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ORIGINAL ARTICLE

# Analysis the Role of Arabidopsis *CKRC6/ASA1* in Auxin and Cytokinin Biosynthesis

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**Abstract** The crosstalk between auxin and cytokinin (CK) is important for plant growth and development, although the underlying molecular mechanisms remain unclear. Here, we describe the isolation and characterization of a mutant of Arabidopsis *Cytokinin-induced Root Curling 6* (*CKRC6*), an allele of *ANTHRANILATE SYNTHASE ALPHA SUBUNIT 1* (*ASA1*) that encodes the á-subunit of AS in tryptophan (Trp) biosynthesis. The *ckrc6* mutant exhibits root gravitropic defects and insensitivity to both CK and the ethylene precursor 1-aminocyclopropane-1-carboxylicacid (ACC) in primary root growth. These defects can be rescued by exogenous indole-3-acetic acid (IAA) or tryptophan (Trp) supplementation. Furthermore, our results suggest that the *ckrc6* mutant has decreased IAA content, differential expression patterns of auxin biosynthesis genes and CK biosynthesis isopentenyl transferase (*IPT*) genes in comparison to wild type. Collectively, our study shows that auxin controls CK biosynthesis based on that CK sensitivity is altered in most auxin-resistant mutants and that CKs promote auxin biosynthesis but inhibit auxin transport and response. Our results also suggest that CKRC6/ASA1 may be located at an intersection of auxin, CK and ethylene metabolism and/or signaling.

**Keywords:** Auxin, Biosynthesis, Cytokinin, Ethylene, *IPTs*

## Introduction

Plant survival depends on the regulated signaling of phytohormones, such as auxin and cytokinin (CK), which are involved in diverse processes including embryogenesis (Muller and Sheen 2008), post-embryonic root and shoot development (Werner and Schmulling 2009), shoot branching (Ongaro and Leyser 2008; Shimizu-Sato et al. 2009), lateral root initiation and development (Casimiro et al. 2003; De Smet et al. 2006; Osmont et al. 2007), and *in vitro* organogenesis (Skoog and Miller 1957). Homeostasis of auxin and CK in plants is necessary and regulated by a number of factors such as biosynthesis, transport, signaling and the interaction of the two hormones (Di et al. 2015a).

The primary form of auxin active in a plant is indole-3-acetic acid (IAA), which is mainly synthesized by the tryptophan (Trp)-dependent (TD) pathway and the Trp-independent (TI) pathway (Chandler 2009; Di et al. 2015b; Normanly 2010; Woodward and Bartel 2005). Four proposed TD pathways depending on the first step after Trp have been identified including the indole-3-pyruvic acid (IPyA) pathway, the indole-3-acetamide (IAM) pathway, the tryptamine (TAM) pathway and the indole-3-acetaldoxime (IAOx) pathway. However, no known genes or enzymes involved in the TI pathway have been identified, yet the branch point of the TI and TD pathways is identified as indole (Ouyang et al. 2000). Recently, a gene that encodes indole synthase (INS) has been suggested to belong to the TI pathway (Wang et al. 2015). Trp synthesis in plants is well characterized, which starts with the synthesis of anthranilate from chorismate (Radwanski and Last 1995). The *ASA1* (also known as *TRYPTOPHAN BIOSYNTHESIS5* (*TRP5*) or *WEAK ETHYLENE INSENSITIVE2* (*WEI2*)) (Barczak et al. 1995; Li and Last 1996; Stepanova et al. 2005) and *ANTHRANILATE SYNTHASE BETA SUBUNIT 1 (ASB1)* (also known as *WEAK ETHYLENE INSENSITIVE7* (*WEI7*) or *TRYPTOPHAN BIOSYNTHESIS4* (*TRP4*) (Niyogi et al. 1993; Barczak et al. 1995; Stepanova et al. 2005) genes respectively encode the α- and β-subunit of anthranilate synthase (AS), which catalyzes

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the conversion of chorismate to anthranilate, a rate-limiting step in Trp biosynthesis (Stepanova et al. 2005). Disruption of this rate-limiting step of Trp biosynthesis can result in defect in the biosynthesis of IAA (Stepanova et al. 2005).

Compared with auxin biosynthesis, the biosynthesis of CK is more clearly understood. An adenine moiety accepts the isoprenoid side chain from dimethylallyldiphosphate (DMAPP) by one of two classes of isopentenyl transferases (IPTs): ATP/ADP IPTs (IPT 1, 3, 4 and 8) and tRNA IPTs (IPT 2 and 9) (Takei et al. 2001; Kakimoto 2001, 2003; Miyawaki et al. 2006; Sakakibara et al. 2006). ATP/ADP IPTs primarily synthesisize $N^6$-isopentenyladenine (iP)-type and trans-zeatin (tZ)-type CKs and are likely responsible for the bulk of CK biosynthesis. The tRNA IPTs are indispensable for biosynthesis of cis-zeatin (cZ)-type CKs (Miyawaki et al. 2006). Each ATP/ADP *IPT* has a specific spatial expression pattern (Miyawaki et al. 2004), while tRNA *IPT*s are ubiquitously expressed in all tissues (Miyawaki et al. 2004). Over-expression of all known ATP/ADP *IPT*s results in CK overproduction and the *IPT*s exhibit redundant function in the plant (Miyawaki et al. 2004).

The interaction between auxin and CK is important for plant growth and development (Muller and Sheen 2008; Moubayidin et al. 2009). In transport level, CK can regulate the polar auxin transport by modulating transcription of several PIN-FORMED (PIN) auxin efflux carriers (Dello Ioio et al. 2008; Ruzicka et al. 2009; Zhou et al. 2011). Both auxin and CK regulate the expression of the CK biosynthesis *IPT* genes (Miyawaki et al. 2004). CK can modify auxin biosynthesis derived through TRYPTOPHAN AMINOTRANSFERASE1 (TAA1)/CYTOKININ INDUCED ROOT CURLING1 (CKRC1) (Tao et al. 2008; Zhou et al. 2011); and auxin can rapidly downregulate CK biosynthesis (Nordstrom et al. 2004). The mutation of *AUXIN RESPONSE FACTOR3* (*ARF3*) causes ectopic CK biosynthesis via the mis-expression of *IPT5* (Cheng et al. 2013). The CK-response transcription factor, ARABIDOPSIS RESPONSE REGULATOR 1 (ARR1), negatively regulates the PIN auxin transport facilitator genes by activating *SHORT HYPOCOTYL 2/ INDOLE-3-ACETIC ACID INDUCIBLE 3* (*SHY2/IAA3*), a repressor of auxin signaling (Dello Ioio et al. 2008). Both auxin and CK levels are balanced by an active homeostatic feedback loop involving both auxin and CK signaling, biosynthesis and transport (Jones et al. 2010). In addition, activating *CYTOKININ OXIDASE/DEHYDROGENASE 4* (*CKX4*) in rice substantially alters expression of auxin-related genes, including those involved in biosynthesis and transport (Gao et al. 2014). Here, we characterize the Arabidopsis *cytokinin induced root curling 6* (*ckrc6*) mutant. Our results suggest that this mutation is involved in Trp/IAA biosynthesis and may influence the biosynthesis of CK.

## Results

### Identification of *ckrc* Mutants and Positional Cloning of the *ckrc6* Locus

To obtain additional auxin-deficient mutants, the CK-induced root curling (CKRC) screening system was established in our previous studies (Zhou et al. 2011; Wu et al. 2015). By screening an additional mutant population, another *ckrc* mutant, *ckrc6* (*cytokinin induced root curling 6*), was isolated (Fig. 1A, B), which showed similar curling phenotype to *ckrc1-1* after 0.1 μM tZ treatment (Fig. 1A) (Zhou et al. 2011). The whole genome resequencing (WGRS) identified a 2999-bp deletion in the upstream of *CKRC6/ASA1/TRP5/WEI2*, rendering *CKRC6/ASA1/TRP5/WEI2* completely inactive (Fig. 1C). Gene cloning indicated that the *ckrc6* mutant is a new allele of *TRP5/WEI2/ASA1* (Fig. 1C, D).

To further confirm the curling root phenotype in *ckrc6* mutant is resulted from *CKRC6* gene mutation, we treated the other three allelic mutants of *ASA1* including *wei2-1*, *wei2-2* and *trp5-1* with 0.1 μM tZ (Fig. 1A, B, D). Similar to *ckrc6*, the loss-of-function alleles *wei2-1* and *wei2-2* exhibited the curling root phenotype on MS containing 0.1 μM tZ (Fig. 1A) and decreased primary root and hypocotyl length phenotypes on MS plate (Fig. 1E, F). Conversely, *trp5-1*, a mutant allele with elevated accumulation of soluble L-Trp (Li and Last 1996), showed no root curling on tZ medium (Fig. 1A). The previous study showed that root curling in tZ treatment and the short primary root and hypocotyl are typical auxin-deficient phenotypes (Zhou et al. 2011). In addition, *ckrc6* responds to exogenous 5-methyl tryptophan (a toxic Trp analog) (Zhao et al. 2001) and L-Kynurenine (inhibitor of auxin synthesis) (He et al. 2011) added to MS in similar ways to the *wei2-1* and *wei2-2* alleles, indicating that the mutation of *CKRC6* influences Trp levels (Fig. S1), and thus possibly affecting auxin levels.

### The Content of IAA is Decreased in *ckrc6* Mutant

To determine whether the decreased primary root length and the disruption of root gravitropism in *ckrc6* were resulted from reduced Trp and/or auxin content, we observed the root phenotype after application of 0.01 μM IAA or 3 μM Trp in medium containing 0.1 μM tZ (Zhou et al. 2011). The root curling phenotype and defective root gravitropic response (GR) of *ckrc6* mutant on 0.1 μM tZ-containing medium were rescued after supplying exogenous IAA or Trp (Fig. 2A, B). In addition, application of a low concentration of IAA or Trp result in longer primary root in the *ckrc6* mutant, but not in wild type (Col) (Fig. 2C, D). Taken together, our results suggest that the decreased primary root length and defective root gravitropism phenotypes in *ckrc6* mutant were caused

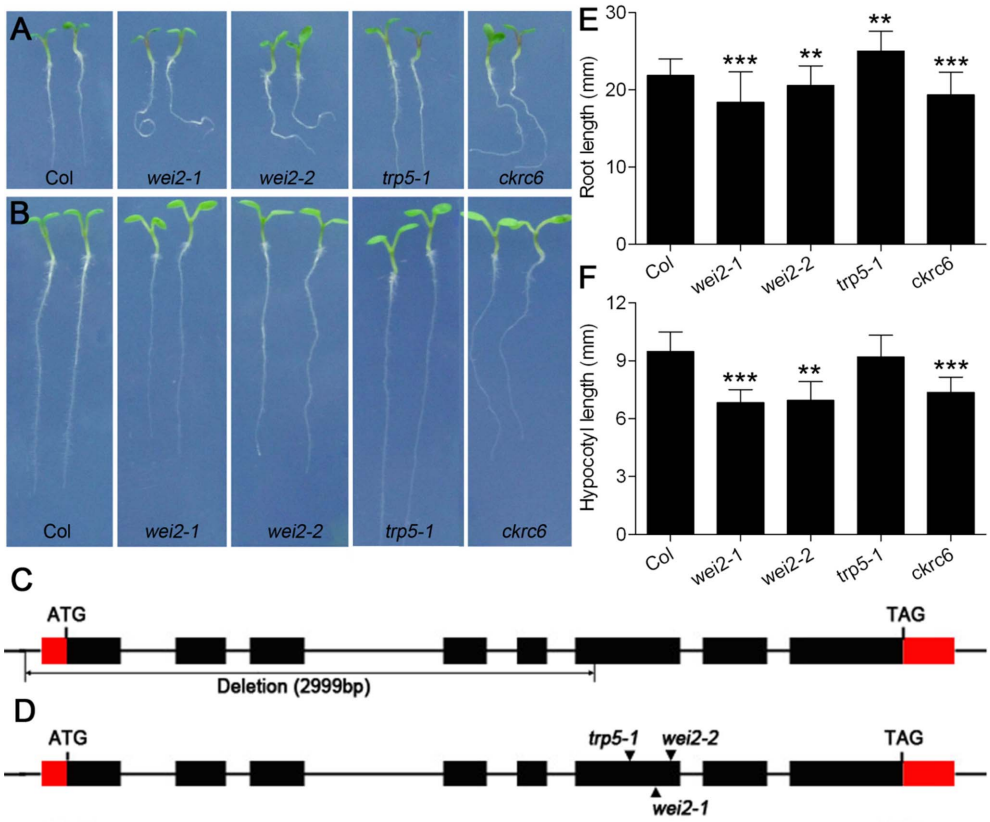


**Fig. 1.** Phenotype of *ckrc6* mutant on medium with or without 0.1 μM tZ. The root phenotype on 0.1 μM tZ-containing medium (A) and on MS (B); the *ckrc6* allele (C) and previously reported alleles of *ASA1* (D); the length of primary root (E) and hypocotyl (F) on MS. Data are shown as average values ± SD of mean (n = 25-40). Asterisks indicate statistically significant difference in the mutant lines (Student's t-test; ** and *** correspond to *P* values of $0.01 > P > 0.001$, and $0.05 > P > 0.01$, respectively).

by reduced levels of endogenous Trp or IAA.

To evaluate the endogenous auxin content, we compared the GUS activities in *ckrc6/Dr5::GUS* and *ASA1/Dr5::GUS* plants. GUS staining in *ckrc6* roots was decreased in mutants compared with Col (Fig. 2F). However, the *ckrc6* mutant supplemented with exogenous IAA or Trp had similar GUS staining to wild type (Fig. 2F). The transcript levels of GUS were lower in the *ckrc6* mutants compared to in Col (Fig. 2E). In addition, *ckrc6* showed decreased sensitivity to N-1-napthylphthalamic (NPA), an auxin transport inhibitor (Fig. S2), in both root length inhibition and *DR5* promoter induction (Fig. S2). Resistance to NPA indicates that the mutant is defective in auxin transport, synthesis or response (Yamada et al. 2009). Together, our results suggest that the endogenous IAA level in *ckrc6* mutant is in fact decreased (Fig. 2).

Since *ckrc6* mutant is insensitive to treatment with high levels of tZ (Fig. 1A, B), dosage responses of primary root length to tZ were assayed for *ckrc6* and other known CK-related mutants (Fig. 3). Primary roots of *ckrc6* seedlings were less sensitive than those of Col to all tZ concentrations tested (Fig. 3A). The mutants *amp1* (high CK levels) (Chaudhury et al. 1993), *ckrc1-1* (low IAA levels) (Zhou et al. 2011), *tir1-1* (defective auxin transport) (Ruegger et al. 1998) and *arr1-3/12-1* (reduced CK signaling) (Mason et al. 2005) are all less sensitive than Col to CK treatment, while *ipt3,5,7* (low CK levels) had Col-like CK sensitivity (Gray et al. 1999; Helliwell et al. 2001; Miyawaki et al. 2004) (Fig. 3, Fig. S3). The combination of the normal response of *ckrc6* to auxin and CK and the *amp1*-like response to exogenous CK suggest that the CK-insensitive phenotype in *ckrc6* may arise from the altered content of auxin and CK (Fig. 3, Fig. S3).

## Altered Expression of Auxin and CK Biosynthesis Genes in *ckrc6* Mutant

Thus, we further investigated the role of *CKRC6* mutation in gene expression involved in auxin and CK biosynthesis. The results showed that inactivation of *CKRC6* influenced the expression of numerous auxin biosynthesis-related genes in *ckrc6* mutant compared to Col in whole seedlings (Fig. 4). *TRP1*, *2* and *3*, *TRP4/WEI7/ASB1*, *CKRC1/TAA1*, *VAS1*, *YUCCA4* (*YUC4*), *YUC5*, *YUC9*, *AMI1*, *CYP79B2*, *CYP79B3* and *SUR2* were up-regulated, and *YUC2*, *3*, *7* and *11* were

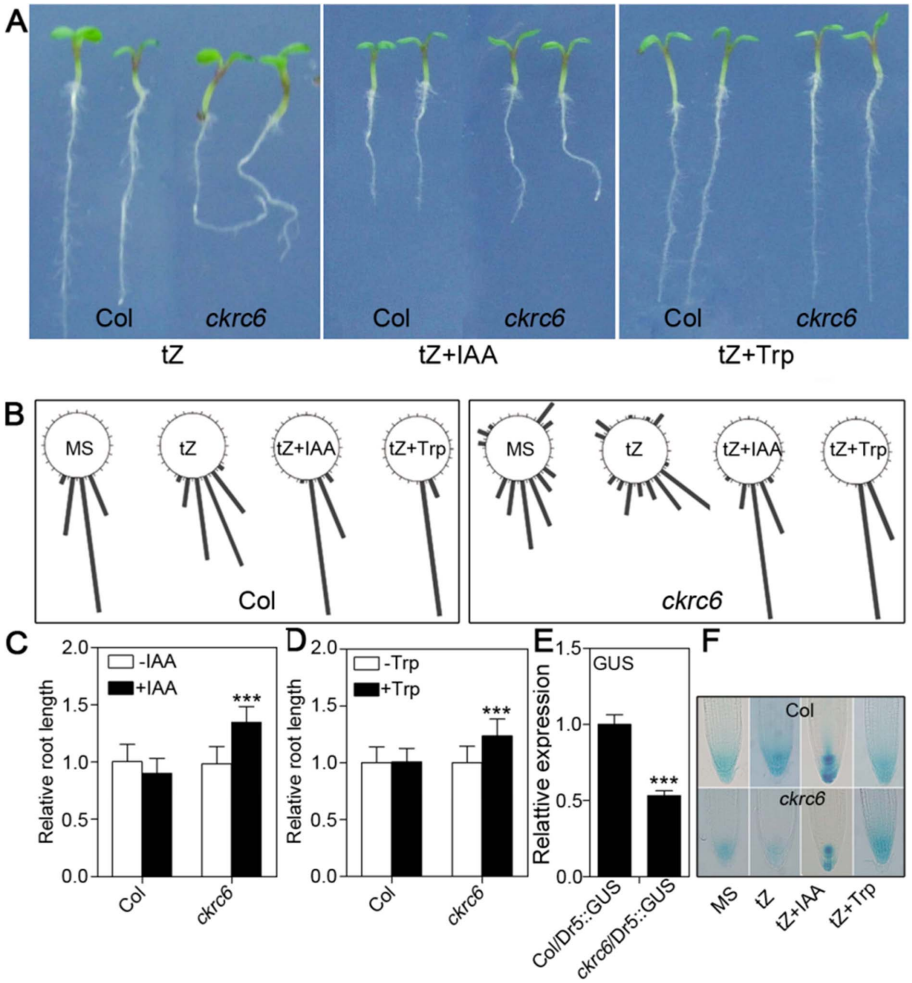


**Fig. 2.** Effect of endogenous auxin on the *ckrc6* mutant. The root phenotype (A) and gravitropism analysis (B) of Col and ckrc6 grown on MS medium containing 0.1 μM tZ, 0.1 μM tZ + 0.01 μM IAA, or 0.1 μM tZ + 3 μM L-Trp. Primary root length of of Col and ckrc6 grown on medium with 0.01 μM IAA (C) and 3 μM L-Trp (D). GUS expression was measured by qRT-PCR analysis (Student' s t-test, P***<0.001 ) (E). GUS staining of 7-d-old seedlings grown on medium with 0.1 μM tZ alone or in combination with 0.01 μM IAA or 3 μM L-Trp (F).

down-regulated in *crkc6*. *YUC10* could not be detected in either plant line (Fig. 4). The altered expression levels of auxin biosynthesis genes in *ckrc6* reflect the existence of auto-regulation of auxin biosynthesis. Meanwhile, the different responses of *YUC* family genes (*YUC 4*, *5, 9* vs *YUC 2*, *3*, *7*, *11* vs *YUC 1*, *6*, *8*) reflect the key roles of YUCs in maintaining *in vivo* auxin biosynthesis balance (Suzuki et al. 2015). Due to the self-regulation of the IAA biosynthesis pathway, any mutation in one step that results in decreased IAA requires coordinated alterations in other compensating steps.

However, the expression patterns of CK biosynthesis-related genes were different (Fig. 4E). *IPT5* was down-regulated, while *IPT1*, *IPT3* and *IPT7* were significantly up-regulated. In addition, *IPT4* and *IPT6*, two genes mainly expressed in floral buds, open flowers and siliques, could be detected in *ckrc6* but not in Col (Data not shown) (Miyawaki et al. 2004). In conclusion, we supposed that the *CKRC6* gene mutation can result in decreased endogenous auxin, which may up-regulate expression of *IPT1*, *3*, *4*, *6* and *7* to promote CK biosynthesis, therefore down-regulating *IPT5* expression. Collectively, the plant can regulate the phyto-hormone, especially auxin and CK, by the interaction and self-regulation.

## The Reason Why the ckrc6 Mutants Have the Curling Root on tZ Containing Medium

To clarify why *ckrc6* mutants exhibit root curling on tZ-containing medium, we tested all Trp biosynthesis mutants on 0.1 μM tZ medium (Fig. 5A). All five Trp biosynthesis mutants displayed root curling on tZ containing medium (Fig. 5A). The relative expression levels of Trp biosynthesis

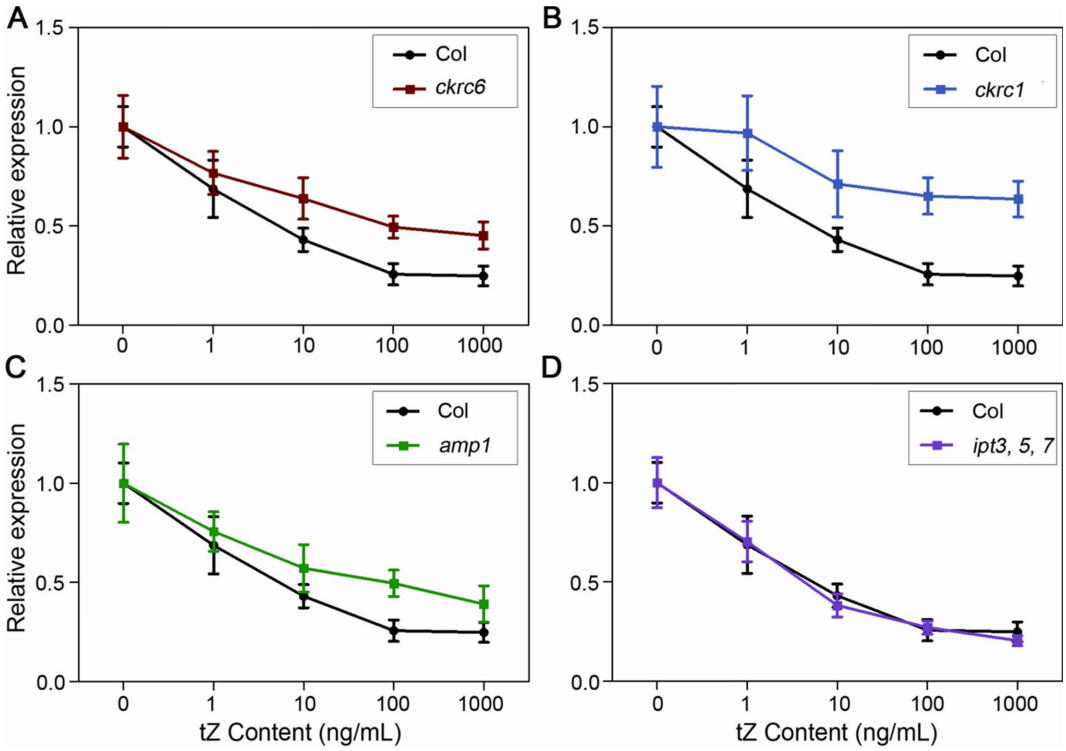


**Fig. 3.** The tZ dose response curve of primary root length in *ckrc6* mutant. Col and the *ckrc6* (A), *ckrc1-1* (B), *amp1* (C) and *ipt3, 5, 7* (D) mutants were grown on medium with various concentrations of tZ for 7 d, when the primary root length was measured. All experiments were repeated three times. Values are means ± SD (n = 45-60 for each experiment).

genes in untreated roots were all higher, similar to the levels in the *ckrc1-1* mutant (Fig. 5B) (Zhou et al. 2011). However, the *yuc6* mutant, although it had the high expression level in root, did not exhibit the curling root phenotype on tZ-containing medium (Fig. 5A). Since *CKRC1* was induced after tZ treatment (Zhou et al. 2011), we analyzed the expression of Trp biosynthesis genes after 7 d of tZ treatment. All Trp biosynthesis genes and *YUC2* were induced after tZ treatment, but not *YUC6* (Fig. 5C). These results suggested that the high induction by tZ of genes in this pathway was necessary for root curling in all auxin-deficient mutants (Fig. 5D).

## Discussion

Plant growth and development are driven by different hormones, with both auxin and CK fairly important. Although an individual hormone may play a predominant role in certain processes or organs, the interaction between auxin and CK controls many processes, particularly in root and shoot development (Skoog and Miller 1957; Nordstrom et al. 2004). Recent studies have provided important information to further understand the molecular mechanisms of auxin-CK interaction and regulation: CKs regulate the polar auxin transport by regulating the auxin efflux carriers and by promoting auxin biosynthesis through CKRC1/TAA1 (Zhou et al. 2011); Auxin regulates CK biosynthesis by regulating *IPT* gene expression (Miyawaki et al. 2004; Nordstrom et al. 2004). In fact, both auxin and CK keep in balance by an active homeostatic feedback loop (Jones et al. 2010). However, the regulations of the auxin and CK biosynthesis levels in an auxin-deficient mutant have not been reported. In this study, we found that the auxin-deficient mutant *ckrc6* response to tZ was most similar to that of the CK-overproducing mutant *amp1*, but not to that of the auxin biosynthesis deficient mutant *ckrc1-1* (Fig. 3B, C) (Helliwell et al. 2001; Zhou et al. 2011). Further study showed the *CKRC6* mutation affects both auxin- and CK- biosynthesis related genes expression in seedlings. Our results suggested that CKRC6 involved in auxin-CK homeostasis by affecting auxin- and CK- biosynthetic genes expression.

The predominant feature of *ckrc6* are the decreased primary root length, reduced GUS staining compared with Col grown on MS medium, and root curling grown on tZ medium (Fig. 1A, Fig. 2). And these defects could be fully restored and/or rescued after application of the IAA and Trp at low concentrations of 0.01 μM and 3 μM, respectively (Fig. 1A, Fig. 2). These results suggested that *ckrc6* are both Trp- and auxin- biosynthesis defective mutant. Another obvious phenotype of *ckrc6* is the significant decrease in GUS

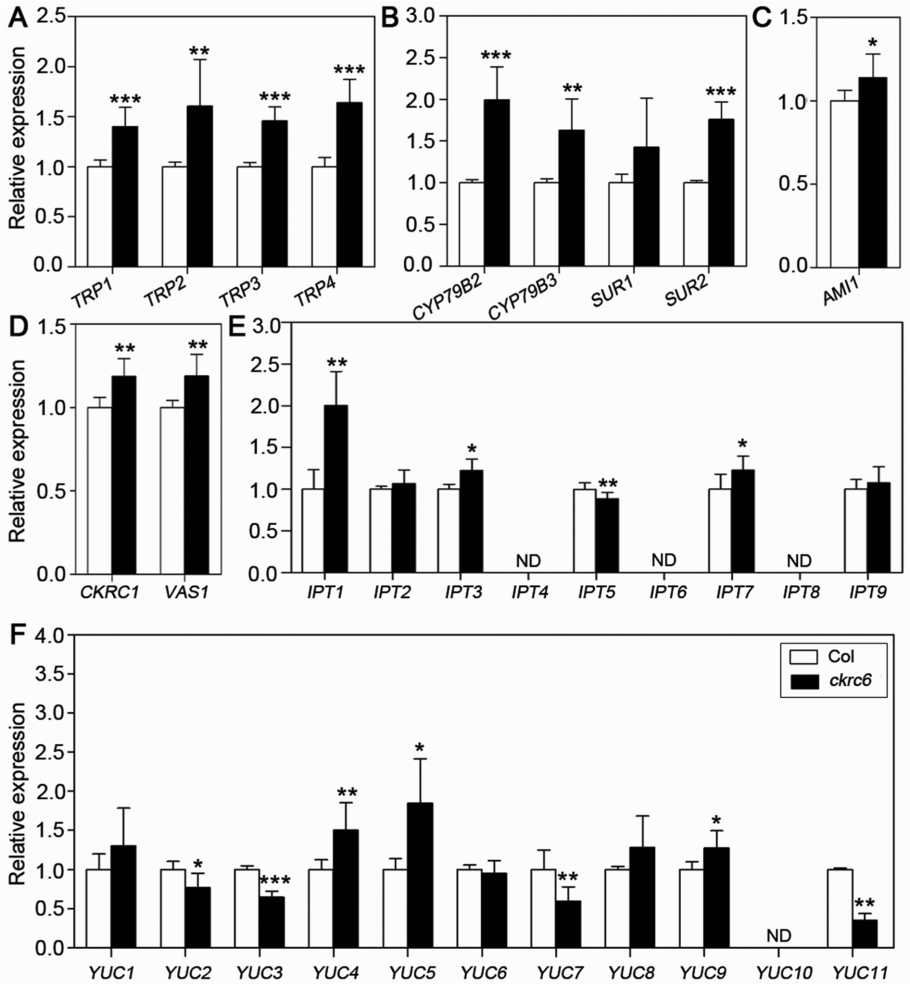


**Fig. 4.** Expression of auxin and CK biosynthetic genes in the *ckrc6* mutant. Seven-d-old seedlings grown on MS medium were used to examine the expression of genes involved in auxin biosynthesis (A-D, F) and CK biosynthesis (E). Whole seedlings were subjected to RNA extraction. Data are mean of 3 replicates. Error bars indicate SD. (Student's t-test; $^{*}$, $^{**}$ and $^{***}$ correspond to *P*-values of $0.05 > P > 0.01$, $0.01 > P > 0.001$, and $P < 0.001$, respectively).

staining after tZ treatment, revealing an important role for CKRC6 in auxin-CK interaction (Fig. 2F). The previous results demonstrated that CKs promote auxin biosynthesis but inhibit its polar transport (Dello Ioio et al. 2008; Ruzicka et al. 2009; Zhou et al. 2011) and that this function depends on CKRC1/TAA1 (Zhou et al. 2011). Interestingly, our results also suggested CKRC6 had a similar role to CKRC1 in CK-auxin crosstalk. In addition, application of ACC, the immediate precursor of ethylene biosynthesis in medium, the GUS staining increased slightly compared with Col (Fig. S2D), suggesting that the ethylene prompts auxin biosynthesis in root dependent on the normal function of CKRC6 (Stepanova et al. 2005). Most of CK biosynthesis related gene expression was increased in *ckrc6* mutant (Fig. 4E), indicating that reduced auxin level impairs CK biosynthesis, resulting in *amp1*-like response to exogenous CK (Fig. 4C). Moreover, CK is partially linked ethylene on regulating root growth (Cary et al. 1995; Rashotte et al. 2005; Kuderova et al. 2008). CKRC6 may, therefore, play an important role in the crosstalk among auxin, CK and ethylene on regulating root growth.

In this article, we identified an auxin-deficient mutant, *ckrc6*, with decreased auxin content. The *ckrc6* mutant had a role in maintaining the balance between CK and auxin by auto-regulation and interaction (Fig. 2, Fig. 4). Mutation of *CKRC6/ASA1/WEI2* influenced IAA biosynthesis and resulted in decreased IAA content, and its mutant is ethylene insensitive (Fig. 2, Fig. S2) (Stepanova et al. 2005). The reason why does mutation of such a key enzyme only partially affect IAA biosynthesis but not block all IAA biosynthesis remains largely unknown (Fig. 2). Previous hypotheses included: the presence of alternative pathways for compounding downstream intermediates (Ouyang et al. 2000; Zhang et al. 2008); and a mutation in *WEI2/ASA1/CKRC6* alters the expression of other auxin biosynthesis-related genes and offsets the auxin deficit (Fig. 4). Based on results in this report, we proposed another possible mechanism:

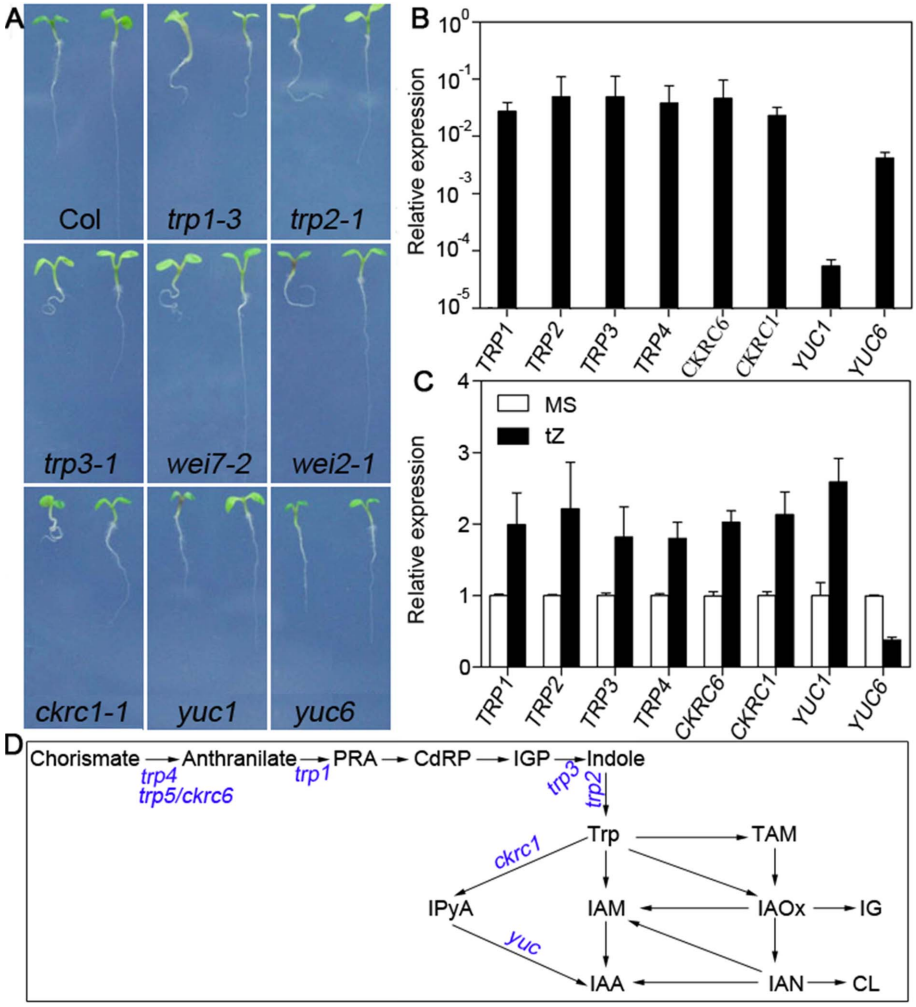


**Fig. 5.** The Trp biosynthesis pathway deficient mutants can be screened from our system. Phenotype of Trp biosynthesis deficient mutants on tZ-containing medium (A). Relative expression of *TRP* genes in root of Col (B). The TRP genes can be induced by tZ treatment (C). Data are mean of 3 replicates. Error bars indicate SD. The biosynthetic pathways of Trp and IAA (D) with key mutants of each biosynthetic enzyme labeled. Relevant enzymes are TRP1, TRP2, TRP3, ASB1/WEI7/TRP4, ASA1/WEI2/TRP5/CKC6, CKRC1/TAA1 and YUCCA.

the *CKRC6* mutation decreased the auxin content, resulting in a feedback mechanism that, possibly regulated by CK, increase auxin biosynthesis (Fig. 4E). In other words, mutation of an auxin biosynthesis gene results in an altered balance of auxin and CK (Fig. 3, Fig. 4) and an altered response to ethylene (Fig. S2) (Stepanova et al. 2005). In summary, *ASA1/WEI2/CKRC6* plays an important role in the crosstalk among auxin, CK and ethylene.

## Materials and Methods

### Plant Material and Growth Conditions

For mutant screening, the *Arabidopsis thaliana* activation-tagged T-DNA pools (CS31100, Col-2 background, composed of approximately 62,000 individual lines) (Weigel et al. 2000) were purchased from the Arabidopsis Biological Resource Center (ABRC) (http://abrc.osu.edu/). Germination and plant growth took place at 25°C with a 16-hours light/8-hours dark cycle. For growth analyses, seedlings were grown on vertical Murashige-Skoog (MS; 1.1% w/v agar and 10 g/L sucrose) plates for 7 d (Zhang et al. 2010).

Arabidopsis accession Col-2 was used as wild-type. Mutants used in this study have been described: *pDR5::GUS* marker line (Ulmasov et al. 1997); *ckrc1-1* (*ckrc1-1/pDR5::GUS*) (Zhou et al. 2011); *wei2-1* (N16397), *trp1-3* (N8159), *trp2-1* (N8327), *trp3-1* (N8331), *arr1-3/12-1* (N6981) and *tir1-1* (N3798), which were purchased from the The Nottingham Arabidopsis Stock Centre (NASC) (http://arabidopsis.info/); *wei2-2* (SALK_071444) and *amp1* (SALK_138749), which were purchased from the ABRC (http://abrc.osu.edu/).

To generate the *ckrc6*/*pDR5:GUS* mutant, the *ckrc6* mutant was crossed with *pDR5:GUS* and the double homozygous mutant was obtained from $F_2$ generation.

### Phenotype Characterization

For root inhibition assays and biochemical complementation, seeds were germinated and grown vertically on MS medium with various phytohormones or compounds at 25°C for 7 d. All the data were the

mean of three separate experiments using at least 40 seedlings.

For analyzing the gravitropism of roots, germinated seedlings were transferred to fresh media with different phytohormones (CK and/or auxin) and grown vertically on MS plate 5 d at 25°C. Three hours after vertically growing, the plates were rotated 90 degrees and cultured for 24 hours. The degree of root curling was measured for approximately 100 seedlings per genotype or treatment.

Gene Cloning

To obtain the mutated gene, whole genome resequencing was carried out (WGRS, provided by Hangzhou Guhe Information and Technology Co., Ltd) (http://www.guheinfo.com/).

RNA Preparation and Real-time qRT-PCR Analysis

RNA was extracted using TRIzol agent (Sangon Biotech, http://www.sangon.com/). First-strand cDNA was synthesized from 1 μg of total RNA pretreated by RNase free-DNase using PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, http://www.takara-bio.com/). The cDNA was diluted 5 times for Real-Time PCR.

For quantitative RT-PCR (qRT-PCR), 20 μL amplication reactions contained 10 μL SYBR Premix Ex Taq (Takara), 0.8 μL of each primer (Table S1) (Lee and Seo 2014), 1.6 μL cDNA and 6.4 μL $ddH_2O$. The results were normalized relative to *ACTIN8* (At1g49240). All real-time qRT-PCR amplifications were performed in a Bio-Rad CFX96™ Real-time System (Bio-Rad, http://www.bio-rad.com) using the following PCR program: An initial denaturation at 95°C for 30 s and 40 cycles of 95°C for 5 s and 60°C for 30 s. PCR reactions were denatured at 95°C for 15 min (Guo et al. 2013) for melting curve analysis. Each experiment was repeated three times, and each reaction was setup in triplicate.

Seedlings were subjected to different treatments prior to RNA isolation. For analyzing the CK- and auxin-induced gene expression, 7-d-old seedlings grown on MS medium were treated in liquid MS medium with 10 μM trans-zeatin (tZ) for 30 minutes (Laxmi et al. 2006) and/or 20 μM IAA for 1.5 hours (Tian et al. 2002). For analyzing CK-induced Trp biosynthesis genes expression, including Trp biosynthesis gene *TRP1-5*, 7-d-old seedlings grown on MS and/or 0.1 μM tZ medium were used.

Histochemical GUS Assay

Seedlings containing *GUS* marker were treated with different phytohormones on MS plates and then incubated in 1 mmol/L X-gluc (5-bromo-4-chloro-3-indolyl-â-D-glucuronide) and 50 mmol/L potassium phosphate buffer, pH7.5, with 0.1% v/v Triton X-100 at 37°C for 20 minutes. Gus staining was observed with microscope (Zeiss AX10, http://www.zeiss.com.cn).

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## Author's Contributions

D.W.D. designed the research and performed major parts of the experiments. P.L. isolated the mutant. L.W., P.L., L.Z., T.Z.Z., X.S., S.D.W. and C.W.A. performed parts of the research. D.W.D. and L.W. analyzed data, tested statistics, and coordinated the figures. D.W.D. wrote the article. G.Q.G participated in discussion and reedited the article. All the authors declare that they have no conflict of interest.

## Supporting Information

**Fig. S1.** L-Trp content in alleles of *ASA1*.
**Fig. S2.** The *ckrc6* mutant exhibits other auxin defect phenotypes.
**Fig. S3.** Detection of CK and auxin response in Col and *ckrc6* mutant.
**Table S1.** PCR primer sequences.

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