A Role of Cytokinin Transporter in *Arabidopsis* Immunity

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Accepted 23 February 2017.

The phytohormone cytokinin (CK) is not only essential for plant growth and development but also impacts plant immunity. A mutant screen in a constitutively active plant immune receptor mutant *snc1* (suppressor of *npr1*, constitutive1) identified a suppressor mutation of *SNC1*-induced defense responses in an ABC transporter coding gene *ABCG14*. *ABCG14* transports CK from roots to the shoots, and the suppression of the *SNC1*-mediated defense response by the loss of *ABCG14* is due to a deficiency of *trans*-zeatin (*tZ*)-type CK in the shoot. In addition, exogenous application of the *tZ*-type CK enhances disease resistance associated with increased expression of the plant immune receptor gene *SNC1*. Taken together, this study further established the role of *tZ*-type CK in disease resistance and suggests a new intersection of CKs with plant immunity at the expression regulation of a plant immune receptor gene.

Plant immunity against pathogens occurs at multiple levels and there are at least two branches of defenses at the molecular level (Jones and Dangl 2006). One is triggered by the perception of pathogen-associated molecular patterns (PAMP) with transmembrane pattern recognition receptors, namely PAMP-triggered immunity (PTI). The other is triggered by the recognition of pathogen-secreted effectors by intracellular immune receptors or resistance (R) proteins, namely effector triggered immunity. Specific recognition of effectors by immune receptor R proteins leads to rapid and efficient defense responses, including a form of programmed cell death named hypersensitive response, which restricts the growth and spread of pathogens (Hammond-Kosack and Jones 1996).

Phytohormones function as intrinsic signals to regulate plant growth and development as they directly regulate cell division and cell expansion. They also respond to biotic and abiotic signals and mediate adaptive responses to environmental changes. With pathogen infection or herbivore attack, a number of hormones have altered levels and are shown to contribute to plant immunity (Bari and Jones 2009; Howe and Jander 2008;

Katagiri and Tsuda 2010; Pieterse et al. 2009). Salicylic acid (SA) and jasmonic acid (JA) are central players in defenses against biotrophic and necrotrophic pathogens, respectively (Browse 2009; Vlot et al. 2009). Other hormones, including ethylene (ET) (van Loon et al. 2006), gibberellins (GAs) (Navarro et al. 2008), abscisic acid (ABA) (Ton et al. 2009), auxins (Kazan and Manners 2009), brassinosteroids (Nakashita et al. 2003), and cytokinins (CKs) (Walters and McRoberts 2006), act as modulators of plant defense responses as well. ET is widely considered to cooperate with JA to induce necrotroph resistance while antagonizing SA-mediated biotroph resistance (Derksen et al. 2013). GA signaling acts as a negative regulator of resistance to the necrotrophic fungus *Alternaria brassicicola*, because the *Arabidopsis* mutants lacking four of the five GA signaling-repressing DELLA proteins showed enhanced susceptibility to the necrotroph pathogen (Achard et al. 2008; Navarro et al. 2008). ABA has multifaceted interaction with plant immunity (Ton et al. 2009), and ABA and SA affect each other at multiple steps from biosynthesis to signaling (Cao et al. 2011; de Torres Zabala et al. 2009; Jiang et al. 2010; Mang et al. 2012; Yasuda et al. 2008). Auxin has a negative role in pathogen resistance. During PTI, induction of the microRNA miR393 by the bacterial PAMP flagellin targets auxin receptor gene *TIR1*, leading to an enhancement of pathogen resistance by suppressing auxin signaling (Navarro et al. 2006).

CKs consist of a highly complex family of *N*⁶-substituted adenine derivatives and chemically unrelated phenylurea-type regulatory molecules, such as *trans*-zeatin (*tZ*), *N*⁶-(Δ^2 -isopentenyl) adenine (iP), dihydrozeatin, and *cis*-zeatin. These compounds are active at the sites of their biosynthesis (Zhao 2008) as well as in distant tissues, in which they arrive via long-distance transport. *ABCG14*, an ABC-type (ATP-binding cassette) transporter, is shown to mediate the translocation of *tZ*-type CK from roots to shoots through xylem and to regulate shoots growth (Ko et al. 2014; Zhang et al. 2014). CKs have long been known to be involved in plant-microbe interactions. Overaccumulation of CKs is associated with disease symptoms and morphological anomalies such as fasciation, senescence, and the formation of tumors, galls, and so-called green islands (Grant and Jones 2009). A dozen bacterial and fungal pathogens can either secrete CKs themselves, induce CK biosynthesis in plants, or both (Naseem et al. 2014; Siemens et al. 2006; Walters et al. 2008). In addition, pathogens may also activate plant CK signaling, leading to the suppression of plant immunity. HopQ1, an effector from *Pseudomonas syringae* induces the expression of type A *Arabidopsis* response regulator (ARR) genes in CK signaling to downregulate expression of the flagellin receptor gene *FLS2* in PTI (Hann et al. 2014).

The levels of CKs are found to impact the strength of plant immunity. Low concentrations of the benzyl adenine (BA) CK enhanced susceptibility of *Arabidopsis* to the biotrophic pathogen *Hyaloperonospora arabidopsidis*, whereas high concentrations of BA enhanced resistance to the pathogen in *Arabidopsis*

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*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary table is published online.

(Argueso et al. 2012). CK has a positive role in resistance to hemibiotrophic *Pseudomonas syringae* pv. *tomato* DC3000. The CK receptor mutant *ahk2ahk3* and transgenic line over-expression of CK oxidases (CKXs) both exhibited enhanced susceptibility to *P. syringae* pv. *tomato* DC3000 (Choi et al. 2010). CK is shown to interact with SA to affect immunity. Synergistic interaction is found between CKs and SA in resistance to *P. syringae* pv. *tomato* DC3000, and a direct interaction was found between the CK-activated transcription factor ARR2 and the SA-response transcription factor TGA3 (TGA1A-related gene 3) (Choi et al. 2010, 2011). Over-expression of a CK response factor 2 (*AtCRF2*) enhances SA biosynthesis and leads to autoimmune response in *Arabidopsis* (Kwon 2016). On the other hand, SA is found to inhibit CK signaling in resistance against *H. arabidopsidis*, which may form a feedback mechanism to fine-tune the effect of CK in plant immunity (Argueso et al. 2012).

Fig. 1. The *int211 snc1-1* double mutant has enhanced dwarf phenotype at 22 and 28°C. **A**, Growth phenotypes of wild-type Col-0, *snc1-1*, and *int211 snc1-1* grown at 22 and 28°C under constant light at 14 days after germination. **B**, Growth of *Pseudomonas syringae* pv. *tomato* DC3000 in Col-0, *snc1-1*, and *int211snc1-1* plants grown under 12 h of light at 22 and 28°C at 0 and 3 days postinoculation from three sets of seedlings for each genotype and temperature combination. Values represent means $\pm$ standard deviation ($n = 3$). Asterisks (**) indicate a significant difference from the wild-type plants ($P < 0.05$ according to a Student's *t* test). Similar results were obtained in three independent experiments.

Here, we report a role of a *tZ*-type CK transporter gene, *AtABCG14*, in plant immunity, through a genetic screen for modifiers of autoimmune mutant *snc1-1*. We found that the loss of *AtABCG14* suppresses *snc1-1* autoimmunity and down-regulates the expression of *SNC1*. In addition, exogenous application of *tZ*-type CK enhances basal resistance to *P. syringae* pv. *tomato* DC3000 and enhances expression of *SNC1*. Together, the results in this study revealed an important role of *tZ*-type CK in plant disease resistance.

RESULTS

The *int211* mutation suppresses *snc1-1* autoimmunity.

The *snc1-1* mutant has a dwarf phenotype at 22°C, resulting from constitutive defense responses, but it is wild type in appearance at 28°C, due to high temperature suppression of defense responses (Yang and Hua 2004). A genetic screen for mutants with dwarf phenotype in the *snc1-1* background at 28°C was carried out to identify *int* mutants that have enhanced resistance at high temperature (Zhu et al. 2010). One putative mutant, *int211snc1-1*, displayed a similar dwarf phenotype at 22 and 28°C (Fig. 1A). However, unlike several other *int snc1* mutants in which the growth defect is correlated with enhanced disease resistance (Mang et al. 2012; Zhu et al. 2010, 2012), this *int211snc1-1* mutant did not have an increased disease resistance at 28°C. These plants were inoculated with virulent pathogen *P. syringae* pv. *tomato* DC3000 and pathogen growth was measured at day 0 and day 3 after inoculation (Fig. 1B). At 22°C, the *snc1-1* plants showed enhanced resistance to the pathogen, while *int211snc1-1* had much reduced resistance compared with *snc1-1* and supported similar levels of pathogen growth as the wild type (Fig. 1B). At 28°C, all the plants supported the same amount of bacterial growth at 3 days after inoculation (Fig. 1B). Therefore, a mutation in the *int211snc1-1* plant or, more likely, the same mutation causing the dwarf phenotype of *int211* is also a suppressor of the enhanced disease resistance phenotype of *snc1-1*.

Cloning of the *INT211* gene as *ABCG14*.

To identify the causal mutation for the growth and defense defects in *int211snc1-1*, we performed next-generation sequencing for mapping and gene identification (Zhu et al. 2012). The *int211snc1-1* double mutant was crossed to the original *snc1-1* mutant, and the *int211snc1-1* double mutant-looking plants segregated at one out of four in the F2 progenies grown at 28°C. Approximately 100 *int211snc1-1*-looking plants and 200 *snc1-1*-looking plants were collected and pooled. Genomic DNAs were extracted and purified from these mutant and non-mutant pools, and libraries constructed from these DNAs were subject to next-generation sequencing on the Illumina Hi-Seq 2500 machine with single-end reads of 100 bp.

We obtained approximately 25 $\times$ coverage of the *Arabidopsis* genome for each of the two samples. By searching for enrichment of mutant alleles in the mutant pool (Zhu et al. 2012), we identified one major peak on chromosome I. Seven consecutive single nucleotide polymorphisms (SNPs) between positions 10,928,363 and 12,450,361 were found to have nonreference alleles in all reads from the mutant pool but not those from the nonmutant pool, indicating that they are *int211* candidate mutations. On the basis of annotation by ANNOVAR (Wang et al. 2010), two of seven SNPs were alterations in the intergenic or noncoding regions and five caused nonsynonymous mutations (Fig. 2A). We subsequently obtained two T-DNA insertion lines (Alonso et al. 2003) for each of the seven candidate genes from the *Arabidopsis* Biological Resource Center. Homozygous mutants were isolated for these 14 lines and only SK_15917 (*abcg14-1*) and SK_15918 (*abcg14-2*), both in *ABCG14* (At1g31770), exhibited a dwarf phenotype. We suspect that this

could be the single mutant phenotype of *int211*, because a similar phenotype segregated in the F2 population of a cross between *int211snc1-1* and Col-0. The *int211snc1-1* contains a transition mutation of C to T, which results in substitution of serine with phenylalanine in the ABCG14 protein (Fig. 2A).

To verify that this mutation in *ABCG14* is indeed the causal mutation of the growth defect of *int211snc1-1*, we crossed *snc1-1* with two *ABCG14* mutant alleles, *abcg14-1* and *abcg14-2*, respectively, and isolated the *abcg14-1snc1-1* and *abcg14-2snc1-1* double mutants. The double mutants exhibited a dwarf phenotype similar to *int211snc1-1* at both 22 and 28°C (Fig. 2B and C). In addition, we introduced a functional enhanced green fluorescent protein (*eGFP*) and *ABCG14* fusion gene *pABCG14::EGFP-ABCG14* to *int211snc1-1* by crossing (Zhang et al. 2014). The transgenic plants of *pABCG14::EGFP-ABCG14* in *int211 snc1-1* exhibited a *snc1-1*-like

phenotype at both 22°C and 28°C (Fig. 2B and C), indicating that *ABCG14* is, indeed, the *INT211* gene for the growth phenotype. We therefore referenced *int211* as *abcg14-3*.

Reduced growth and defense in *abcg14snc1-1* both result from the loss of *ABCG14* function.

The *abcg14* mutants had retarded shoot growth, smaller rosette leaves, and thinner stems. Because cytokine has been shown to promote cell division, we analyzed the expression of some core cell-cycle regulating genes, including *CDT1A*, *MCM2*, *CYCD3;1*, *CDC20.1*, and *CYCA2;1* (Menges et al. 2005). *CDT1* and *MCM2* are predominantly expressed in G1/S transition and early S phase; *CYCD3;1* is in both G1/S and G2/M transitions; *CDC20.1* is in the S and M phase; and *CYCA2;1* is in the G2/M transition. The *abcg14* mutant had increased expression of *CDT1A* and *MCM2* genes and reduced

Fig. 2. Identification of the *int211* mutation. **A**, Annotation of all seven candidate single nucleotide polymorphisms (SNPs) in the defined *int211* region. The base position, sequence of the reference allele, sequence of the nonreference allele, its associated gene, and type of alteration for each SNP are shown. **B** and **C**, Phenotype of *abcg14-1* and *abcg14-2* single mutants, *abcg14snc1-1* double mutants, and complementation lines in *int211snc1-1* grown at 22°C (**B**) and 28°C (**C**) under constant light for two weeks.

expression of the *CYCD3;1*, *CDC20.1*, and *CYCA2;1* genes (Fig. 3A). This suggests that mitosis is reduced in the mutant, which accounts, at least partially, for the growth defect.

We subsequently quantified the biomasses as a readout of growth, to determine how *abcg14* and *snc1-1* interact to impact the growth. The wild type, *snc1-1*, *abcg14-3*, and *abcg14-3snc1-1* plants were grown under constant light for 14 days after germination, before measurement. At 22°C, the *snc1-1* and *abcg14-3* single mutants were 48 and 30% of the wild-type weight and the double mutant was 14% of the wild-type weight (Fig. 3B). At 28°C, *snc1-1* had a similar weight to the wild type, due to a lack of defense activation, and the *abcg14-3* and *abcg14-3snc1-1* mutants were both approximately 28% of the wild-type weight (Fig. 3B). These results indicate that *abcg14*

has an additive interaction with *snc1-1* to affect growth at both 22 and 28°C.

We further asked whether or not the mutation in *ABCG14* is also responsible for the suppressed disease resistance in *int211snc1-1* compared with *snc1-1*. To this end, we analyzed bacterial growth of *P. syringae* pv. *tomato* DC3000 in the double mutants *snc1-1* with two *abcg14* alleles. Both the *abcg14-1* and the *abcg14-2* mutations suppressed the disease resistance phenotype of *snc1-1* at 22°C (Fig. 4A). In addition, the transgene *pABCG14::EGFP-ABCG14* that rescued the growth defect of *int211snc1-1* also rescued its defense phenotype. The transgenic line *pABCG14::EGFP-ABCG14 int211snc1-1* showed enhanced resistance to pathogen *P. syringae* pv. *tomato* DC3000 when compared with *int211snc1-1*, to a similar resistance level as that of *snc1-1* (Fig. 4A). Consistent with the disease resistance phenotype, accumulation of *SNC1* and *PR1* transcripts is also affected by *ABCG14*. Analysis by quantitative reverse transcription-polymerase chain reaction (PCR) revealed that both *SNC1* and *PR1* genes had a higher expression level in the *snc1-1* single mutant and were reduced to the wild-type levels in the *abcg14-3snc1-1* and *abcg14-1snc1-1* double mutants (Fig. 4B and C). Therefore, *ABCG14* is the *INT211* gene and it is a positive regulator for both plant growth and *SNC1*-mediated disease resistance.

Exogenous application of *tZ*-type CK rescues growth defects of *abcg14* but not *abcg14snc1-1*.

ABCG14 was shown to mediate the translocation of *tZ*-type CK from roots to shoots via xylem, to regulate shoot growth (Ko et al. 2014; Zhang et al. 2014). We hypothesized that the enhanced growth defect of *abcg14-3snc1-1* compared with that of *snc1-1* is due to a deficiency of *tZ*-type CK. To test this, we applied iP and *tZ* types of CK externally to the plants every day. Application of the *tZ* type but not much of the iP type of CK induces a slight elongation of petioles in both the wild type and *abcg14-3* mutant at 22°C (Fig. 5A and C). Among the different concentrations tested, 10 µM of *tZ*-type CK had the best effect in rescuing the smaller rosette phenotype of the *abcg14-3* single mutant at 22°C, and the *abcg14-3* mutant grew similarly to the wild type with this application (Fig. 5A and C). Surprisingly, CK application did not revert the *abcg14-3snc1-1* double mutant phenotype to the *snc1-1* single mutant phenotype, and the *abcg14-3snc1-1* remained the same dwarf phenotype with or without CK application (Fig. 5A and C). At 28°C, the *tZ*-type (not the iP-type) CK partially rescued the growth defects of the *abcg14-3* and the *abcg14-3snc1-1* mutants, and these two mutants grew larger with *tZ*-type CK application than without application but were smaller than wild type or *snc1-1* without CK application (Fig. 5B and C).

The apparent lack of effect of CK application on *abcg14-3snc1-1* growth phenotype at 22°C prompted us to investigate the effect of CK application on defense responses independent of its promoting effect on plant growth in *snc1-1*. Indeed, the *snc1-1* single mutant plants were smaller with the treatment of *tZ*-type CK and their leaves had more pronounced water-soaked phenotype associated with defense activation at 22°C and constant light (Fig. 5A). The *abcg14-3snc1-1* double mutant plants developed leaves that were more curved and water-soaked with *tZ*-type CK application (Fig. 5A). We also analyzed plants grown under a 12-h light and 12-h dark photoperiod, in which defense activation in *snc1-1* is milder than under constant light. Indeed, the *abcg14-3snc1-1* double mutant has a milder growth defect grown under 12 h of light than under constant light, likely due to a more complete inhibition of defense activation from *snc1-1* by the *abcg14-3* mutation (Fig. 5D). Again, *tZ*-type CK rescued the *abcg14-3* single mutant to the size to the wild type, but it did not rescue the *abcg14-3snc1-1* double mutant to the size of *snc1* (Fig. 5D). Furthermore,

Fig. 3. *ABCG14* mutations have an additive effect on *snc1-1*. **A**, The expression of cell cycle-related genes in *abcg14* mutants and wild type. **B**, Fresh weight of Col-0, *abcg14-3*, *snc1-1*, and *abcg14-3snc1-1* mutants grown at 22 and 28°C under constant light for 2 weeks after germination (mean value $\pm$ standard deviation [$n = 20$]).

application of *tZ*-type CK enhanced the water-soaked and curved-down phenotype in leaves of *snc1-1* and *abcg14-3snc1-1* grown under 12 h of light, similarly to those seen under constant light (Fig. 5D). These observations suggest that exogenous application of *tZ*-type CK may enhance plant immune responses in *snc1-1*. We hypothesized that deficiency of *tZ*-type CK simultaneously reduces growth and suppresses immune responses in *snc1-1*, and application of *tZ*-type CK not only brings back but, also, further enhances immunity in *abcg14-3snc1-1* leading to growth inhibition.

Exogenous application of *tZ*-type CK enhances plant disease resistance.

To further test the above hypothesis, we performed a pathogen growth assay after spraying with CK under the 12-h light and 12-h dark condition. The plants of Col-0, *snc1-1*, *abcg14-3*, and *abcg14-3 snc1-1* were sprayed with CK daily for 10 days

before inoculation with the virulent pathogen *P. syringae* pv. *tomato* DC3000 (Fig. 6A). The four concentrations (0.01, 0.1, 1, and 10 μ M) used gave similar results, and the 10- μ M data are presented here. The *tZ*-type CK conferred a significant reduction of pathogen growth in all four genotypes compared with plants sprayed with iP-type CK or the solvent control. The enhancement of resistance was most drastic in the *abcg14-3snc1-1* double mutant, with a more than 38-fold decrease in pathogen growth (Fig. 6B).

We subsequently analyzed the expression of defense-related genes with application of the *tZ*-type CK. Daily spray with *tZ*-type CK increased expression of *PR1* by fourfold compared with the control. Interestingly, *SNC1* expression is also increased by about fourfold (Fig. 6C). This result indicates that application of *tZ*-type CK enhances basal disease resistance and increases *SNC1* gene expression.

Fig. 4. Loss-of-function mutation in *ABCG14* suppresses the constitutive defense response in *snc1-1*. **A**, Growth of *Pseudomonas syringae* pv. *tomato* DC3000 in Col-0, *abcg14*, *snc1-1*, *abcg14snc1-1*, and complementation lines grown under a 12-h light condition at 22 and 28°C, at 0 and 3 days postinoculation, from three sets of seedlings in each sample. Values represent means $\pm$ standard deviation ($n = 3$). Similar results were obtained in three independent experiments. Asterisks (**) indicate a significant difference from wild-type plants ($P < 0.05$ according to a Student's *t* test). **B**, Expression of *SNC1* and **C**, *PR1* in above-mentioned plants grown under constant light for 2 weeks, as assayed by reverse transcription-polymerase chain reaction. *Actin* was used as internal control. Three biological repeats were performed and similar results were obtained.

DISCUSSION

CKs act as both long-range and local signals to regulate cell division and differentiation throughout plant development, such as chloroplast development, leaf senescence, and root- and shoot apical dominance (Chory et al. 1994; Davies et al. 1966; Matsumoto-Kitano et al. 2008). Recently, a role of CKs in plant immunity has also been discovered and investigated. Exogenous application of *tZ*-type CK or overexpression of CK biosynthetic enzyme genes exhibited enhanced resistance to virulent bacterial pathogen *P. syringae* pv. *tomato* DC3000 in *Arabidopsis* (Choi et al. 2010; Naseem et al. 2012). In contrast, CK receptor mutant *ahk2ahk3* and transgenic line overexpression of CK-degrading enzyme genes such as *CKX2* and *CKX4* displayed enhanced susceptibility to *P. syringae* pv. *tomato* DC3000 (Choi et al. 2010). In this study, we found further evidence for the role of *tZ*-type CK in resistance against *P. syringae* pv. *tomato* DC3000 and revealed a new target of CK in plant immunity.

ABCG14 is an ABC transporter, shown to translocate the *tZ*-type of CK from roots to shoots through xylem (Ko et al. 2014; Zhang et al. 2014). Plants without *ABCG14* showed abnormal expression of key cell cycle-related genes, which likely accounts, at least partially, for the growth defect in the *abcg14* mutant (Fig. 3A). The *abcg14* and *snc1-1* mutations displayed an additive effect on growth inhibition in terms of biomass and plant size, indicating that CK and plant immune receptor *SNC1*-mediated autoimmunity affect plant growth independently (Figs. 2B and C and 3B). CK has numerous effects on plant growth, including the development of xylem and phloem cells in the

vascular bundle (Zhang et al. 2014). It would be interesting to determine, in the future, how other aspects of growth besides biomass and size are affected by the combination of immune activation and CK pathway perturbation.

The *tZ*-type CK is required for defense responses mediated by *SNC1*, as autoimmunity of *snc1-1* is suppressed by the deficiency of *tZ*-type CK in the *abcg14* mutation. Interestingly, a gain-of-function mutant of another immune receptor, UNI (*uni-1D*), showed higher accumulation of *tZ*-type CK but not *iP*-type CK (Hwang et al. 2012; Igari et al. 2008). The *rin4* knockdown mutant, in which the two immune receptor proteins RPS2 and RPM1 are activated, also had upregulation of CK ARR transcription factor genes (Igari et al. 2008). The CK pathway activation was postulated to mediate, at least partially, the growth effect from immunity activation. It is not yet determined whether either the CK amount, the CK response, or both are activated in the *snc1-1* mutant. On the other hand, application of *tZ*-type CK enhances disease resistance to pathogen *P. syringae* pv. *tomato* DC3000 in wild type and *snc1-1*, indicating that CK activation might contribute to defense activation in the autoimmune mutants and during defense responses against pathogens. CK (BA type) was shown to have a concentration-dependent effect on resistance to *H. arabidopsidis* (Argueso et al. 2012). We did not observe an obvious concentration-dependent effect of *tZ*-type CK on resistance to *P. syringae* pv. *tomato* DC3000. Future study is needed to measure endogenous CK concentrations after exogenous application, to determine whether or not there is a difference in concentration effect of CK between resistance to the

Fig. 5. Exogenous application of *tZ*-type cytokinin rescues the *abcg14* single but not the *abcg14snc1-1* double mutant growth phenotype. **A** and **B**, Phenotype of wild type, *snc1-1*, *abcg14-3*, and *abcg14-3snc1-1* plants grown under constant light and sprayed with 0.1% dimethyl sulfoxide (control) solution, 10 μ M *iP*-type, and 10 μ M *tZ*-type cytokinin once a day for 10 days, starting from 5 days after sowing at 22°C (**A**) and 28°C (**B**) respectively. **C**, Quantification of rosette areas of plants in **A** and **B**. The area is calculated from the smallest circle encompassing the rosette. Values are mean $\pm$ standard deviation ($n = 10$). Asterisks (*) indicate a significant difference from each control plant ($P < 0.05$ according to a Student's *t* test). **D**, Phenotype of the same plants as in (**A**) but grown under a 12-h light and 12-h dark photoperiod.

hemibiotrophic *P. syringae* pv. *tomato* DC3000 and that to the biotrophic *H. arabidopsidis* pathogens.

Earlier studies found a synergistic effect of CK and SA in the plant immune signaling network and the effect was attributed to CK response transcription factors that also regulate plant immunity genes. An increase or a decrease of the positive CK-regulating transcription factor ARR2 lead to enhanced or decreased resistance to bacterial pathogen *P. syringae* pv. *tomato* DC3000, respectively. ARR2 is shown to interact directly with the SA transcription factor TGA3, to positively regulate *PR1* gene expression (Choi et al. 2010, 2011). The negative CK-regulating type A ARR transcription factors are found to

be negative regulators of resistance to biotrophic pathogen *H. arabidopsidis* Noco2 (Argueso et al. 2012). Overexpression of a CK response factor 2 (*AtCRF2*) leads to autoimmune response by enhancing SA biosynthesis in *Arabidopsis* (Kwon 2016). The direct binding targets of these transcription factors in the later cases were not known.

Here, we found a deficiency in *tZ*-type CK led to the reduction of expression of *R* gene *SNC1* (Fig. 4B) and exogenous application of *tZ*-type CK upregulated *SNC1* expression (Fig. 6C). Therefore, expression of plant immune receptor genes could be another target of CK intersection with plant immunity. It will be interesting to test whether or not CK-response

Fig. 6. Exogenous application of *tZ*-type cytokinin enhances plant disease resistance. **A**, Phenotype of wild-type, *snc1-1*, *abcg14-3*, and *abcg14-3snc1-1* plants grown at 22°C under a 12-h light and 12-h dark photoperiod. They were sprayed with control solution, 10 μ M iP-type, and 10 μ M *tZ*-type cytokinin once a day for 10 days, starting from 5 days after sowing. **B**, Growth of bacterial strains of *Pseudomonas syringae* pv. *tomato* DC3000 at 0 and 3 days postinoculation in plants in **A**. Each genotype had three biological repeats in each experiment. Values represent means $\pm$ standard deviation ($n = 3$). An asterisk (*) indicates a significant difference from each control plant at $P < 0.05$ and two asterisks (**) indicate a difference at $P < 0.005$, according to a Student's *t* test. Similar results were obtained in three independent experiments. **C**, Expression of *SNC1* and *PR1* is up-regulated in Col-0 plants sprayed with 10 μ M *tZ*-type cytokinin once a day for 14 days, starting from 5 days after sowing and grown at 22°C under a 12-h light photoperiod. *Actin* was used as internal control. Three biological repeats were performed and similar results were obtained.

transcription factors directly regulate expression of plant immune receptor genes. Future study should reveal the impact of immune receptor activation and CK responses on each other in both plant growth and plant immunity.

MATERIALS AND METHODS

Plant material and growth conditions.

Arabidopsis thaliana plants were grown on soil at 22 and 28°C, with light intensity at 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and relative humidity at 50 to 70%. Plants were grown under constant light for morphological phenotypic and gene expression analysis unless stated otherwise. Plants were grown under the condition of 12 h of light and 12 h of darkness for pathogen growth tests.

The *pABCG14::EGFP-ABCG14* transgenic line (Zhang et al. 2014) was crossed to *int211* single and *int211snc1-1* double mutants, respectively, for complementation.

Mutant screen and next-generation sequencing.

Mutant screen was carried out as described by Mang et al. (2012) and Zhu et al. (2013). The *snc1-1* seeds were treated with 0.25% ethane methylsulfonate for 12 h. Approximately 30,000 M2 plants (derived from 3,000 M1 plants) were screened at 28°C for *int* mutants with the 22°C *snc1-1*-like dwarf phenotype. For mapping, F2 progenies from a cross of *int211 snc1-1* and *snc1-1* were grown and were scored at 28°C. Equal amounts of leaf tissues were collected from each plant and tissues from mutant and nonmutant plants were pooled separately for genomic DNA extraction, using the E.Z.N.A. SP plant DNA midi kit (Omega Bio-tek, Inc.). DNA libraries for Hi-Seq were constructed using NEBNext Ultra II DNA library prep kit (New England Biolabs). The libraries were run on Illumina Hi-Seq with single reads of 100 bp.

Pathogen resistance assay.

Pseudomonas syringae pv. *tomato* DC3000 was grown 2 to 3 days on King's B medium with 50 mg of kanamycin per liter and were resuspended at 10^5 CFU ml^{-1} in a solution of 10 mM MgCl_2 and 0.02% Silwet L 77. Two-week-old seedlings (grown under 12 h of light and 12 h of dark) were dip-inoculated with bacteria and were kept covered for 1 h, before being slowly uncovered. The amount of bacteria in plants was analyzed at 1 h after dipping (day 0) and 3 days after dipping (day 3). The aerial parts of three inoculated seedlings were pooled per sample, and three samples were collected for each genotype and time point. Seedlings were ground in 1 ml of 10 mM of MgCl_2 , and serial dilutions of the ground tissue were used to determine the number of CFU per gram of fresh leaf tissue.

Real-time quantitative PCR and gene expression.

Total RNA was extracted with TRIzol reagent (Invitrogen) from leaves of 2- to 3-week-old plants, according to the manufacturer's protocol. To remove any contaminating DNA, the samples were treated with DNase (RNase-free DNase kit; Promega). cDNAs were synthesized from total RNA by using AffinityScript QPCR cDNA synthesis kit (Agilent Technologies). Real-time quantitative PCR was performed on the *BIO-RAD* PCR system using iQ SYBR Green supermix (Bio-Rad). Primers used in this study are presented in Supplementary Table S1.

Exogenous CK application.

Seeds were directly sowed on the soil. After 5 days of growth, the plants were sprayed with 0.01, 0.1, 1, and 10 μM of *tZ*-type or *iP*-type CK or 0.1% dimethyl sulfoxide + 0.02% Silwet 77 solution (control) every day.

ACKNOWLEDGMENTS

We thank the *Arabidopsis* Biological Resource Center for the *Arabidopsis* mutants and C. Liu for the *pABCG14::EGFP-ABCG14* transgenic plant. This work was supported by grants from the National Science Foundation (IOS-0919914 and IOS-1353738) to J. Hua. S. Wang was supported by a fellowship from the China Scholarship Council. J. Hua and Shuai Wang designed the experiments; Shuai Wang, Shu Wang, L. Yang, and Y. Zhu performed experiments; Shuai Wang, Q. Sun, and J. Hua analyzed data; Y. Yuan provided supervision; and Shuai Wang and J. Hua wrote the paper.

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