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Analysis of Cytokinin Mutants and Regulation of Cytokinin Metabolic Genes Reveals Important Regulatory Roles of Cytokinins in Drought, Salt and Abscisic Acid Responses, and Abscisic Acid Biosynthesis [C][W]

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Cytokinins (CKs) regulate plant growth and development via a complex network of CK signaling. Here, we perform functional analyses with CK-deficient plants to provide direct evidence that CKs negatively regulate salt and drought stress signaling. All CK-deficient plants with reduced levels of various CKs exhibited a strong stress-tolerant phenotype that was associated with increased cell membrane integrity and abscisic acid (ABA) hypersensitivity rather than stomatal density and ABA-mediated stomatal closure. Expression of the *Arabidopsis thaliana ISOPENTENYL-TRANSFERASE* genes involved in the biosynthesis of bioactive CKs and the majority of the *Arabidopsis CYTOKININ OXIDASES/DEHYDROGENASES* genes was repressed by stress and ABA treatments, leading to a decrease in biologically active CK contents. These results demonstrate a novel mechanism for survival under abiotic stress conditions via the homeostatic regulation of steady state CK levels. Additionally, under normal conditions, although CK deficiency increased the sensitivity of plants to exogenous ABA, it caused a downregulation of key ABA biosynthetic genes, leading to a significant reduction in endogenous ABA levels in CK-deficient plants relative to the wild type. Taken together, this study provides direct evidence that mutual regulation mechanisms exist between the CK and ABA metabolism and signals underlying different processes regulating plant adaptation to stressors as well as plant growth and development.

INTRODUCTION

Water deficit and high-salinity stress limit plant productivity worldwide. Plants have developed elaborate and sensitive protection systems that enable them to rapidly signal, respond, and properly adapt to various stresses, including drought and high salinity (Yamaguchi-Shinozaki and Shinozaki, 2006; Tran et al., 2007a, 2007b). Phosphorylation, which is catalyzed by protein kinases, is one of the key mechanisms for intracellular signal transduction in both eukaryotic and prokaryotic cells. Typically, His kinases sense extracellular stimuli by autophosphorylation and transfer a phosphoryl group to the response regulator, resulting in the activation of downstream proteins that elicit a specific response (Mizuno, 2005; Schaller et al., 2008).

Various phytohormones regulate the protective responses in plants to abiotic and biotic stresses. Increasing evidence suggests that cytokinins (CKs) are involved in stress responses (Tran et al., 2007b; Havlová et al., 2008; Argueso et al., 2009). CKs have been recognized as an important signal that travels from roots to shoots (Letham, 1994). Recent data implied that abscisic acid (ABA):CK ratios in xylem sap are important for stress signaling (Alvarez et al., 2008; Schachtman and Goodger, 2008). Generally, stresses, such as drought, decrease the production and transport of CKs from roots. Additionally, application of exogenous CKs can increase stomatal apertures and transpiration of many plants (Davies and Zhang, 1991; Pospisilova and Batkova, 2004; Pospisilova et al., 2005). In plants such as *Arabidopsis thaliana*, the key enzymes involved in CK metabolism are adenosine phosphate-isopentenyltransferases (IPTs) and CK oxidases/dehydrogenases (CKXs) (Hirose et al., 2008; Werner and Schmülling, 2009). *Arabidopsis* plants have two classes of IPTs

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acting on the adenine moiety, with seven genes for ATP/ADP IPTs (*IPT1*, *IPT3*, *IPT4*, *IPT5*, *IPT6*, *IPT7*, and *IPT8*) and two genes for transfer RNA IPTs (*IPT2* and *IPT9*). Functional analysis of various *ipt* mutants indicated that ATP/ADP IPTs are responsible for the synthesis of isopentenyladenine (iP)- and *trans*-zeatin (*t*Z)-type CKs, whereas transfer RNA IPTs are required for the biosynthesis of *cis*-zeatin (*c*Z)-type CKs (Miyawaki et al., 2006). The iP- and *t*Z-type CKs are the major forms and are more physiologically active than *c*Z-type CKs in many plant species, including *Arabidopsis*; however, in some other species, predominantly monocotyledonous ones, *c*Z is an abundant and biologically active CK (Sakakibara, 2006). Formation of *t*Z by the hydroxylation of iP requires cytochrome P450 monooxygenases (CYP735A1 and CYP735A2) (Takei et al., 2004). Irreversible CK degradation is catalyzed by CKX, which selectively cleaves unsaturated isoprenoid side chains, resulting in the formation of adenine/adenosine and the corresponding side chain aldehyde (Sakakibara, 2006; Werner et al., 2006). Therefore, CKX enzymes are important players in regulating CK concentrations and thereby influence plant growth and development. In *Arabidopsis*, the CKXs are encoded by a family of seven genes (*CKX1* to *CKX7*). It is hypothesized that CKX1, CKX3, and CKX5 proteins share similar biochemical characteristics because overproduction of these three enzymes in *Arabidopsis* leads to similar phenotypes compared with overproduction of other CKX members (Werner et al., 2003). Studies on the substrate specificity of the CKXs suggested that CKX2 and CKX4 possess the highest enzymatic activity with iP, *t*Z, and their ribosides. In comparison, turnover rates of *Arabidopsis* CKX1, CKX3, CKX5, and CKX7 in tobacco (*Nicotiana tabacum*) plants are substantially lower, and activity of *Arabidopsis* CKX6 is almost undetectable (Galuszka et al., 2007).

In *Arabidopsis*, CK signaling is mediated by a multistep phosphorelay, which is comprised of sensor histidine kinases (AHKs), histidine phosphotransfer proteins (AHPs), and response regulators (ARRs) (To and Kieber, 2008). Functional analysis of *ahk2*, *ahk3*, and *ahk4* single, double, and triple mutants suggests that AHK2, AHK3, and AHK4/CRE1 function as CK receptor AHKs and act as positive regulators in CK signaling and plant growth (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006). Increasing evidence indicates that CK signaling is involved in response to environmental stimuli and ABA signaling (Tran et al., 2007b; Jeon et al., 2010). In *Arabidopsis*, alterations of external osmolarity induce changes in the transcript levels of CK-responsive *AHK2*, *AHK3*, and *AHK4*, suggesting a functional importance of these AHKs for the efficient sensing of environmental signals. The altered expression of *AHK* receptor genes may have important effects on receptor output following exposure to CK (Tran et al., 2007b, 2010). In planta studies have subsequently demonstrated that AHK2, AHK3, and AHK4 function as negative regulators in ABA signaling and osmotic stress signaling in both abscisic acid (ABA)-dependent and ABA-independent pathways (Tran et al., 2007b). It is important to note that AHK4 exhibits a dual function that is dependent upon the presence or absence of CKs. Specifically, AHK4 phosphorylates AHPs in the presence of CKs, whereas it removes phosphate from AHPs in the absence of CKs (Mähönen et al., 2006). Therefore, AHK4 has been shown to require CKs to function as a negative regulator in stress responses. As a result, this requirement of CKs for AHK4 function provides direct evidence for the involvement of CK in mediating stress responses (Tran et al., 2007b). These aforementioned studies have provided strong lines of evidence highlighting the existence of crosstalk among CK, ABA, and stress signaling pathways. It is becoming evident that CKs play a crucial regulatory role not only in plant growth and development but also in ABA and stress signaling via CK-mediated signaling (Tran et al., 2007b, 2010).

It has been postulated that CK and ABA exert antagonistic activities during a number of growth and physiological processes, including plant adaptation to environmental stresses (Zdunek and Lips, 2001; Chang et al., 2003; Chow and McCourt, 2004; Werner et al., 2006). When plants are exposed to stress, the accumulation of ABA helps plants to avoid stress by several different mechanisms. Specifically, the accumulation of ABA promotes stomatal closure to minimize water loss, accelerates leaf senescence, downregulates plant growth, and induces the biosynthesis of protective substances (e.g., late embryogenesis-abundant proteins). On the other hand, CKs trigger responses to delay both stomatal closure and leaf senescence (Finkelstein et al., 2002; Hansen and Dörffling, 2003; Pospisilova, 2003; Chow and McCourt, 2004; Pospisilova and Batkova, 2004; Pospisilova et al., 2005). Many studies have focused on the molecular functions of ABA in relation to abiotic stress and vice versa and the influence of environmental stresses on ABA metabolism (Bartels and Sunkar, 2005; Hirayama and Shinozaki, 2010). However, similar studies are lacking for CKs. Therefore, we designed a series of experiments to investigate the relationship of CK metabolism, environmental stresses, and ABA biosynthesis. We used both gain- and loss-of-function studies to investigate the consequence of CK deficiency on stress responses as well as on ABA biosynthesis and ABA response. We performed functional and expression analyses of genes encoding the key CK biosynthetic IPT and the degradative CKX enzymes to elucidate the effect of CKs on plant stress responses and the effect of stresses on CK metabolism. Collectively, these studies have contributed to a better understanding of these aforementioned impacts and have also provided insight into the crosstalk among different hormone pathways that regulate plant adaptation to adverse conditions.

RESULTS

Concentration of CKs in *35S:CKX3* and *35S:CKX4* Plants

It is well known that CKX enzymes catalyze CK breakdown, and overexpression of *Arabidopsis CKX* genes results in the reduction of endogenous CK contents (Werner et al., 2001, 2003). Therefore, transgenic plants individually overexpressing *Arabidopsis CKX1, CKX2, CKX3*, and *CKX4* were selected for evaluation of the consequences of decreased CK content on the adaptation of plants to stresses (see Supplemental Figure 1A online). Although all four of the *CKX* overexpressers showed retarded shoot growth, but enhanced root development in comparison with the wild type, they represented two different developmental phenotypes. Overexpression of *CKX1* and *CKX3* resulted in stronger developmental changes than that of *CKX2*

and *CKX4* (Werner et al., 2003). Total CK content and concentration of some individual CKs, showing significant reduction relative to wild type, were previously reported for *35S*:*CKX1* and *35S*:*CKX2* plants, but not yet for *35S*:*CKX3* and *35S*:*CKX4* (Werner et al., 2003). Thus, before the evaluation of stress tolerance, we determined the concentrations of all CKs in *35S*:*CKX3* and *35S*:*CKX4* plants. For comparison, we also included the *35S*:*CKX1* and *35S*:*CKX2* into the quantification, which would eventually enable us to explain the difference in the shoot growth phenotype observed between two different groups of overexpression plant lines: *35S*:*CKX1* and *35S*:*CKX3* and *35S*:*CKX2* and *35S*:*CKX4*. Data shown in Table 1 indicated that the *35S*:*CKX1* and *35S*:*CKX3* group displayed a similar CK profile, as did the *35S*:*CKX2* and *35S*:*CKX4* group of plants. These similarities in their CK profiles were in good accordance with the consistency of their respective phenotypic traits. With respect to the concentration of CKs, the levels of the bioactive iP- and *t*Z-type CKs were reduced in both groups of *CKX* overexpressers, with a higher degree of reduction observed in *35S*:*CKX1* and *35S*:*CKX3* than in *35S*:*CKX2* and *35S*:*CKX4* plants (Table 1). This difference may explain the more severe shoot growth retardation of the *35S*:*CKX1* and *35S*:*CKX3* plants in comparison with that of the *35S*:*CKX2* and *35S*:*CKX4* plants.

The *Arabidopsis* CK-Deficient *CKX* Overexpressers Display an Enhanced Salt- and Drought-Tolerant Phenotype

Next, we examined stress tolerance of the four CK-deficient *CKX* overexpressers. To carry out the salt tolerance test, 10-d-old wild-type and *CKX* plants were transferred onto agar plates containing 200 mM NaCl and maintained for a period of 6 d. All of the *CKX* overexpressers exhibited a salt stress tolerant phenotype with significantly enhanced survival rates (Figures 1A to 1C). The highest elevation of stress tolerance was observed in the case of *CKX1* plants, which exhibited the slowest shoot growth rate among all of the *CKX* overexpressers. For a drought tolerance study, 2-week-old plants grown on germination medium (GM) agar plates were transferred to soil and grown for 1 additional week, and water was subsequently withheld. The "same-tray" method was used to ensure a valid comparison of genotypes with different growth rates (Verslues et al., 2006). After this long-term drought stress treatment, which spanned flowering and silique ripening stages, the plants were rewatered and allowed to grow for an additional period of 3 d. A remarkably high number of *CKX* overexpresser plants was found to survive the exposure to drought in comparison with the wild type (Figures 1D to 1F). For comparison, plants were also grown in well-watered conditions in parallel with the long-term drought stress test (see

Table 1. Concentration of CK Metabolites in *CKX* Overexpresser and *ipt1 3 5 7* Plants

	Wild Type		*35S:CKX1*		*35S:CKX3*		*35S:CKX2*		*35S:CKX4*		*ipt1 3 5 7*	
Hormones	Average	SE	Average	SE	Average	SE	Average	SE	Average	SE	Average	SE
*t*Z	1.69	0.31	N.D.		0.19	0.01	N.D.		0.52	0.01	0.27	0.02
*t*ZR	1.98	0.02	0.16	0.00	1.14	0.25	0.69	0.02	0.88	0.04	0.31	0.02
*t*ZRPs	19.97	0.49	0.22	0.01	2.31	0.40	4.58	0.18	8.85	0.29	1.89	0.06
*t*Z7G	23.44	0.96	N.D.		0.63	0.04	1.71	0.05	7.02	0.18	1.78	0.07
*t*Z9G	22.47	0.68	N.D.		1.14	0.03	1.33	0.18	8.55	0.39	0.80	0.07
*t*ZOG	4.72	0.17	N.D.		N.D.		N.D.		N.D.		N.D.	
*t*ZROG	1.08	0.05	0.23	0.02	0.24	0.03	0.29	0.06	0.46	0.03	0.22	0.01
*t*ZRPsOG	0.10	0.01	N.D.		N.D.		N.D.		N.D.		N.D.	
iP	0.79	0.06	N.D.		0.25	0.04	0.33	0.04	0.55	0.08	0.26	0.01
iPR	0.13	0.01	0.08	0.00	0.25	0.02	0.13	0.01	0.11	0.00	0.02	0.00
iPRPs	17.30	0.39	9.77	0.37	10.99	0.37	30.35	1.91	20.09	0.42	3.64	0.12
iP7G	40.71	1.03	1.05	0.04	3.91	0.22	8.79	0.18	21.17	0.44	12.23	0.04
iP9G	3.07	0.07	N.D.		0.22	0.02	0.59	0.01	1.43	0.07	0.93	0.02
*c*Z	0.25	0.03	N.D.		N.D.		N.D.		0.29	0.00	0.49	0.08
*c*ZR	1.25	0.01	0.76	0.03	0.97	0.02	1.24	0.02	1.13	0.03	1.40	0.08
*c*ZRPs	5.96	0.15	0.52	0.04	1.64	0.08	7.36	0.23	5.54	0.25	6.24	0.16
*c*ZOG	N.D.		N.D.		N.D.		N.D.		N.D.		2.14	0.14
*c*ZROG	3.20	0.22	0.75	0.01	1.11	0.02	3.10	0.06	3.20	0.12	2.32	0.06
*c*ZRPsOG	0.09	0.01	N.D.		0.05	0.02	0.08	0.01	0.08	0.01	0.06	0.01
DZ	N.D.		N.D.		N.D.		N.D.		N.D.		N.D.	
DZR	N.D.		N.D.		N.D.		N.D.		N.D.		N.D.	
DZRPs	N.D.		0.29	0.01	0.79	0.10	0.43	0.01	N.D.		N.D.	
DZ9G	0.19	0.01	0.16	0.00	0.29	0.02	0.26	0.02	0.35	0.04	N.D.	

One hundred milligrams of *Arabidopsis* seedlings (10 d old) per sample were pooled, and three independent biological samples were taken for each genotype.

Data shown are pmol/g fresh weight (n = 3). *t*ZR, *t*Z riboside; *t*ZRPs, *t*ZR 5′-phosphates; *c*ZR, *c*Z riboside; *c*ZRPs, *c*ZR 5′-phosphates; DZ, dihydrozeatin; DZR, DZ riboside; DZRPs, DZR 5′-phosphates; iP, N^6-(Δ^2-isopentenyl) adenine; iPR, iP riboside; iPRPs, iPR 5′-phosphates; *t*Z7G, *t*Z-7-*N*-glucoside; *t*Z9G, *t*Z-9-*N*-glucoside; *t*ZOG, *t*Z-*O*-glucoside; *c*ZOG, *c*Z-*O*-glucoside; *t*ZROG, *t*ZR-*O*-glucoside; *c*ZROG, *c*ZR-*O*-glucoside; *t*ZRPsOG, *t*ZR 5′-phosphate-*O*-glucoside; *c*ZRPsOG, *c*ZR 5′-phosphate-*O*-glucoside; DZ9G, DZ-9-*N*-glucoside; iP7G, iP-7-*N*-glucoside; iP9G, iP-9-*N*-glucoside; N.D., not detected.

Supplemental Figure 1B, Supplemental Data Set 1 online). Taken together, our results indicate that *CKX* overexpresser plants are better suited to survive under both salt and drought stresses, suggesting that a reduction in CK contents significantly improves tolerance to both stressors. Therefore, CKs appear to play an integral role in the regulation of tolerance to abiotic stresses by acting as negative regulators in stress response.

The *Arabidopsis* CK-Deficient *ipt1 3 5 7* Mutant Possesses Enhanced Salt and Drought Tolerance

CK metabolism is regulated not only by *CKX* but also by *IPT* genes (Takei et al., 2001; Miyawaki et al., 2004, 2006; Werner and Schmülling, 2009). In the *ipt1 3 5 7* mutant, levels of *t*Z riboside 5′-phosphates, *t*Z riboside, iP riboside 5′-phosphates, iP riboside, *t*Z-7-*N*-glucoside, *t*Z-9-*N*-glucoside, and *t*Z-*O*-glucoside were all reduced to <20%, and those of the free-base iP and *t*Z were decreased to less than 50% of the wild-type values, whereas the concentration of *c*Z, *c*ZR, and *c*ZRPs was slightly increased (Miyawaki et al., 2006) (Table 1). To confirm whether the improved salt and drought tolerances of the CK-deficient plants were the result of reduced endogenous CK contents, especially of the physiologically active iP and *tZ* and their corresponding ribosides, the survival rate of the *ipt1 3 5 7* mutant was compared with that of the wild type under stress conditions. Our results indicated that under salt stress, similar to the *CKX* overexpressers, the survival of *ipt1 3 5 7* plants at various concentrations of NaCl was significantly greater relative to wild-type plants (Figures 2A to 2C; see Supplemental Figure 2A online). The enhanced salt tolerance of the CK-deficient plants

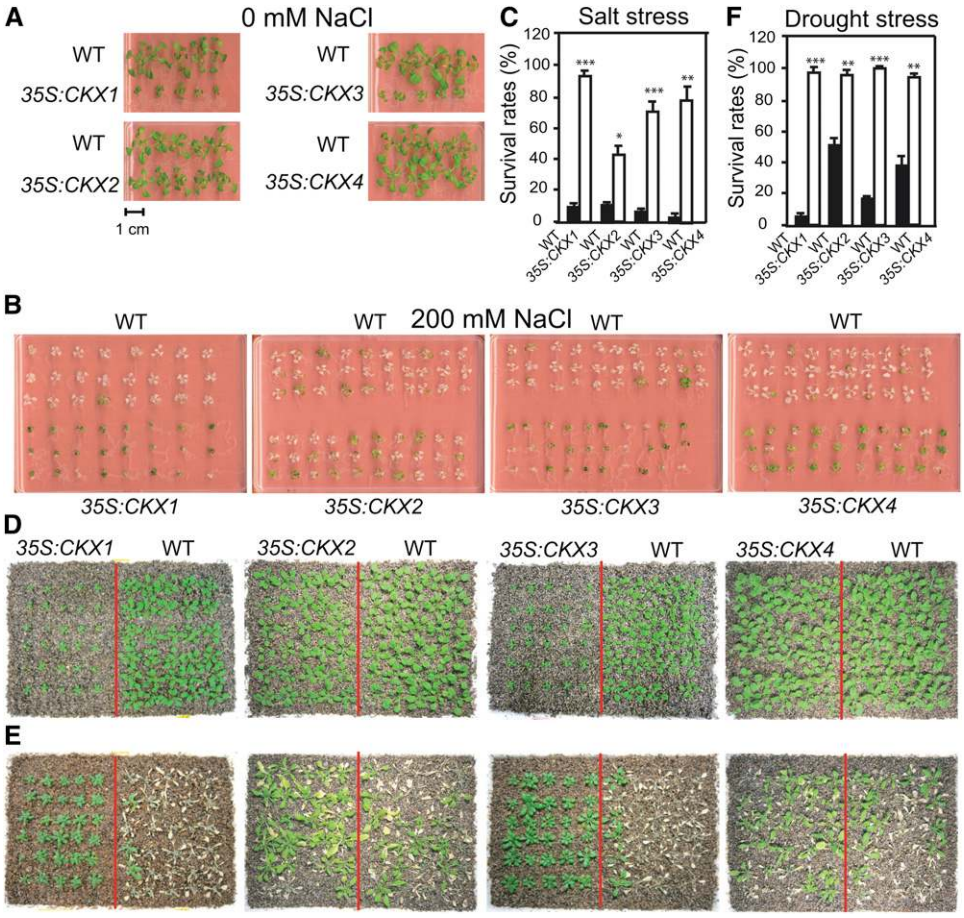

Figure 1. Salt- and Drought-Tolerant Phenotypes of the CK-Deficient *CKX* Overexpresser Plants.

(A) Plants were grown on GM plates for 10 d and were transferred on 0.5× MS/0 mM NaCl medium for 6 d. WT, wild type.
(B) Plants were grown on GM plates for 10 d and were transferred on 0.5× MS/200 mM NaCl medium for 6 d.
(C) Survival rates and SE values (error bars) were calculated from the results of three independent experiments (n = 30 plants/genotype).
(D) Two-week-old wild-type and *CKX* overexpresser plants were transferred from GM plates to soil and grown for 1 additional week.
(E) Three-week-old plants were exposed to drought stress for 14 d and plants were photographed 3 d subsequent to rewatering and after removal of inflorescences.
(F) Survival rates and SE values (error bars) were calculated from the results of three independent experiments (n = 30 plants/genotype). Asterisks indicate significantly higher survival rates than the wild type as determined by Student's t test (* $P < 0.01$; ** $P < 0.005$; *** $P < 0.001$).

was also reflected in a root growth assay. The dose–response experiment shown in Supplemental Figure 2B online demonstrated that the growth of the primary roots of the CK-deficient plants was more resistant to the saline environments than was that of the wild-type plants.

As for the evaluation of drought stress tolerance, the *ipt1 3 5 7* plants were subjected to a long-term drought stress treatment similar to the treatment applied to the *CKX* overexpressers. In comparison with wild-type plants, the *ipt1 3 5 7* mutant showed enhanced survival under drought stress (Figures 2C to 2E). Because of retardations in plant growth, a significant difference in the plant sizes of wild type, *35S*:*CKX1*, *35S*:*CKX3*, and *ipt1 3 5 7* was observed (Figures 1D and 2D). To determine whether the difference in plant growth rates influenced water usage and affected the analysis of drought tolerance for the dwarf CK-deficient plants, we first quantified soil moisture levels on both sides of the tray used for the drought tolerance test of the dwarf *ipt1 3 5 7* and wild-type plants (Figures 2D and 2E). Our data demonstrated that although there were significant differences in plant sizes, only minimal differences in soil moisture content were detected between the wild-type (right) and *ipt1 3 5 7* (left) sides of the trays (Figure 2F). These observations confirmed that the same-tray method exposed both genotypes to the same water availability, even if one genotype might use water more quickly than the other (Verslues et al., 2006). Second, we employed an even more stringent approach for using the same-tray method for evaluating the drought tolerance of the *ipt1 3 5 7* mutant. In this method, *ipt1 3 5 7* and wild-type plants were grown in an alternate order as shown in Supplemental Figure 3A online and subjected to long-term drought stress. Under this condition, the wild-type plants exhibited severely wilted or dead leaves, whereas the *ipt1 3 5 7* plants remained turgid (see Supplemental Figure 3B online). Under well-watered conditions, both the mutant and wild-type plants grew normally and exhibited their typical phenotypes (see Supplemental Figure 3C online). Collectively, the loss-of-function studies of the *ipt1 3 5 7* mutant strengthened the results of the studies of *35S*:*CKX* overexpressers. Taken together, our data support a negative regulatory role for the bioactive CKs in stress tolerance.

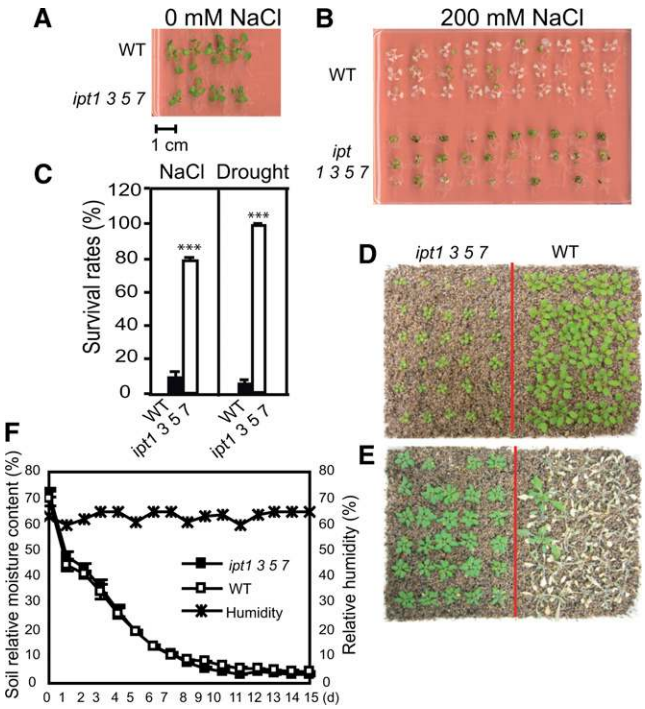

Figure 2. Salt- and Drought-Tolerant Phenotypes of the CK-Deficient *ipt1 3 5 7* Mutant.

(A) Plants were grown on GM plates for 10 d and were transferred to 0.5× MS/0 mM NaCl medium for 6 d. WT, wild type.

(B) Plants were grown on GM plates for 10 d and were transferred to 0.5× MS/200 mM NaCl medium for 6 d.

(C) Survival rates and SE values (error bars) were calculated from the results of three independent experiments (n = 30 plants/genotype). Asterisks indicate significantly higher survival rates than wild type as determined by Student's t test (*** $P < 0.001$).

(D) Two-week-old wild-type and *ipt1 3 5 7* plants were transferred from GM plates to soil and grown for 1 additional week.

(E) Three-week-old plants were exposed to drought stress for 14 d and plants were photographed 3 d subsequent to rewatering and after removal of inflorescences.

(F) Soil relative moisture contents were monitored during the drought tolerance test of the *ipt1 3 5 7* mutant.

Stress Tolerance Mediated by Downregulation of CKs Is Associated with Maintenance of Cell Membrane Integrity

We next sought to explore the mechanisms by which downregulation of CKs functions to mediate drought stress tolerance. We found that under drought stress, reduction of CK levels enhanced survival rates of the CK-deficient plants by minimizing water loss (Figure 3A). To assess whether altered transpiration rates, which are regulated by ABA-mediated stomatal closure, contribute to the lower water loss rates in the CK-deficient plants, we analyzed stomatal movement in these plants. In comparison with the wild type, the CK-deficient plants did not exhibit smaller stomatal apertures when plants were treated with or without exogenous ABA applications (Figure 3B). Another decisive factor affecting transpiration rates is stomatal density. However, we did not observe any significant differences in stomatal density between the wild-type and CK-deficient plants as well (Figure 3C). Collectively, these data suggest that the drought-tolerant phenotype of the CK-deficient plants is not associated with modulated stomatal density or with ABA-regulated stomatal closure. Reduction of CKs may result in plant tissues that are enhanced in sensing and responding to general water deficit.

It is generally accepted that the maintenance of cell membrane integrity and stability under water deficit is a major component of drought tolerance in plants. The degree of cell membrane injury induced by drought stress can be quantified by measuring intracellular electrolyte leakage (Bajji et al., 2001; Verslues et al., 2006). To characterize differences in drought tolerance between the wild-type and CK-deficient plants, we measured electrolyte leakage in the wild-type and CK-deficient plants after exposure to drought stress. Data shown in Figure 3D indicated that the CK-deficient plants exhibited significantly lower electrolyte leakage than wild type after 12 d of water deficit. Taken

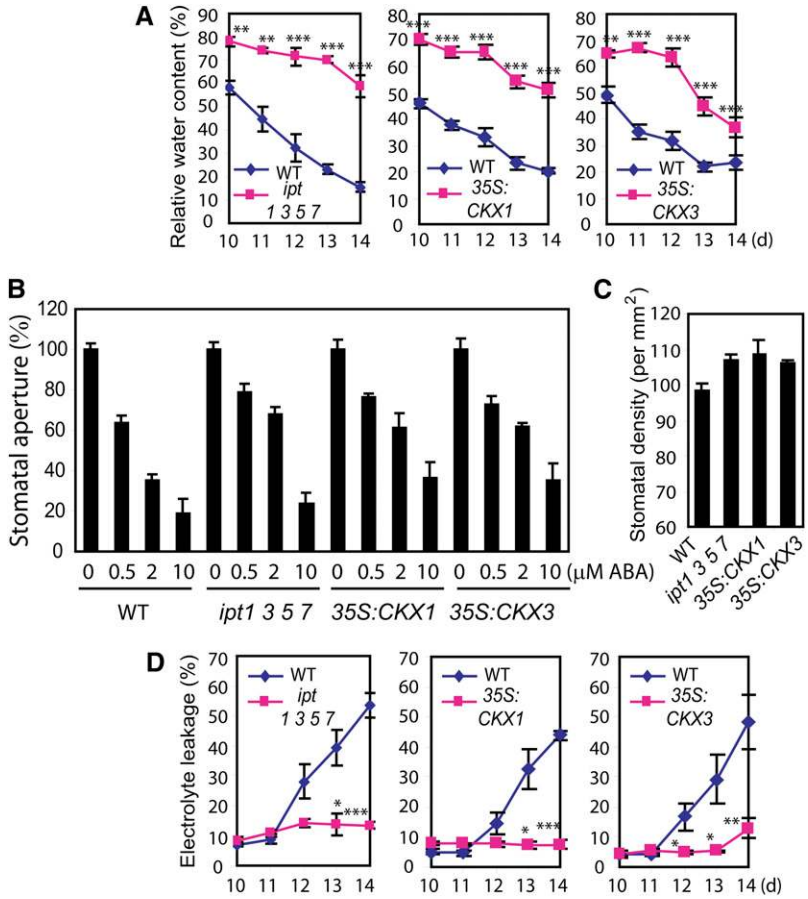

Figure 3. RWC, Electrolyte Leakage, Stomatal Aperture, and Stomatal Density of the CK-Deficient Plants.

(A) The wild type (WT), *ipt1 3 5 7*, *35S:CKX1*, and *35S:CKX3* plants were grown and exposed to drought stress as described in Figures 2D and 2E. At the indicated time points, plants were harvested for measurement of RWC. Error bars = SE values (n = 5).

(B) Average stomatal aperture of rosette leaves from 4-week-old wild-type and CK-deficient plants in the presence or absence of ABA. Error bars = SE values (n > 17).

(C) Average stomatal density of rosette leaves from 4-week-old wild-type and CK-deficient plants. Error bars = SE values (n > 3).

(**D**) The wild-type and CK-deficient plants were grown and exposed to drought stress as described in Figures 2D and 2E. At the indicated time points, plants were harvested for measurement of relative electrolyte leakage. Error bars = SE values (n = 5). Asterisks indicate significant differences as determined by a Student's t test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

[See online article for color version of this figure.]

together, the enhanced stress tolerance of the CK-deficient plants can be attributed to increased cell membrane integrity and stability rather than to differences in stomatal density and ABA-mediated stomatal closure.

ABA Biosynthesis in CK-Deficient Plants in Well-Watered Conditions

A number of studies postulated that CKs and ABA exert antagonistic activities during several developmental and physiological processes, including the regulation of plant growth, leaf senescence, and stomatal closure (Gan and Amasino, 1995; Cowan et al., 1999; Zdunek and Lips, 2001; Chang et al., 2003; Chow and McCourt, 2004). In line with these findings, we performed direct measurements of ABA levels in CK-deficient plants and quantitative measurements of transcript levels of the key ABA biosynthetic genes to determine whether a reciprocal relationship exists between ABA and CK contents. Data shown in Figure 4A indicated that ABA levels in all of the CK-deficient plants, either the *ipt1 3 5 7* mutant or *CKX* overexpressers, significantly decreased in comparison with wild type. These data suggest that a decrease in CK content does not necessarily lead to an increase in ABA content.

As a means to identify how CK deficiency affects ABA biosynthesis, we investigated the expression of several key ABA biosynthetic genes, such as *ABA1* (zeaxanthin epoxidase), *ABA2* (xanthoxin dehydrogenase), aldehyde oxidase 3 (*AAO3*), and 9-cis-epoxycarotenoid dioxygenase (NCED3), which regulate the key steps of ABA biosynthesis (Nambara and Marion-Poll, 2005; Barrero et al., 2006). The result of quantitative RT (qRT)-PCR

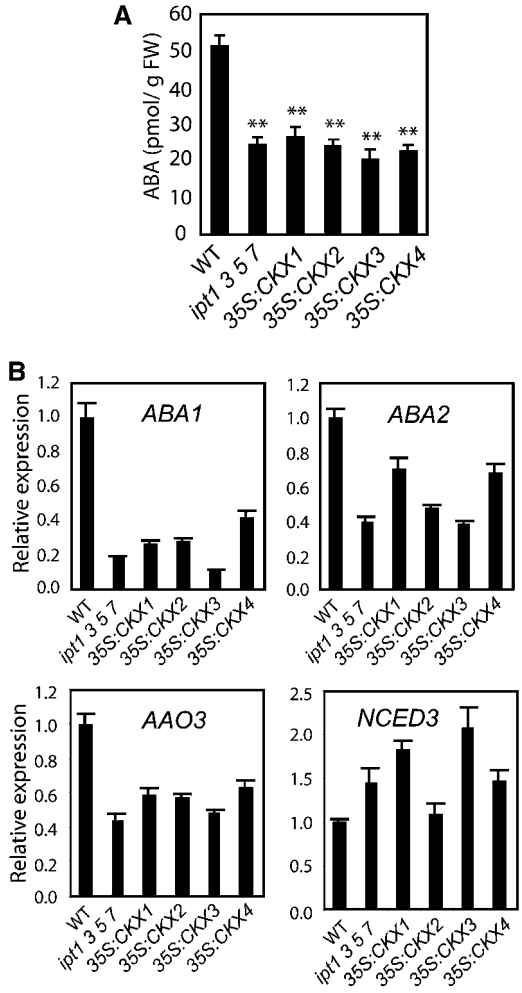

Figure 4. Altered ABA Biosynthesis in CK-Deficient Plants.

(A) Average levels of ABA in 10-d-old plants grown on GM plates. Error bars = SE values of three biological replications. Asterisks indicate significant differences as determined by a Student's *t* test (** $P < 0.01$). FW, fresh weight; WT, wild type.

(B) Expression of ABA biosynthetic genes *ABA1*, *ABA2*, *AAO3*, and *NCED3* in 10-d-old wild-type and CK-deficient plants grown on GM plates. Mean relative expression levels were normalized to a value of 1 in wild-type plants. Error bars = SE values of three biological replicates.

analysis indicated that the mRNA levels of the examined ABA biosynthetic genes, with the exception of *NCED3*, were well correlated with hormone levels among all CK-deficient plants (Figure 4B). These data suggest that the reduction in ABA levels in CK-deficient plants was the consequence of decreased expression of *ABA1*, *ABA2*, and *AAO3* rather than reduced expression of *NCED3*.

Decreased CK Content Increases ABA Sensitivity and ABA Response

We have shown that a reduction of the CK content results in a lower ABA content. Interestingly, it has been reported that CK-responsive His kinase mutants displayed hypersensitivity to exogenous ABA (Tran et al., 2007b; Jeon et al., 2010). Therefore, we determined whether CK-deficient plants exhibit an altered ABA response. To address this question, we subjected the *ipt1 3 5 7* mutant and *CKX* overexpressers to both growth inhibition and germination assays to assess their ABA sensitivity in the absence or presence of ABA. As shown in Figures 5A and 5B and Supplemental Figure 4 online, all of the examined CK-deficient plants were more sensitive to exogenous ABA than the wild type. Because the endogenous ABA levels in all of the examined CK-deficient plants were reduced to approximately one-half of the wild-type levels under normal conditions (Figure 4A), the CK-deficient plants might possess an improved ability to sense and/or respond to extracellular ABA as a means to compensate for the reduction of endogenous ABA levels. This hypothesis was further supported by comparison of the expression levels of several well-known ABA-responsive marker genes, as well as the ABA contents in wild-type and CK-deficient plants under stress conditions. We found that although the induced ABA levels in the CK-deficient plants in most cases did not exceed wild-type levels under drought and salt stresses (Figure 5C), the expression of the ABA-responsive marker genes examined was more strongly induced in CK-deficient plants than in wild-type plants under the same stress conditions (Figure 5D). It is also noteworthy that the degrees of ABA induction were higher in the CK-deficient plants than in wild-type plants (Figure 5C).

Expression of *IPT* and *CKX* Genes under Dehydration and Salt Stresses

As a next step to study the relationship between CK metabolism and stress response, we examined the expression profiles of all *Arabidopsis IPT* genes, which are involved in the synthesis of the bioactive *tZ*- and iP-type CKs (*IPT1*, *IPT3*, *IPT4*, *IPT5*, *IPT6*, *IPT7*, and *IPT8*), and all *Arabidopsis CKX* genes (*CKX1-7*) in the wild-type plants under dehydration and salt stress conditions to determine how stresses influence the CK metabolism at the transcriptional level. Expression of *IPT4*, *IPT6*, and *IPT8* was undetectable in vegetative tissues as previously reported (Miyawaki et al., 2006). Results of qRT-PCR demonstrated that expression of the examined *IPT* and *CKX* genes was influenced by NaCl, dehydration, and ABA treatments (Figure 6). With the exception of *IPT5*, which was slightly induced after 10 h of dehydration, all of the remaining *IPT* genes were repressed in the 2-week-old *Arabidopsis* seedlings during dehydration treatment. *IPT1* and *IPT3* were repressed in the *Arabidopsis* seedlings during NaCl treatment, whereas expression of *IPT5* and *IPT7* was slightly induced after 1 and 2 h of salt stress, respectively, but their expression returned to the basal level after 5 h of treatment. Interestingly, with the exception of *IPT7*, whose mRNA was slightly increased after 1 h but rapidly decreased below levels of the control after 5 h of exogenous ABA treatment, all of the remaining *IPT* genes were suppressed by treatment with exogenous ABA, suggesting that excess ABA might inhibit CK biosynthesis. Expression of the *CKX* genes was also suppressed by stress treatments with a few exceptions. Transcription of all of the *CKX* genes was significantly decreased during exposure to drought stress. Similarly, ABA treatment reduced expression of

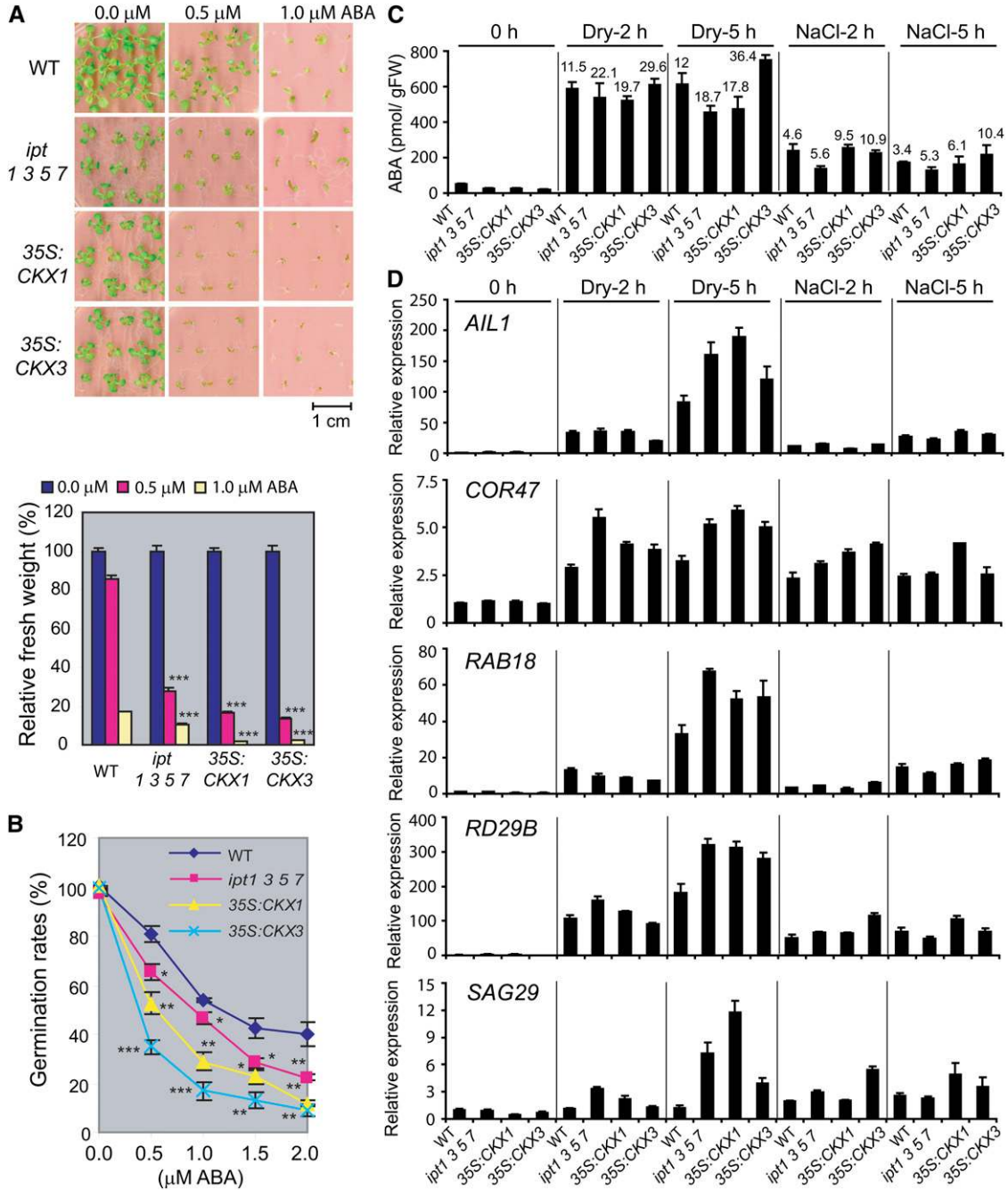

Figure 5. ABA Response of CK-Deficient Plants.

(A) Response of the CK-deficient plants to exogenous ABA as determined by a growth inhibition assay. Relative fresh weights of plants were determined after 14 d of incubation at 22°C. Errors bars = SE values (n = 7, where each measurement represents the weight of six plants). WT, wild type.

(B) Response of the CK-deficient plants to exogenous ABA as determined by a germination assay. Germination rates were determined after 6 d of incubation at 22°C by counting the number of open cotyledons. SE values (error bars) were calculated from the results of four independent experiments. Asterisks indicate significant differences as determined by a Student's t test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

(C) Endogenous ABA levels in the CK-deficient plants under dehydration and salt stresses. Error bars = SE values of three biological replications. Printed numbers represent the fold change over the untreated sample from the same genotype. FW, fresh weight.

(D) Expression of ABA signaling marker genes in CK-deficient plants under 2- and 5-h dehydration and salt stress. Data represent the means and SE values of three independent biological replicates.

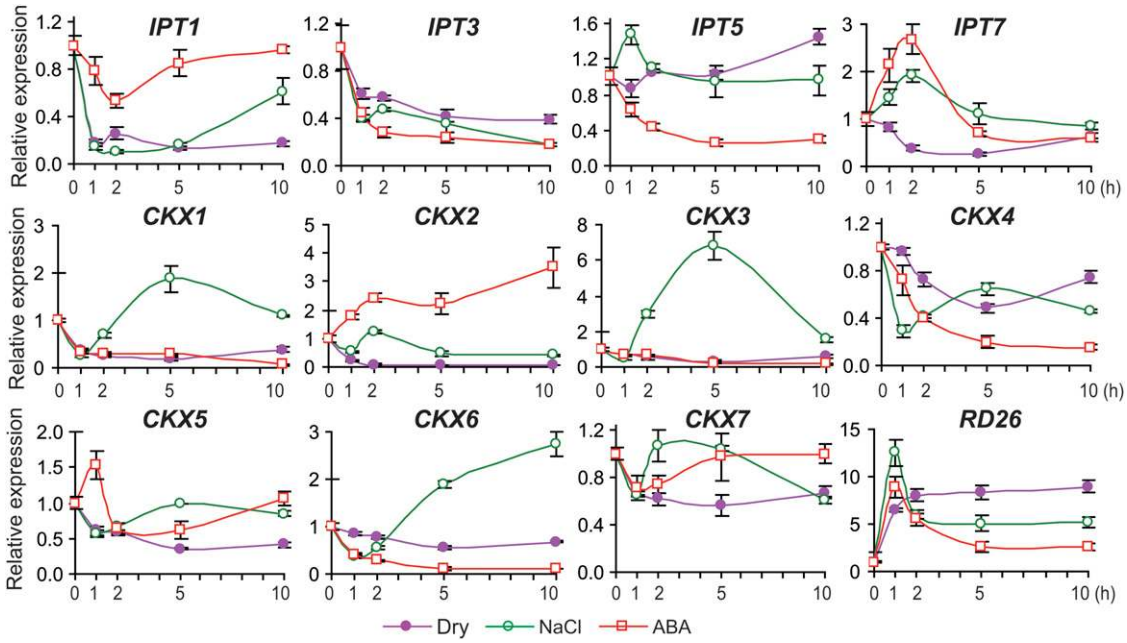

Figure 6. Expression of *Arabidopsis IPT* and *CKX* Genes in 2-Week-Old Wild-Type Plants under Various Stress and Hormone Treatments.

Relative expression levels for *IPT* and *CKX* genes were normalized to a value of 1 in the respective mock control plants. Data represent the means and SE values of three independent biological replicates. The stress-inducible *RD26* gene was used to confirm that the treatments were effective in stimulating stress-responsive gene expression.

the *CKX* genes, with the exception of *CKX2* and *CKX5*. *CKX2* transcription was continuously induced after 1 h of ABA treatment, wheras expression of *CKX5* was transiently induced after 1 h and then quickly reduced to the basal level at 2 h. As the ABA treatment continued, expression of *CKX5* was significantly repressed. With NaCl treatment, expression of the majority of the *CKX* family, including *CKX2*, *CKX4*, *CKX5*, and *CKX7*, was decreased, whereas expression of the remaining three genes (*CKX1*, *CKX3*, and *CKX6*) was increased, suggesting that these genes encode CKX proteins that may help plants reduce the accumulated CKs as quickly as possible upon exposure to environmental stresses. *RD26* was utilized as an ABA, NaCl, and dehydration stress marker to confirm the efficacy of our stress treatments.

The Contents of the Bioactive CKs Are Reduced under Stresses

Expression analysis data suggest that stresses alter the expression of *IPT* and *CKX* genes, leading to a reduced CK content. We examined this hypothesis by determining the CK levels in *Arabidopsis* plants under stress conditions. We observed that under drought stress, the content of *t*Z-type CKs was significantly reduced but that of the iP- and *c*Z-type CKs remained relatively unaffected (Figure 7A). These data demonstrate that in *Arabidopsis*, among the most physiologically active CKs, the *t*Z-type CKs rather than the iP-type CKs act as negative regulators in drought stress response. On the other hand, under salt stress, both the *t*Z- and iP-type CKs are remarkably decreased, indicating that not only *t*Z-type CKs but also iP-type CKs are involved in the regulation of salt stress response. Although the levels of the *t*Z- and/or iP-type CKs were reduced in the stressed wild-type plants, these reduced levels were still higher than those in nonstressed CK-deficient plants. Furthermore, we did not observe an additional decrease in CK levels in CK-deficient plants under stress conditions (see Supplemental Data Set 2 online). In addition, because the expression of *CKX* genes is regulated by CKs (Werner et al., 2006), a reduction in CK content would subsequently cause downregulation of *CKX* genes. To confirm this hypothesis, we examined the expression of the *CKX* genes in the CK-deficient plants. As expected, the expression of *CKX* genes was markedly reduced as a long-term adaptive reaction to CK deficiency in the CK-deficient plants in comparison with wild type, with one exception; the expression of *CKX5* was relatively unchanged in the *35S*:*CKX2* and *35S*:*CKX4* plants (Figure 7B).

DISCUSSION

In this study, we have explored the function of CKs in stress responses by investigating the consequences of a reduced CK content on the adaptation of plants to stresses, ABA metabolism, and the ABA response. Additionally, by examining the stress-related expression of key genes involved in CK metabolism, such as the *CKX* and *IPT* genes, we have revealed a link between abiotic stress and CK metabolism. The results of our gain- and loss-of-function studies suggested that the CKs exert a negative role on stress responses. Constitutive overexpression of the *CKX*

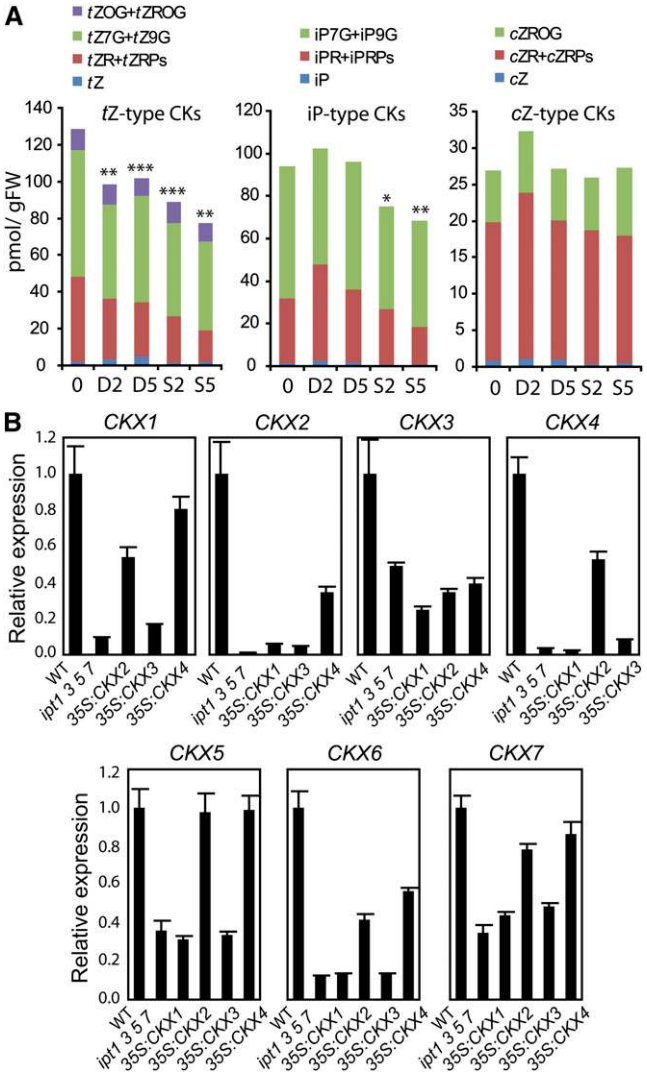

Figure 7. CK Contents in Stressed Wild-Type Plants and Expression of CKX Genes in CK-Deficient Plants.

(A) Endogenous CK levels in the wild-type *Arabidopsis* plants under dehydration (D2, 2-h dehydration; D5, 5-h dehydration) and salt stress (S2, 2-h salt stress; S5, 5-h salt stress). Data were calculated from three independent biological replicates. SE values for each CK metabolite are available in Supplemental Data Set 2 online. Asterisks indicate significant differences as determined by a Student's *t* test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). FW, fresh weight.

(B) Downregulated expression of the *CKX* genes in CK-deficient plants. Ten-day-old plants were grown on GM plates and were harvested for expression analysis. Relative expression levels of the *CKX* genes were normalized to a value of 1 in the wild-type (WT) plant. Data represent the means and SE values of three independent biological replicates.

genes resulted in CK deficiency and enhanced drought- and salt stress-tolerant phenotypes (Figure 1). In accordance with the results of gain-of-function studies of *CKX* genes, the reduction of bioactive CK levels by loss-of-function of *IPT1*, *IPT3*, *IPT5*, and *IPT7*, which are involved in biosynthesis of the bioactive CKs, also led to a stress-tolerant phenotype (Figure 2). The enhanced drought tolerance of the CK-deficient plants was attributed to their capacity to maintain higher water content under stress (Figure 3A), which was associated with their intact membrane structure, as evidenced by lower electrolyte leakage (Figure 3D). Reduction of CK content, therefore, might coincide with an increase of structural protective proteins and/or intracellular solute concentrations; as a consequence, higher water content was maintained because of the protection of membrane structures (Verslues et al., 2006).

We observed that both the wild-type and CK-deficient plants responded in a similar manner to ABA-mediated inhibition of stomatal opening (Figure 3B). In addition, although the size of the epidermal cells of the CK-deficient plants was increased (Werner et al., 2003), there were no significant differences in stomatal density between the wild-type and CK-deficient plants (Figure 3C). These data indicate that the increased drought tolerance of the CK-deficient plants was not a result of either the inhibition of stomatal opening or reduced stomatal density. Several studies have reported that alteration in drought phenotype of plants is not always a consequence of alteration in stomata-related traits (Bartels and Sunkar, 2005; Fujita et al., 2005; Hirayama and Shinozaki, 2010). For instance, constitutive overexpression of the ABA-responsive *AREB1ΔQT* gene in *Arabidopsis* resulted in better performance under drought stress, independent from the status of stomatal closure. The improved drought tolerance of the *AREB1ΔQT* transgenic plants was shown to be because of their increased ABA sensitivity, which led to the induction of downstream genes within the ABA signaling cascade (Fujita et al., 2005). Consistent with this report, the CK-deficient plants are more sensitive to ABA in relative comparison with the wild type. In turn, this leads to a higher induction of ABA signaling genes by stresses and subsequently to enhanced stress tolerance, despite the fact that the ABA contents in the wild-type and CK-deficient plants were comparable under stress conditions (Figure 5). Moreover, given that the CK levels in CK-deficient plants were lower than those in wild-type plants under stress conditions (see Supplemental Data Set 2 online), the increased ABA:CK ratio appeared to be favorable for CK-deficient plants to better tolerate adverse conditions than wild-type plants.

In good accordance with the negative regulatory role of CKs in stress responses, expression of the majority of examined *Arabidopsis IPT* genes was repressed by the stresses in the wild-type plants (Figure 6). These data are consistent with the reduced bioactive CK contents observed under the same conditions (Figure 7A). Furthermore, our data also indicated that the expression of the majority of *CKX* genes was downregulated during stress treatments (Figure 6), suggesting that a reduction in CK levels under stress conditions might subsequently lead to suppression of most of the *CKX* genes. A reduction in CK levels as a consequence of loss-of-function of *IPT* genes lowered *CKX* expression (Figure 7B). Additionally, altered expression of the CK metabolic genes after exogenous ABA treatments suggests that ABA might participate in the control of CK metabolism similarly as CKs affect ABA metabolism under normal conditions (Figure 4). This hypothesis requires further confirmation by analysis of the CK levels in ABA-deficient and/or ABA-overproducing plants. A reduction of ABA levels observed in the CK-deficient plants under well-watered conditions suggests the existence of a

fine-tuning mechanism that maintains the appropriate ABA:CK ratio. Consistent with our finding, Havlová et al. (2008) detected an increase in ABA levels in the CK-overproducing *35S:ZOG1* plants under nonstressed conditions. It is possible that an increase of ABA:CK ratio caused by CK deficiency might result in constitutive stomatal closure under well-watered conditions, leading to reduced transpiration, which inhibits CO_2 uptake and thereby negatively impacts photosynthesis (Cornic and Fresneau, 2002). To avoid these unfavorable consequences, the adjustment of ABA:CK ratio was observed in the CK-deficient plants, which coincided with the absence of any significant alterations in stomatal aperture in relative comparison with wild-type plants under normal conditions. Thus, it is apparent that mutual regulatory mechanisms exist between CK metabolism and ABA metabolism. These mechanisms mediate fine-tuning of the ABA:CK ratio, as well as CK and ABA responses, as a means to ensure ideal homeostasis for plant growth and development. The fact that the expression of *NCED3* was unaffected in the CK-deficient plants might reflect the key role of the NCED3 enzyme in quick osmotic stress responses (Barrero et al., 2006). The maintenance of a high *NCED3* expression level might be necessary to preserve the ability of CK-deficient plants to induce ABA synthesis upon exposure to stress to survive adverse conditions.

The elevated stress tolerance of the CK-deficient plants may be attributed to their ability to react faster to the ABA and stresses by the repression of CK signaling. Strong lines of evidence have demonstrated that various members of CK signaling, including CK receptor AHKs and a number of ARRs, such as ARR5 and ARR7, function as negative regulators in osmotic and cold stress responses (Tran et al., 2007b; Wohlbach et al., 2008; Jeon et al., 2010). Furthermore, all of the *ahk2*, *ahk3*, and *ahk4* single mutants and the *ahk2 3* double mutant showed an ABA-hypersensitive phenotype (Tran et al., 2007b; Jeon et al., 2010). Taken together, we suggest a mechanism in which plants adapt to adverse conditions by triggering an efficient regulation of CKs and CK signaling. We propose that during stress, the stress-dependent downregulation of transcription of CK biosynthetic *IPT* genes occurs. As a result of the repression of *IPT* genes, CK biosynthesis is reduced; as a consequence, the expression of *CKX* genes is inhibited (Figures 6 and 7). On the other hand, the repression of CK metabolic genes by exogenous ABA treatment (Figure 6) suggests that the stress-induced ABA accumulation might also play a role in the downregulation of CK levels by stresses. In agreement with our observation, it was reported that treatment of plants with exogenous ABA decreased CK contents (Vaseva et al., 2008). Therefore, the ABA:CK ratio changed by stresses in favor of ABA may facilitate plant adaptation to adverse environmental conditions. As shown in our model (Figure 8), a reduction in CK contents to a critical threshold level may repress the CK signal transduction pathway, as indicated by downregulation of the primary-response *ARR5* and *ARR7* genes in the CK-deficient plants (see Supplemental Figure 5 online). As a result, its negative regulatory function on the expression of stress- and/or ABA-responsive genes is alleviated, resulting in better plant survival under stress conditions.

The morphological adjustment that optimizes growth in response to environmental cues was shown to be an important mechanism of plant survival (Sharp et al., 2004; Achard et al., 2006; Achard et al., 2008). We observed that both the stress-tolerant CK-deficient and CK receptor His kinase mutant plants have enhanced root growth but retarded shoot growth (Figures 1 and 2) (Werner et al., 2003; Higuchi et al., 2004; Nishimura et al., 2004; Miyawaki et al., 2006; Riefler et al., 2006; Tran et al., 2007b; Jeon et al., 2010). Root enhancement is one of the key traits that is correlated with drought resistance (Sharp et al., 2004). On the other hand, restraint of shoot growth has been regarded as an advantage in adverse environments and is an integral part of plant stress tolerance to promote plant survival (Achard et al., 2006, 2008; Huang et al., 2009). Therefore, it is possible that a complex process regulates CK metabolism to ensure maintenance of an appropriate homeostasis, by which CK signaling would enable CK-deficient plants and CK receptor kinase mutants to survive stress exposure via enhancement of root growth but restraint of shoot growth.

It is well established that the leaf senescence strategy, which is used by plants to adapt to drought, is associated with a decrease in CK content and CK signaling suppression (Davies and Zhang, 1991; Gan and Amasino, 1995; Kim et al., 2006; Riefler et al., 2006). Transgenic plants expressing the *Agrobacterium tumefaciens IPT* gene exhibited CK overproduction and some delay of leaf senescence, suggesting that CKs may have applications in agriculture. However, excessive overproduction of CKs above a

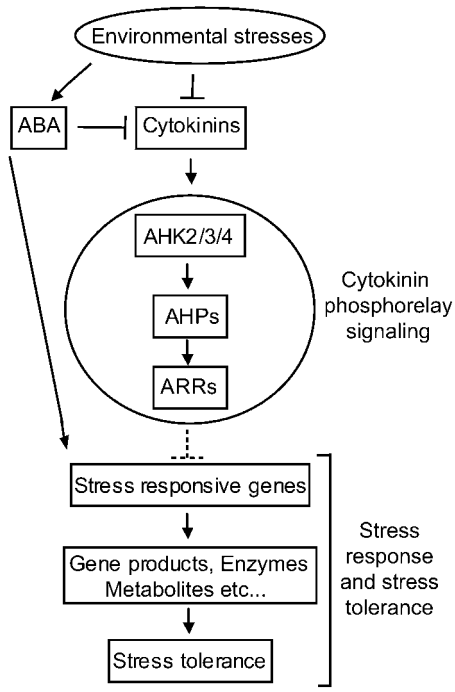

Figure 8. Model for the Role of Bioactive CKs under Stresses.

Upon stress, *IPT* gene expression is reduced, leading to a decrease in CK accumulation. The stress-induced ABA levels also downregulate the expression of *IPT* genes, which results in a further decrease in CK contents. Because of a reduction in CK content, the inhibitory effect of CK signaling on the expression of stress responsive genes is alleviated (dotted bar), leading to enhanced plant survival.

threshold also caused stunted plant growth, abnormal tissue development, and sensitivity but not tolerance to drought (Li et al., 1992; Hewelt et al., 1994; Wang et al., 1997; Synkova et al., 1999; Havlová et al., 2008). Therefore, appropriate manipulation of CK levels is necessary to increase leaf longevity and photosynthetic capacity under drought stress, taking into account the dosage effect of CKs on plant growth and stress responses. Carefully designed transgenic tobacco plants containing the drought/senescence-dependent P_{SARK}:*IPT* construct exhibited enhanced drought survival with minimal yield loss because of a preprogrammed delay of drought-induced senescence (Rivero et al., 2007, 2009), which diminished the negative impact of excessively overproduced CKs on plant growth and stress responses. Thus, CK biology seems to represent a promising tool for agronomy and can provide multiple biotechnological strategies to maintain agriculture in a sustainable fashion.

METHODS

Plant Materials and Stress Treatments

Arabidopsis thaliana plants (Columbia ecotype) were grown on GM agar plates for 2 weeks (22°C, 16-h-light/8-h-dark cycle, 60 μmol m^{-2} s^{-1} photon flux density) and treated with dehydration, water (hydroponic control), 100 μM ABA, or 250 mM NaCl for the indicated time periods as previously described (Tran et al., 2004). The *ipt1 3 5 7* mutant in the Columbia ecotype was previously constructed (Miyawaki et al., 2006). Detailed methodology for the construction and characterization of the *35S*:*CKX1-11*, *35S*:*CKX2-9*, *35S*:*CKX3-9*, and *35S*:*CKX4-41* transgenic plants was previously described (Werner et al., 2003).

Transcriptional Analyses

Total RNA was extracted with TRIzol Reagent (Invitrogen) according to the supplier's instructions (Invitrogen). cDNA synthesis and qRT-PCR were performed according to previously described methods (Tran et al., 2009). The primer pairs that were used in qRT-PCR reactions are listed in Supplemental Table 1 online. *UBQ10* was used as internal control for expression analysis.

Quantification of Endogenous CKs and ABA

Ten-day-old *Arabidopsis* plants were used for ABA and CK measurements according to previously described procedures (Kojima et al., 2009). For quantification of ABA and CK contents under salt stress (250 mM NaCl) and dehydration, the fresh weight of each plant sample was determined before stress treatments.

Stress Tolerance Tests

Drought and salt stress tolerance tests of transgenic or mutant plants were conducted as previously described (Tran et al., 2007b). For a long-term drought stress treatment that spanned flowering and silique ripening stages, we utilized the same-tray method to ensure valid comparisons of genotypes with different growth rates (Verslues et al., 2006). In brief, plants were grown on GM medium for 2 weeks and subsequently transferred to trays of soil and grown in a greenhouse (24 to 25°C, 50 to 60% relative humidity, 16-h-light/8-h-dark cycle, 65 μmol m^{-2} s^{-1} photon flux density) for 1 additional week before exposure to drought stress. Drought stress was imposed by withholding water until the lethal effect of dehydration was observed on the majority of control plants. Relative soil moisture content was monitored using a HydroSense soil moisture probe (Campbell Scientific Inc.). Measurements were taken for each side of the tray and repeated five to seven times at different places in the tray (n = 5 to 7) and averaged. After resuming watering for 3 d, the number of plants that survived and continued to grow was quantified. Inflorescences were removed from all plants before photographing them. For comparison, plants were also grown in well-watered conditions in parallel with the long-term drought-tolerant test (see Supplemental Figures 1B and 1C online).

For analysis of salt stress tolerance, 10-d-old plants grown on GM medium were transferred onto 0.5× Murashige and Skoog (MS) plates containing either 0 or 200 mM NaCl. The plates were then maintained at 22°C under a 16-h-light/8-h-dark cycle until visual symptoms of salt stress were observed. A root growth assay under salt stress was performed as previously described. Four-day-old plants germinated on GM medium were transferred onto 0.5× MS plates containing either 0 mM or the indicated concentration of NaCl. The root length (from root tip to hypocotyl base) of at least eight vertically grown seedlings was measured 7 d after transfer. A dose–response curve was expressed as a percentage of the untreated control (Achard et al., 2006; Verslues et al., 2006).

Bolting, Flowering, and Silique Ripening Times

Wild-type and CK-deficient plants were grown in well-watered conditions as described in the long-term drought stress tolerance test (see Supplemental Figures 1B and 1C online). Bolting was scored every day by counting the number of rosette leaves and noted as the date when an inflorescence of ~0.5 cm was apparent in the center of the rosette. Flowering time was determined as previously described (Gazzani et al., 2003). A plant was regarded as "ripened" when the first opened brown silique was observed. Bolting time, flowering time, and silique ripening time were determined as the number of days after germination when more than one-half of the population (n = 30/genotype) bolted, flowered, and ripened, respectively.

Measurement of Relative Water Content in Drought-Stressed Plants

Measurement of relative water content (RWC) during long-term drought stress was taken using the method adapted from Barrs and Weatherley (1962). Detached aerial parts of stressed plants (n = 5) were individually weighed to determine sample weight (W) at various time points. After the initial determination of the sample fresh weight, individual samples were placed into 50-mL tubes and hydrated overnight in 40 mL of deionized water to full turgidity under normal room light and temperature. The samples were then removed from water, residual leaf moisture was gently removed with filter paper, and samples were immediately weighed to obtain a fully turgid weight (TW). Subsequently, the plants were dried in an oven at 65°C for 48 h, and dry weight was measured (DW). RWC was calculated as RWC (%) = [(W − DW)/(TW − DW)] × 100.

Measurement of Electrolyte Leakage

Electrolyte leakage was determined from the detached aerial parts of drought-stressed plants at various time points as indicated. The detached plants (n = 5) were individually placed in 50-mL tubes containing 40 mL of deionized water and gently shaken for 3 h. The percentage of electrolyte leakage was determined as the percentage of the conductivity before boiling and after boiling of the detached plants.

Stomatal Movement Assays

Stomatal movement assays were conducted as described previously with minor modifications (Fujita et al., 2009). In brief, rosette leaves of 4-week-old wild-type and CK-deficient plants grown on soil at 22°C, 50

μmol m^{-2} s^{-1} photon flux density, and 60% relative humidity under a 16-h light/8-h dark photoperiod were harvested and incubated for 2 h in a solution containing 10 mM KCl, 0.2 mM $CaCl_2$, and 10 mM MES-KOH (pH 6.15) under white light. These leaves were subsequently transferred to a solution containing the same buffer and ABA and incubated for an additional 2 h. Guard cells were photographed under a color laser three-dimensional profile microscope (Keyence).

Determination of Stomatal Density

Rosette leaves of 4-week-old wild-type and CK-deficient plants grown on soil at 22°C, 50 μmol m^{-2} s^{-1} photon flux density, and 60% relative humidity under a 16-h-light/8-h-dark photoperiod were harvested and photographed under a color laser three-dimensional profile microscope (Keyence, Japan) for determination of stomatal density.

Assays for Sensitivity to ABA

Growth Inhibition Assay

Seeds were sterilized and plated on GM medium containing 1% sucrose and 0 or 0.5 or 1.0 μM ABA. After 4 d of stratification at 4°C in the dark, plates were incubated at 22°C under a 16-h-light/8-h-dark cycle. Plant fresh weights were measured at 14 d after incubation and seven independent measurements (n = 7) were taken for each genotype. Each measurement represents the pooled fresh weight obtained from six plants using an electronic balance with a resolution of 0.00001 g (Sefi IUW-200D, Shimadzu, Kyoto, Japan).

Germination Assay

Seeds were sterilized and plated on GM medium containing 1% Suc and various concentrations of ABA. After 4 d of stratification at 4°C in the dark, plates were incubated at 22°C under a 16-h-light/8-h-dark cycle. The germination time was considered as the time of appearance of opened cotyledons.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *IPT1* (At1g68460), *IPT3* (At3g63110), *IPT5* (At5g19040), *IPT7* (At3g23630), *CKX1* (At2g41510), *CKX2* (At2g19500), *CKX3* (At5g56970), *CKX4* (At4g29740), *CKX5* (At1g75450), *CKX6* (At3g63440), *CKX7* (At5g21482), *ABA1* (At5g67030), *ABA2* (At1g52340), *AAO3* (At2g27150), *NCED3* (At3g14440), *AIL1* (At3g17520), *COR47* (At1g20440), *RAB18* (At5g66400), *RD29B* (At5g52300), *SAG29* (At5g13170), *ARR5* (At3g48100), *ARR7* (At1g19050), *RD26* (At4g27410), and *UBC10* (At4g05320).

Supplemental Data

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

Supplemental Figure 1. Wild Type and CK-Deficient Plants Grown in Well-Watered Conditions.

Supplemental Figure 2. Salt-Tolerant Phenotypes of the CK-Deficient Plants.

Supplemental Figure 3. Evaluation of the Drought Tolerance of the *ipt1 3 5 7* Mutant.

Supplemental Figure 4. Response of the CK-Deficient Plants to Exogenous ABA.

Supplemental Figure 5. Decreased Expression of the Type A *ARR5* and *ARR7* Genes in CK-Deficient Plants.

Supplemental Table 1. Primers Used for qRT-PCR.

Supplemental Data Set 1. Bolting, Flowering, and Ripening Times of Wild-Type and CK-Deficient Plants.

Supplemental Data Set 2. Concentration of CK Metabolites in CK-Deficient Plants under Drought and Salt Stresses.

ACKNOWLEDGMENTS

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Analysis of Cytokinin Mutants and Regulation of Cytokinin Metabolic Genes Reveals Important Regulatory Roles of Cytokinins in Drought, Salt and Abscisic Acid Responses, and Abscisic Acid Biosynthesis

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