWF1} \)) and \( \text{cyclopropyl isomerase} \) (\( \text{CPI1} \)), genes involved in sterol synthesis (Fujioka and Yokota 2003), displayed higher transcript abundance in the transgenic plants compared with the WT under both control and restricted water conditions. Several other genes involved in BR biosynthesis (e.g. BR-6-oxidase 1) and BR signaling (\( \text{BIN2} \)) were also differentially regulated. Almost all of the expression differences persisted between the WT and transgenic plants irrespective of the presence of stress, suggesting that the increased CK levels activated the transcription of genes associated with the biosynthesis of sterols and their derivative hormone. The positive interaction between CKs and BRs was further supported by a 2.3-fold up-regulation of cyclin D3 genes in transgenic plants.
Cyclin D3 is a plant cyclin gene that mediates CK-induced cell division that was reported to be induced by BR treatment (Hu et al. 2000, Mussig et al. 2002). Collectively, our data suggest that CKs activate BR biosynthesis and BR-activated pathways while negatively affecting the carotenoid pathway leading to ABA biosynthesis. The interhormonal interactions suggested by our data are consistent with the putative antagonism between ABA and CKs, and ABA and BR, and the synergism between CKs and BR (Wolters and Jurgens 2009). An antagonistic relationship between ABA and CKs has been postulated (Pospisilova and Dodd 2005), and the expression of genes associated with ABA synthesis was repressed in P$_\text{SARK}_{\text{ipt}}$ transgenic plants (Supplementary Fig. S1; Rivero et al. 2009), consistent with the increased CK content in the transgenic plants. Recently, cross-talk between ABA and BR has been postulated (Zhang et al. 2009). It has been shown that the ABA and BR interaction requires the ABA signaling elements ABI1 and ABI2, as well as the BR signaling component BIN2 (Zhang et al. 2009). Notably, the expression of HAB1 and HAB2, homologs of ABI1 and ABI2, respectively, was relatively higher in WT plants (Supplementary Fig. S1), while the expression of BIN2 was higher in the transgenic plants (Fig. 2), thus supporting an antagonistic relationship between ABA and BR. Although our data suggest an interaction between CKs and BR, whether this interaction is direct, or indirectly mediated by ABA, is not clear.

**Increased endogenous CK results in transcriptional activation of Chl biosynthesis**

Transgenic plants expressing P$_\text{SARK}_{\text{ipt}}$ maintained a higher photosynthetic activity during drought (Rivero et al. 2009). Consistent with this observation, we found the transcriptional activation of several genes coding for enzymes involved in Chl biosynthesis in the transgenic plants (Fig. 3). Some of them (e.g. porphobiligen deaminase and protochlorophyllide oxidoreductase, among others) displayed higher relative expression in P$_\text{SARK}_{\text{ipt}}$ plants irrespective of the watering regimes. However, the expression of a gene coding for glutamyl-tRNA reductase, which has a role during early stages of tetrapyrrole synthesis, was repressed in the WT plants during growth under water deficit but remained unchanged in the transgenic plants. Interestingly, the expression of genes coding for Mg protoporphyrin chelatase (MgCh) was at least 2-fold higher in the WT plants relative to transgenic plants grown under control conditions, and no significant expression difference was detected between the genotypes growing under water deficit. MgCh catalyzes the first committed step in Chl synthesis and is also considered to be an important component of the regulatory mechanism for Chl synthesis (Rissler et al. 2002). In addition to its role in Chl biosynthesis, the Arabidopsis ortholog of MgCh, genomes uncoupled 5 (GUNS), has also been proposed to play an important role in plastid to nucleus signaling as well as ABA signaling (Mochizuki et al. 2001, McCourt and Creelman 2008). Furthermore, plants overexpressing the major subunit of MgCh were reported to be hypersensitive to ABA, whereas knockout mutants showed ABA insensitivity.

![Fig. 2](image-url)  
**Fig. 2** Brassinosteroid (BR) pathway. The sterol and BR biosynthesis pathway is differentially regulated between the wild type and P$_\text{SARK}_{\text{ipt}}$. (A) Heat map of a selected set of BR-related genes derived from hierarchical cluster analysis. Red color represents higher relative expression and blue represents lower relative expression when compared with the mean expression value across all samples. Scale is the log$_2$ of mean expression values. The figure is representative of two independent experiments. (B) Validation of a subset of BR-related genes using a quantitative PCR assay. Wild-type plants growing under control conditions (1,000 ml d$^{-1}$) were used as the reference sample (log$_2$(WT) = 0). Each data point represents the mean±SE (n=6). Two internal controls (rRNA 18S and UBQ2) were used for data normalization. FACKEL (HYD2), cyclopropyl isomerase (CPI1), sterol-4-α-methyl oxidase 1–1 (SMO1-1), sterol methyl transferase 2 (SMT2), farnesyl diphosphate synthase 1 (FPS1), brassinosteroid insensitive 2 (BIN2), C-5 sterol desaturase (DWARF7), DWARF1/DIMINUTO1, brassinosteroid-6-oxidase 1, brassinosteroid-responsive ring H2 (BRH1), Arabidopsis shaggy-related protein kinase DZETA (ASKDZETA).

The GUNS expression was strongly repressed by ABA and drought in the Arabidopsis expression atlas data set (www.arabidopsis.org), and the potential regulatory role of MgCh in the context of CK-mediated Chl biosynthesis during water stress should be further investigated.

**Transcriptional activation of light reaction-related genes under water deficit**

Genes that were differentially expressed in WT and P$_\text{SARK}_{\text{ipt}}$ plants grown under water deficit were imported into MapMan software...
To obtain a transcriptional overview of the pathways that could be differentially regulated by CKs, this analysis indicated that several genes encoding proteins associated with light reactions, the Calvin–Benson cycle and photorespiration were up-regulated in the PSARK::IPT transgenic plants (Fig. 4, Supplementary Tables S8, S9). Notably, we found increased transcript abundance for genes associated with PSII, PSI, NADH oxidoreductase and ATPase complexes in PSARK::IPT plants when compared with wild type. Each data point corresponds to the log₂ of the mean value from three biological replicates.
The expression of most of the photosynthesis-related genes listed was repressed in the WT, but induced in the $P_{SARK} \cdot IPT$ plants in response to water deficit (Fig. 5A). We further assayed the expression of a subset of these genes using quantitative PCR (Fig. 5B). All of the 16 genes tested were down-regulated in the WT and 15 of these genes had higher expression in $P_{SARK} \cdot IPT$ plants in response to water deficit.

### Table: Photosynthetic Complexes and Genes

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<td>-1.55</td>
<td>1.44</td>
<td>1.69</td>
</tr>
<tr>
<td><strong>ATP Synthase</strong></td>
<td>β-ATPase</td>
<td>Les:4995.1.1 <em>st</em></td>
<td>*</td>
<td>-3.03</td>
<td>1.77</td>
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<tr>
<td></td>
<td>γ-ATPase</td>
<td>Les:6867.1.1 <em>st</em></td>
<td>*</td>
<td>-2.56</td>
<td>2.09</td>
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<tr>
<td></td>
<td>δ-ATPase</td>
<td>Les:1260.1.1 <em>st</em></td>
<td>*</td>
<td>-1.58</td>
<td>-1.3</td>
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</tr>
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</table>

Fig. 5 Analysis of genes associated with the photosynthesis complexes. (A) Classification and differential expression (log$_2$) of photosynthesis-related genes. The analysis of the DNA microarrays was performed as described in Materials and Methods for three biological replicates. (B) Validation of the microarray expression by quantitative PCR. Wild-type plants growing under control conditions (1,000 mL d$^{-1}$) were used as the reference sample (log$_2$ = 0). Each data point represents the mean ± SE (n = 6). Two internal controls (rRNA 18S and UBQ2) were used for data normalization.
We also tested the protein levels of several of the photosynthetic complex proteins in WT and \textit{P SARK ::IPT} plants under control and water deficit conditions (Fig. 6). The PSII reactions center proteins D1 and D2 bind to electron transfer prosthetic groups such as P680, and to PQ. At the protein level, the expression of D1 was significantly reduced in WT plants under stress. However, the \textit{P SARK ::IPT} plants maintained a higher level of D1 irrespective of soil moisture status (Fig. 6). The protein levels of D2 did not change significantly in the WT plants under stress, but displayed a slight increase in the \textit{P SARK ::IPT} plants in response to water deficit. During water deficit conditions, the phosphorylation index of the PSII proteins increased and a reorganization of the photosystems occurred (Giardi et al. 1995). D1 levels have been reported to be more sensitive to long-term drought stress in pea seedlings (Giardi et al. 1995), and a high rate of D1 protein turnover was shown to stabilize thylakoid membranes and the electron transport chains against oxygen free radicals that accumulate under water deficit (Guseynova et al. 2006). A similar expression pattern was seen with antibodies raised against the OEC of PSII, cyt \textit{b}6/\textit{f}, Fd, \textit{α}-ATPase and PsaB (core protein of PSI) and the ATPase \textit{α}-subunit, with decreased protein levels in the WT plants exposed to water deficit, but unchanged and sometimes increased protein levels in the \textit{P SARK ::IPT} plants (Fig. 6). The cyt \textit{b}6/\textit{f} complex-related genes encode proteins that transfer electrons between the two reaction center complexes (PSI and PSII). They also participate in the formation of the transmembrane electrochemical proton gradient by transferring protons from the stroma to the internal lumen compartment. Senescence and stress lead to the degradation of the photosynthetic apparatus and the cyt \textit{b}6/\textit{f} complex (Hortensteiner 2009), and the relatively stable protein levels seen in the transgenic plants grown under water deficit could be a consequence of the CK-induced delayed senescence observed in the transgenic plants (Rivero et al. 2007).

In contrast to the other photosynthesis-related protein tested, Fd protein levels increased in the WT plants grown under water deficit (Fig. 6), and a similar increase was seen in the transgenic plants grown under water deficit. During stress, the energy that is not used to drive photosynthesis is dissipated as heat to avoid the generation of ROS. In this situation, Fd drives the CET reactions, avoiding damage to PSI (Arnon et al. 1954). Using Chl fluorescence measurements of WT and \textit{P SARK ::IPT} plants, we determined the electron transport rate carried out by the cyt \textit{b}6/\textit{f} complex (ETR) and the energy driven to photosynthesis (photochemical quenching; \textit{qP}) or dissipated as heat (non-photochemical quenching; NPQ) (Fig. 7). Under well-watered conditions, no significant differences were observed between WT and transgenic plants. However, during water deficit the WT plants displayed a marked reduction in ETR and \textit{qP}, and an increase in NPQ (Fig. 7). The decrease in D1 protein levels and OEC in WT plants (Fig. 6), together with the reduction in ETR and \textit{qP} and the increase in NPQ, suggest a decrease in energy transfer to the PSII core complexes and a possible increase in CET during water deficit. In spite of the decrease in PsaB protein content and PsaN and...
PsaH gene expression, the increase in PSI activity (Supplementary Table S11) seen in WT plants during stress would suggest an increase in CET and would support the notion of a role for CET as a source of ATP for C3 plants under stress conditions, where CO2 or RuBP can be limiting (Golding and Johnson 2003, Nandha et al. 2007). The occurrence of CET in the WT plants during water deficit was also supported by the enhanced expression of PGRS (PROTON GRADIENT REGULATION 5). PGRS have been shown to be important for the activity of Fd-dependent CET (Long et al. 2008, Munekage et al. 2008). Although PsaARC::IPT plants also displayed an increase in Fd protein levels during water deficit (Fig. 6) and a relatively higher PSI activity (Supplementary Table S11), these plants did not show an increase in NPQ or a decrease in qP or increased PGRS transcription during water deficit. Moreover, PsaARC::IPT plants displayed an increased RuBP regeneration capacity during water deficit (Rivero et al. 2009). These results would suggest that the increased Fd levels during water deficit are not likely to be related to increased CET levels in the transgenic plants. The increase in Fd levels in PsaARC::IPT plants during water deficit cannot be explained within the frame of this study and requires further investigation.

In conclusion, during water deficit, WT plants showed activated ABA biosynthesis and ABA signaling pathways, a typical drought stress response. On the other hand, increased CK levels in the transgenic plants expressing PsaARC::IPT resulted in the activation of BR synthesis and BR signaling and in the repression of drought-induced ABA responses. Recent reports have indicated that treatment of plants with 24-epibrassinolide (an active BR) increased CO2 assimilation and quantum yield of PSII in Cucumis sativa (Xia et al. 2009), and BR pre-treatment protected PSII in Vicia faba treated with a herbicide (Pinol and Simon 2009). This notion is supported by the data presented here. Taken together, the transcriptome analyses suggest that the prevention of the degradation of the photosynthetic protein complexes, and the maintenance of photosynthesis during prolonged water deficit in the transgenic PsaARC::IPT plants resulted in the activation of BR-associated pathways. Whether the activation of BR-associated genes was directly affected by CKs or indirectly affected by the negative interaction between CKs and ABA is not clear and is presently under investigation.

Materials and Methods

Experimental design

Seeds of WT tobacco (N. tabacum ‘SR1’) and transgenic plants expressing PsaARC::IPT were sown in soil (Metro-Mix 200; Sun Gro) in a growth chamber (500 µmol photons m−2 s−1, 16 h photoperiod, 25°C) for 15 d until the emergence of the first two true leaves. Initially, five seeds per pot were germinated in the growth chamber. Fifteen days after germination, plants with two true leaves of similar size were chosen and one plant per pot was selected for experiments and continuously grown. During this period, no differences in germination rate and development between the WT and PsaARC::IPT were observed. Fifty plants of each genotype were transferred and transplanted (1 liter pots) to a greenhouse, where they were grown for 1 week to allow acclimation of the plants to the new conditions (1,000 µmol photons m−2 s−1, 16 h photoperiod, 28–30°C/23–25°C day/night). At this point, half of the WT plants and half of the PsaARC::IPT plants were selected to receive 1,000 ml of water per day (the amount of water necessary for tobacco plants to...
maintain cell turgor, designated as optimal watering conditions) (Rivero et al. 2007), whereas the other half of the plants received 300 ml of water per day (restricted watering regime). This amount of water was considered to induce a water deficit, because it produced a 50% yield reduction in the WT plants (Rivero et al. 2007). For the transcriptome analyses, plants were grown for 45 d under a restricted watering regime. For physiological measurements, we used plants that were grown for 70 d at 300 ml d$^{-1}$. No water was allowed to drain from the pots in any of the treatments. For microarray analysis, as well as for physiological and biochemical measurements, leaves at positions 7 and 8 (corresponding to leaves at the middle position) from WT and $P_{sark}::$IPT plants were harvested. The expression profile of IPT was taken into consideration for the selection of these particular leaf positions (see Rivero et al. 2007). On day 67, WT and transgenic plants did not show any sign of natural senescence and all the leaves collected at positions 7 and 8 were green. IPT expression profiles and CK contents of these leaves have been reported previously (Rivero et al. 2007, Rivero et al. 2009).

**RNA preparation and GeneChip hybridization**

RNA extraction, purification, labeling and hybridization to GeneChips were performed using standard protocols. Briefly, total RNA was isolated from leaf tissue using the TRIzol reagent. The RNA was cleaned by passing through an RNeasy spin column (Qiagen). To eliminate traces of DNA, the RNA was treated with DNase I (Fermentas Life Sciences) according to the manufacturer’s protocol. The quality of the RNA was assessed using the RNA Lab-On-A-Chip (Caliper Technologies Corp.) on an Agilent Bioanalyzer 2100 (Agilent Technologies). A 2 μg aliquot of total RNA was used for each sample. Further labeling and hybridization steps were performed as recommended by Affymetrix, Inc. (Affymetrix Genechip Expression Analysis Technical Manual). Each biological replicate was hybridized to an array to obtain a total of three replicates for each genotype and treatment.

**Real-time quantitative PCR**

cDNAs were obtained from two independent RNAs (2 μg of total RNA) using the SuperScript VILO cDNA synthesis kit (Invitrogen). The tissue materials were obtained from WT plants and $P_{sark}::$IPT plants growing under control (1,000 ml d$^{-1}$) and water deficit (300 ml d$^{-1}$) conditions. Every sample was represented by two independent cDNAs. The RNA used in the quantitative PCR assays was obtained from the same pool that was used for array hybridizations.

From each cDNA, three technical replicates were used, so that every sample was represented by six replicates in total. ABI Primer Express software was used for primer design. The genes assayed for expression and the primer sequence used are listed in Supplementary Table S10.

Two independent internal controls (18S rRNA and $Ubiquitin-Conjugated Protein2$ (UBQ2)) whose expression did not change across different samples were used. A total reaction volume of 20 μl was used. Reactions included 2 μl of template, 10 μl of Fast SYBR Green Master Mix, 0.9 μl of forward primer, and 0.9 μl of reverse primer. Amplification and data analysis were carried out using the following conditions: 95°C for 10 min followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. A melting curve analyses for all targets was carried out under the following conditions: 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. Amplification and data analysis were carried out using the ABI StepOne Plus real-time PCR system (Applied Biosystems) using internal controls 18S rRNA and/or UBQ2. The relative fold change was measured against the control WT samples.

**Thylakoid membrane isolation**

Intact chloroplasts were isolated from WT and $P_{sark}::$IPT tobacco plants. A 20 g sample of leaves (the same leaves were used for thylakoid membrane isolation and DNA microarrays) were cut into small pieces and homogenized in a blender (3 x 5 s) with 100 ml of grinding buffer containing 50 mM potassium phosphate (pH 7.5), 100 mM NaCl, 1 mM MgCl$_2$, 1 mM MnCl$_2$, 300 mM sorbitol, 10 mM potassium ascorbate, 2 mM EDTA and 50 mM HEPES buffer (pH 7.5). The resulting homogenate was filtered through Miracloth and the homogenate centrifuged at 200 x g for 2 min. The supernatant was then centrifuged at 5,000 x g for 10 min and the pellet resuspended in 15 ml of a wash buffer containing 50 mM potassium phosphate (pH 7.5), 50 mM NaCl, 1 mM MgCl$_2$, 1 mM MnCl$_2$, and 300 mM sorbitol. The suspension was centrifuged at 200 x g for 2 min and the supernatant centrifuged at 5,000 x g for 10 min. The pellet was resuspended in 1–2 ml of the same medium. Isolated chloroplasts were osmotically shocked by a 10-fold dilution with 50 mM potassium phosphate buffer (pH 7.5), 10 mM NaCl and 1 mM MgCl$_2$, and incubation in ice for 3 min. After centrifugation at 5,000 x g for 5 min the thylakoid pellet was washed with the same buffer and centrifuged again under the same conditions. The pellet was resuspended in 1–2 ml of washing buffer. The Chl concentration was calculated using 80% acetone as described by Arnon (1949). All procedures were carried out at 4°C and preparations were protected from light. The isolated thylakoid membranes were used for photosynthetic activity measurements or for Western blots within the next 2 h after isolation.

**Immunoblotting**

For Western blots, thylakoid membranes from frozen leaves (the same leaves were used for thylakoid membrane isolation and DNA microarrays) were used. Thylakoid membrane isolation from frozen tissue was achieved according to Rintamäki et al. (1996) with some modifications; frozen leaves were homogenized in a mortar with liquid nitrogen and ice-cold isolation buffer containing 50 mM HEPES-NaOH, pH 7.5, 300 mM sucrose, 5 mM MgCl$_2$, 1 mM Na-EDTA, 10 mM NaF and 1% (w/v) bovine serum albumin. The homogenates were filtered through Miracloth and centrifuged at 1,500 x g for 4 min. The pellets were washed with 10 mM HEPES-NaOH (pH 7.5),
Measurements of Chl fluorescence were performed on the eighth leaf of WT and transgenic plants by using a portable fluorometer (LI-6400-40; LICOR Biosciences). The maximal photochemical efficiency of PSII was estimated by the fluorescence ratio \( F_v/F_m \) calculated from \( F_v \) (basal fluorescence) and \( F_m \) (maximal fluorescence), with \( F_v \) being the variable fluorescence. The intrinsic efficiency of open PSII reaction centers \( (F_v/F_m)_{sat} \) ratio was calculated by measuring the same parameters as above, but under natural irradiance \( (F_v') \) and \( (F_m') \). After a saturating flash of 0.8 s and the determination of \( F_m'\) and \( F_v'\) (steady-state fluorescence), a black cloth was placed over the leaf for 20 min and a 3 s far-red pulse was applied to oxidize PSII fully during \( F_v'\) measurement. The photochemical efficiency of PSII in light-adapted leaves \( (\Psi_{PSII}) \) was calculated by the \( F_v'-F_o'/F_m'=\Delta F/F_m' \) ratio (Genty et al. 1989). The \( q_P \) which was used as an estimate of the fraction of open reaction centers, was calculated as the ratio: \( q_P=1−(F_o−F_o')/(F_m−F_o') \) (Bilger and Schreiber 1986). The thermal energy dissipation or NPQ at the PSI level was estimated by using the Stern–Volmer equation: \( NPQ=(F_m/F_m')−1 \) (Cornic 1994). The ETR was estimated as described by Krall and Edwards (1992): \( \Delta F/F_m'=\text{ETR}×0.5×0.84 \). We used the common \( C_3 \) leaf absorbance value of 0.84 ( Bjorkman and Demmig 1987 ).

Measurement of PSI activity
Photosynthetic oxygen uptake rates were determined using the photochemical efficiency of PSII was estimated by the fluorescence ratio \( F_v/F_m \) calculated from \( F_v \) (basal fluorescence) and \( F_m \) (maximal fluorescence), with \( F_v \) being the variable fluorescence. The intrinsic efficiency of open PSII reaction centers \( (F_v/F_m)_{sat} \) ratio was calculated by measuring the same parameters as above, but under natural irradiance \( (F_v') \) and \( (F_m') \). After a saturating flash of 0.8 s and the determination of \( F_m'\) and \( F_v'\) (steady-state fluorescence), a black cloth was placed over the leaf for 20 min and a 3 s far-red pulse was applied to oxidize PSII fully during \( F_v'\) measurement. The photochemical efficiency of PSII in light-adapted leaves \( (\Psi_{PSII}) \) was calculated by the \( F_v'-F_o'/F_m'=\Delta F/F_m' \) ratio (Genty et al. 1989). The \( q_P \) which was used as an estimate of the fraction of open reaction centers, was calculated as the ratio: \( q_P=1−(F_o−F_o')/(F_m−F_o') \) (Bilger and Schreiber 1986). The thermal energy dissipation or NPQ at the PSI level was estimated by using the Stern–Volmer equation: \( NPQ=(F_m/F_m')−1 \) (Cornic 1994). The ETR was estimated as described by Krall and Edwards (1992): \( \Delta F/F_m'=\text{ETR}×0.5×0.84 \). We used the common \( C_3 \) leaf absorbance value of 0.84 ( Bjorkman and Demmig 1987 ).

Statistical analysis
Scanned GeneChip images were examined for visual aberrations. All the images obtained were of high quality. Further pre-processing and analysis was performed using the CEL files. The CEL files were imported into RMA (Irizarry et al. 2003) for further processing. The background adjustment and quantile normalization were performed using the default settings. The log-transformed values from RMA were imported into SAM software ( Tusher et al. 2001 ). To perform differential expression analysis we used the two-class unpaired selection analysis method to determine genes differentially regulated. The \( FDR \) was set at 0.001. Pearson’s correlation was used for placing neighboring probe sets.

Data availability
All microarray data from this work are available from NCBI GEO (www.ncbi.nlm.nih.gov/geo) under the series entry GSE19787.

Supplementary data
Supplementary data are available at PCP online.

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References


