Plant Physiology and Biochemistry 153 (2020) 1–10

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Expression and functional analysis of VviABCG14 from *Vitis vinifera* suggest the role in cytokinin transport and the interaction with VviABCG7

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ARTICLE INFO

Keywords:
Grapevine
ABCG transporters
Gene expression
Cytokinin transport
ABCG dimer

ABSTRACT

Cytokinins are important hormones involved in many aspects of plant growth and development. However, there remain many knowledge gaps with regard to their metabolism and transport mechanisms. Here, we characterise a half-size ATP binding cassette G (ABCG) transporter gene, also called white-brown complex transporter, *VviABCG14*, from grapevine (*Vitis vinifera* L. cv. Pinot noir). Quantitative real-time PCR analysis shows the expression of *VviABCG14* gene is significantly increased after grape berries are treated with exogenous N-(2-chloro-4-pyridyl)-N′-phenylurea (CPPU) and *trans*-zeatin (*tZ*). Significant differences in phenotype were observed between overexpressing *VviABCG14* transgenic and wild-type *Arabidopsis* lines grown for 12 days. The fresh weight of transgenic *Arabidopsis* was greater than of wild-type plants, and root lengths were greater. After growing in soil for 26 days, the vegetative growth of transgenic lines significantly greater than the wild-type and the bolting rate was lower. Hormone content analysis indicates the levels of *tZ* in the shoots of overexpressing transgenes are higher than in wild-types. Using the split-ubiquitin yeast membrane system and bimolecular fluorescence complementation assay we show VviABCG14 and VviABCG7 transporter can form a heterodimer. Meanwhile, *VviABCG7* is also significantly induced by exogenous CPPU and *tZ* in grape berries. Altogether, our results suggest VviABCG14 may affect the phenotype of *Arabidopsis* by transporting cytokinins and VviABCG14 interacts with VviABCG7 to form a heterodimer.

1. Introduction

Phytohormones are trace organic substances (1 μmol/L or less) that are synthesized in plants and transported from the sites of synthesis to other sites, which have a significant effect on growth and development (Pan, 2004). Cytokinins are a class of plant hormones that promote cell division, induce bud formation and promote their growth. Letham (1963) was first to isolate and identify zeatin in corn and demonstrate its involvement in seed development. Subsequent studies have shown that cytokinins also play essential roles in fruit and seed development (Cheng et al., 1996; Rijavec et al., 2010; Ruan et al., 2010). In the early stages of fruit and seed development in *Arabidopsis thaliana*, cytokinin levels rise transiently and remain correlated with cell nucleus and cell division, ultimately, with seed yield (Zwack et al., 2016). Deletion of *Arabidopsis* half-size ATP binding cassette G 14 (ABCG14) results in a deficiency of *trans*-zeatin (*tZ*) type cytokinin in the stem, thereby inhibiting the *SNC1*-mediated defense response (Wang et al., 2017). It has been shown that cytokinins affect plant immunity (Paul et al., 2018).

There are two types of cytokinins in plants: isoprenoids and aromatics. Isopentenyl cytokinins are the main form and include isopentenyladenine (iP), *tZ*, *cis*-zeatin (*cZ*), dihydrozeatin and its riboside (Stirk et al., 2005; Zhao, 2008). If it is to play its role, the cytokinin synthesized at a specific site must be transported to target cells some way away by diffusion or by active transport. The iP-type cytokinin in the phloem is transferred from the aerial parts of the plant to the root to maintain the vascular structure in the root meristem (Bishopp et al.,

Abbreviations: ABC, ATP-binding cassette protein; ABCG, ATP-binding cassette G; BiFC, bimolecular fluorescence complementary assay; cZ, *cis*-zeatin; ENT, nucleoside transporters; GFP, green fluorescent protein; iP, isopentenyladenine; LC/MS, liquid chromatography-tandem mass spectrometry; NBD, nucleotide binding domain; PDLs, pleiotropic drug resistance; PUP, purine permeases; qRT-PCR, quantitative real-time PCR; TMD, transmembrane domain; *tZ*, *trans*-zeatin; *tZR*, *tZ* riboside; WBCs, white brown complex; YFP, yellow fluorescent protein

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https://doi.org/10.1016/j.plaphy.2020.05.011
Received 23 February 2020; Received in revised form 8 May 2020; Accepted 9 May 2020
Available online 16 May 2020

2011). The *tZ*-type cytokinin is transferred from the root to the aerial parts of the plant through the xylem to regulate shoot growth (Kiba et al., 2013). However, studies on cytokinin transport mechanisms are relatively few. Currently, there are three main types of cytokinin transporters known: purine permeases (PUP) (Kudo et al., 2010), nucleoside transporters (ENT) (Girke et al., 2014) and half-size ABCG transporters (Zhang et al., 2014).

Cytokinin extracellular transporters are poorly understood. Until 2014, it was thought the AtABCG14 transporter could mediate the extracellular transport of cytokinins in the root xylem, and play a role in long-distance transport from the roots to the shoots (Ko et al., 2014; Zhang et al., 2014). AtABCG14 transporter is localized on the plasma membrane and is expressed mainly in the vascular tissues such as in the middle column and the middle column sheath cells in the root elongation zone; Knockout of *AtABCG14* gene leads to defects in long-distance transport of cytokinins, further resulting in abnormal distributions of *tZ*-type cytokinins in the roots (Ko et al., 2014). Meanwhile, it has been demonstrated by isotope labelling that AtABCG14 is an efflux pump of *tZ*-type endogenous cytokinin (Zhang et al., 2014).

ATP-binding cassette protein (ABC) is the oldest, and one of the largest, protein superfamily known. It has been shown that ABC transporters responsible for transmembrane input substrates have substrate-binding proteins that are responsible for transporting substrates to transporters, while ABC transporters responsible for exporting substrates do not (Procko et al., 2009; Berntsson et al., 2010). The ABCG subfamily is the largest subfamily of ABC transporters in plants. It is divided into two classes: half-transporters WBCs (white brown complex) and full-transporters PDLs (pleiotropic drug resistance) (Andolfo et al., 2015). The complete ABC transporters need to contain two nucleotide binding domains (NBD) and two transmembrane domains (TMD), while half-size ABCG transporters consist of one NBD and one TMD, so they must form homologous or heterodimers to create the TMD-NBD-TMD-NBD structure to complete their biological functions (Velamakanni et al., 2007; Verrier et al., 2008). It has been found that ABCG transporters play important roles in many aspects of plant growth, such as AtABCG25 and AtABCG40 which are involved in ABA transport (Kang et al., 2010; Kuromori et al., 2010) and AtABCG26 which is involved in pollen development (Quilichini et al., 2010) and PhPDR1 which is required to transport strigolactone (Kretzschmar et al., 2012). AtABCG11 is involved mainly in the transport of alkane waxes and oils on the surface of plants (Bird et al., 2007). It has been found that AtABCG14 can only form a heterodimer with AtABCG11 (Le Hir et al., 2013). In the *Arabidopsis abcg9/abcg11/abcg14* mutant, there is a serious defect in the vascular tissue, and mutant plants are weaker than wild type plants (Le Hir et al., 2013). AtABCG14 acts as an efflux pump for endogenous cytokinin and is responsible for its transport from the root to the shoot tip (Zhang et al., 2014). It has further been shown that endogenous cytokinin responsible for transport of AtABCG14 is principally *tZ*-type cytokinins (Ko et al., 2014).

Our previous studies using tissue specificity analysis of *VviABCG14* indicate it is highly expressed in young roots, young stems, young tendrils and flowers, while expressions in leaves, ovules and mature pericarps are relatively low (Tang et al., 2018). The expression level of *VviABCG14* is highest in highly metastatic tissues and organs. This is similar to the tissue expression pattern of *AtABCG14*. Therefore, in the present study, based on the *VviABCG14* gene, we have cloned and analysed the ABCG half-transporters that may interact with VviABCG14 in *Vitis vinifera* L. cv. Pinot noir. Then, the response of *VviABCG14* gene to cytokinins was explored. The *VviABCG14* gene was over-expressed in *Arabidopsis* to explore the function of *VviABCG14*. Meanwhile, we searched for the interaction proteins using the membrane protein yeast two-hybrid assay and the bimolecular fluorescence complementary assay (BiFC). Through this work we hope to be able develop a theoretical basis for the study of grapevine cytokinin transport mechanisms.

2. Materials and methods

2.1. Plant materials and exogenous hormone treatments

The *Vitis vinifera* L. cvs. Pinot noir, Youngle and Thompson seedless were planted in the Grape Germplasm Resources of the College of Horticulture, Northwest A&F University in Yangling, Shaanxi, China. In this study, all the grapes used are perennial. Seeds of *Nicotiana tabacum* (tobacco) and Wild-type (Col-0) *Arabidopsis* were obtained from the Grape Germplasm Resources and Breeding Laboratory. *Arabidopsis* and tobacco seeds were placed in phytotron using a 2: 1: 1 mixture of soilrite, perlite and vermiculite. Four seeds of *Arabidopsis* and one tobacco were placed in per pot (8 × 8 cm). Plants grown in phytotron set to 23 °C, 16 h day/18 °C, 8 h night dark photoperiod and relative humidity 70%.

- Fruit clusters on the Thompson seedless and Youngle vines were dipped in 100 μM *tZ* and N-(2-chloro-4-pyridyl)-N′-phenylurea (CPPU), shake for 5 s to ensure the fruits were equally covered, then immediately covered by fruit bags (28 × 36 cm, Guokang, Qingdao, China) and kept on the vines until harvest. Berries were treated with water as a control. The berries were harvested at 0, 0.5, 3, 6, 12, 24, 48 h and 7 days after treatment and stored at −80 °C pending analysis. Treatments were replicated three times. CPPU is a cytokinin-like compound with plant growth regulator activity. The origin of *tZ* and CPPU was Yuanye Bio-Technology, Shanghai, China.

2.2. RNA extraction and synthesis of cDNAs

An EZNA Plant RNA Kit (R6827-01, Omega Bio-tek, USA) was used to isolate total RNA from grape berries and leaves according to the plant RNA difficult sample protocol. A ND-2000 spectrophotometer and 1.0% (w/v) agarose gel electrophoresis were used to check RNA quality. Total RNA (1.5 μg) was used for first-strand cDNAs synthesis using the FastQuant RT Kit (with gDNase) (TIANGEN Biotechnology, Beijing, China). Synthesis of cDNAs was carried out for gene cloning and expression analysis.

2.3. qRT-PCR analysis

The cDNA templates for quantitative real-time PCR (qRT-PCR) were grape berries. Primers for qRT-PCR were designed near the 3' untranslated regions of *VviABCGs* and are shown in Table S1. The qRT-PCR was carried out using a StepOneTM instrument (USA), and each reaction was subjected to three biological replicates, with the *VviActin* gene (AY680701) as an internal reference. The cDNA template was diluted tenfold and used for qRT-PCR analysis. Reaction program was 95 °C for 30 s, 40 cycles at 95 °C for 15 s, and 58 °C for 15 s, then 72 °C for 30 s. The program for melting curve analysis was 95 °C for 15 s and then a constant increase from 60 °C to 95 °C. Relative expression was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.4. Bioinformatics analysis of VviABCG14 gene

In order to identify the potential proteins interact with half-size VviABCG14, a bioinformatics analysis was carried out according to the functional and phylogenetic analysis of AtABCG14. MEGA 6.0 was used to build the phylogenetic tree of several ABCG proteins in *Arabidopsis* and grape, using the neighbour-joining method (Tamura et al., 2013) and a bootstrap test was conducted with 1000 replicates. ClustalX2.1 was used for the multi-sequence alignment of several *Arabidopsis* and *V. vinifera* ABCG proteins (Larkin et al., 2007).

2.5. VviABCG14 over-expression vector construction and Arabidopsis transformation

The ORF was inserted into the vector pCAMBIA2300-GFP, constructing the over-expression vector *35S-VviABCG14-GFP* (primers see Supplemental Table S2). Subsequently, the plasmid *35S-VviABCG14-GFP* was transformed into *Agrobacterium* strain GV3101 using the freeze-thaw method. *Agrobacterium* containing over-expression vector was then transferred to *A. thaliana* by the inflorescence infection method (Clough and Bent, 1998).

The genome DNA of T1 and total RNA of T3 homozygous generation lines were extracted for PCR and qRT-PCR detection respectively. The details of homozygosity analysis are in the Supplemental texts. Wild-type and T3 *Arabidopsis* strains were then cultured under the same conditions and phenotypic traits, such as root length, fresh weight and bolting rate were compared.

2.6. Determination of cytokinin content in Arabidopsis shoots

The shoots of 10 days *Arabidopsis* seedling grown on MS-agar medium were used to extract *tZ*. For extraction and determination of cytokinins, refer to the previous method (Mueller and Munne-Bosch, 2011). Briefly, 100 mg of fresh samples were ground, extracted ultrasonically for 30 min at 4 °C (400 μl methanol/isopropanol/glacial acetic acid (60/39/1, v/v/v), then centrifuged at 4 °C for 15 min. The supernatant was collected and the pellet was re-extracted three times with 200 μl of solvent extract. The supernatants were combined and dried under a nitrogen stream, then re-suspended in 200 μl methanol and filtered for high-performance liquid chromatography-mass spectrometry (HPLC-MS) analysis. The *tZ* was analysed using an AB QTRAP 5500 HPLC-MS equipped with diode array detector and triple quadrupole mass spectrometer detector (ABSCIEX, USA). The content of *tZ* was calculated by external standard method and carried out three biological replicates. *Trans*-Zeatin standards (HPLC ≥ 98%, Yuanye Bio-Technology, Shanghai, China) were used as external standards.

2.7. Yeast two-hybrid assay using the split-ubiquitin yeast membrane system

To confirm protein interactions, the recombinant bait vectors pDHB1-VviABCG14 and pDHB1-VviABCG7, and recombinant prey vector pPR3-N-VviABCG7/14/11.1/11.3/11.4/11.7/15.1/21 were constructed (Fig. S1). Primers related to recombinant vectors construction are presented in Table S3. After co-transformation of the prey and bait plasmids into NMY51 yeast strain using the LiAc method, yeast cells were grown 3–4 days on SD/-Trp/-Leu media, then diluted in 100 μl sterile ddH_2O and coated on SD/-Trp/-Leu/-Ade/-His/x-α-Gal media for colour detection and confirmation of interacting proteins.

2.8. BiFC assay

The ORFs of VviABCG14 and VviABCG7 were cloned into the pSPYCE and pSPYNE vectors to generate pSPYNE(R)173/VviABCG14, pSPYNE(R)173/VviABCG7, pSPYCE(M)/VviABCG14 and pSPYCE(M)/VviABCG7, respectively. Primers are shown in Table S3.

The *Agrobacterium* strains GV3101 carrying different plasmids or empty vectors were transiently co-infiltrated into tobacco leaves. *Agrobacterium*-mediated transient transformation was performed on *N. benthamiana* grown for 6–7 weeks (Sparkes et al., 2006). After 2–3 days of co-infiltrated, the yellow fluorescent protein (YFP) fluorescence was observed using a confocal laser microscope (LEICA TCS SP8, Leica, Germany). The emission wavelength of YFP fluorescence is 527 nm, and the excitation wavelength is 514 nm.

2.9. Subcellular localization

To identify the subcellular location of VviABCG14 and VviABCG7, the *Agrobacterium* strain GV3101 carrying the plasmids *35S-VviABCG14-GFP* and *35S-VviABCG7-GFP* was transiently expressed in *N. benthamiana* leaves. Vector *pCAMBIA2300-GFP* served as a positive control. Green fluorescent protein (GFP) fluorescence was observed by laser confocal microscopy (LEICA TCS SP8, Leica, Germany).

2.10. Statistical analysis

Values shown are means ± SD of three independent biological experiments. Statistical analyses were carried out using Student's t-test (http://www.physics.csbsju.edu/stats/t-test_bulk_form.html), significant differences are indicated by asterisks (*P < 0.05, **P < 0.01).

3. Results

3.1. Bioinformatics analysis of the VviABCG14 gene

Because of AtABCG14 interacts with AtABCG11 to form a heterodimer (Le Hir et al., 2013), we selected several half-size VviABCGs that are closely related to AtABCG11 and AtABCG14 respectively to verify the interaction proteins that form heterodimer with VviABCG14. Multiple sequence alignment analysis showed these proteins have multiple conserved structural sites in the amino acid sequence, which contain the unique characteristic motif or ring of the ABCG half-transporter family: the WalkerA motif, WalkerB motif, ABC signal motif, D loop, H loop and Q loop (Fig. S2). Phylogenetic analysis shows AtABCG14 and VviABCG14 have the highest homology, the homologous sequence of AtABCG11 in grape may include VviABCG11.1, VviABCG11.3, VviABCG11.4, VviABCG11.7 and VviABCG15.1, among these VviABCG11.7 and AtABCG11 have the highest similarity (Fig. 1).

The cDNA sequences of *VviABCG14* and predicted interacting protein genes *VviABCG7*, *VviABCG11.1*, *VviABCG11.3*, *VviABCG11.4*, *VviABCG11.7*, *VviABCG15.1* and *VviABCG21* were then cloned in Pinot noir (*VviABCG9* was also cloned, but the cDNA sequence was not obtained). Primers for cloning *VviABCGs* in grape are shown in Table S4. The PCR amplification bands of *VviABCG14* (1974 bp) and *VviABCG7* (2190 bp) genes are shown in Fig. S3 (the cloning results for the other genes are not shown). The ORFs of *VviABCG* genes were 1968–2190 bp in length, encoding 656–730 aa.

3.2. The expression patterns of VviABCG14 under treatment with exogenous cytokinins

Our previous study cloned the *VviABCG14* gene and found it highly expressed in young tissues (Tang et al., 2018). In order to preliminarily

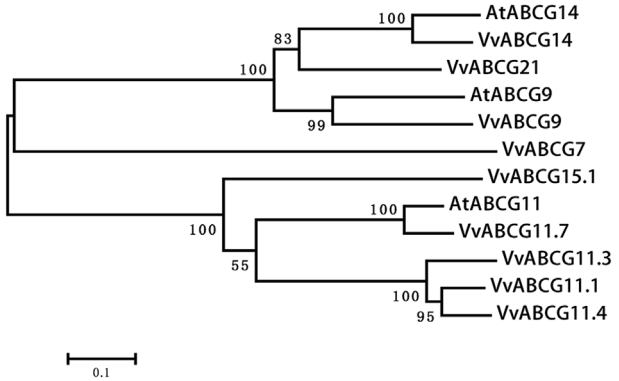

Fig. 1. Phylogenetic analysis of *VviABCG14*. Phylogenetic analysis of *V. vinifera* and *Arabidopsis* ABCGs. MEGA 6.0 was used to build the phylogenetic tree using the neighbour-joining method (Tamura et al., 2013) and a bootstrap test was conducted with 1000 replicates.

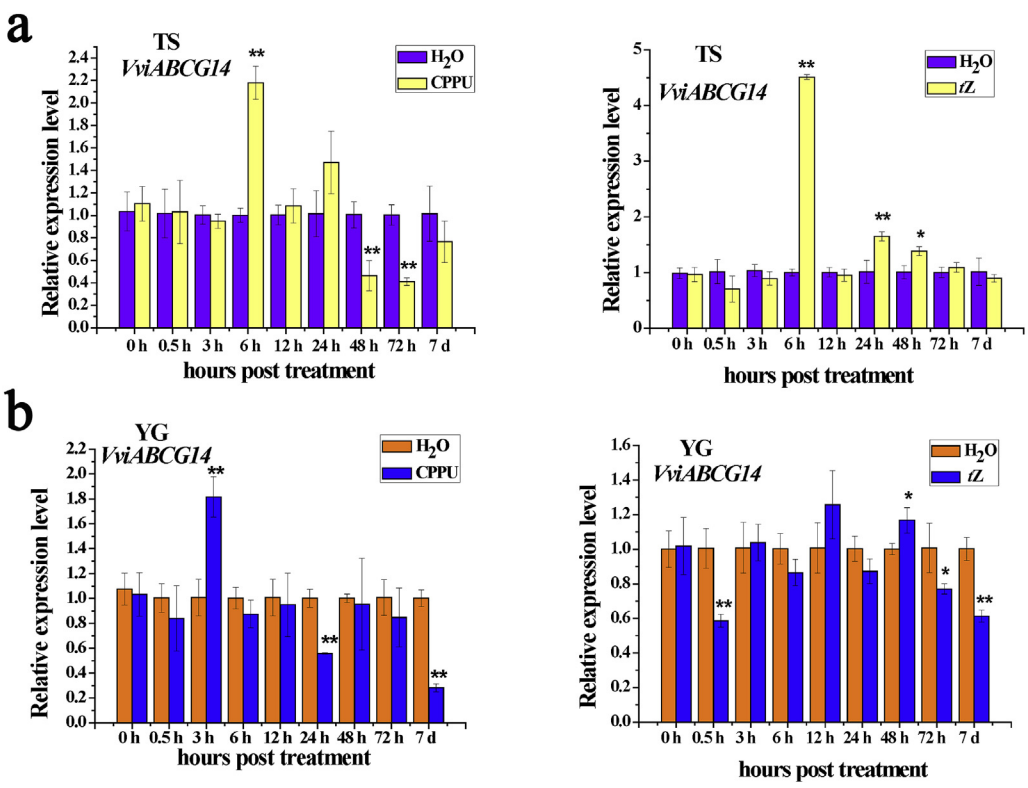

Fig. 2. Expression analysis of *VviABCG14* in response to exogenous cytokinins. a The expression of *VviABCG14* in Thompson seedless berries 7 days after treatment with 100 μM CPPU and *trans*-zeatin (*tZ*). **b** The expression of *VviABCG14* in Youngle berries 7 days after treatment with 100 μM CPPU and *tZ*. For each time, the expression is presented relative to the control (water treatment). Values shown are means ± SD of three independent biological experiments. Significant differences (*t*-test) are indicated by asterisks (*P < 0.05, **P < 0.01). TS, Thompson seedless; YG, Youngle.

determine the response of *VviABCG14* to exogenous cytokinins, grape berries in different varieties were treated by CPPU and *tZ*. In this study, qRT-PCR analysis shows the expression of *VviABCG14* was induced to various degrees at different times after treatment with exogenous hormones CPPU and *tZ* (Fig. 2). In Thompson seedless berries, the expression level of *VviABCG14* was significantly increased at 6 h after treatment with CPPU, at 6 h and 24–48 h after *tZ* treatment (Fig. 2a). In Youngle, *VviABCG14* was significantly induced at 3 h after CPPU treatment and 48 h after *tZ* treatment (Fig. 2b). The results showed that the induction of *VviABCG1*4 by exogenous CPPU and *tZ* was inconsistent in the two varieties, and was larger in Thompson seedless than in Youngle.

3.3. Analysis of phenotypic differences in transgenic plant

In order to study the biological function of VviABCG14 transporter, *VviABCG14* gene was overexpressed in *Arabidopsis*. A target band of about 2000 bp was observed in transgenic strains of pC2300-35S/VviABCG14 (1974 bp) (Fig. 3a). The qRT-PCR showed there was no expression of *VviABCG14* gene in Col-0, and the expression level of *VviABCG14* in strain 5 was significantly higher than in strain 8 or 10 (Fig. 3b). Strains 5, 8 and 10 (35S:VviABCG14#5, 8, 10) were used for the following experiments.

The seeds of T3 *Arabidopsis* lines and Col-0 were sown under the same conditions, the seedlings showed phenotypic differences (Fig. 4 and Fig. 5). The *VviABCG14*-overexpresion seedlings grew faster than Col-0 at 12 days after sowing and the seedlings of line 5 grew most vigorously (Fig. 4a), and there was a significant difference in fresh weight (Fig. 4c). Meanwhile, it was found that the roots of transgenic plant were significantly longer than of Col-0 plant (Fig. 5a and b).

At 26 days, transgenic *Arabidopsis* has larger rosettes than wild-type with significant difference in fresh weight (Fig. 4b and d). The seedlings showed differences in vegetative growth and reproductive growth at 26 days after sowing (Fig. 4b). Overexpression of *VviABCG14* leads to prolonged vegetative growth. Under the same conditions, 60% of wild-type *Arabidopsis* were already bolting but only 16.7% transgenic plants

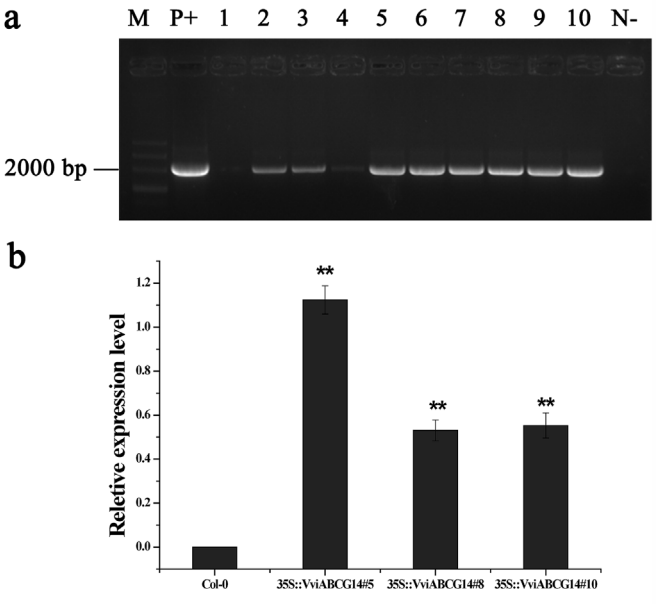

Fig. 3. Screened lines with overexpression of *VviABCG14* in *Arabidopsis thaliana*. a DNA of *VviABCG14* identified in T1 generation. M: DNA Maker III, P +: pC2300-35S-GFP/*VviABCG14*, lane 1-10: *VviABCG14* in T1 lines; N-: wild-type. **b** Transcription level of *VviABCG14* in T3 generation of different lines. The relative expression was to expression of *VviABCG14* in 35S:VviABCG14#5 line.

bolted.

3.4. Determination of tZ in Arabidopsis thaliana shoots

In order to determine whether the accelerated growth rate of the shoots in transgenic *Arabidopsis* was caused by excessive transport of

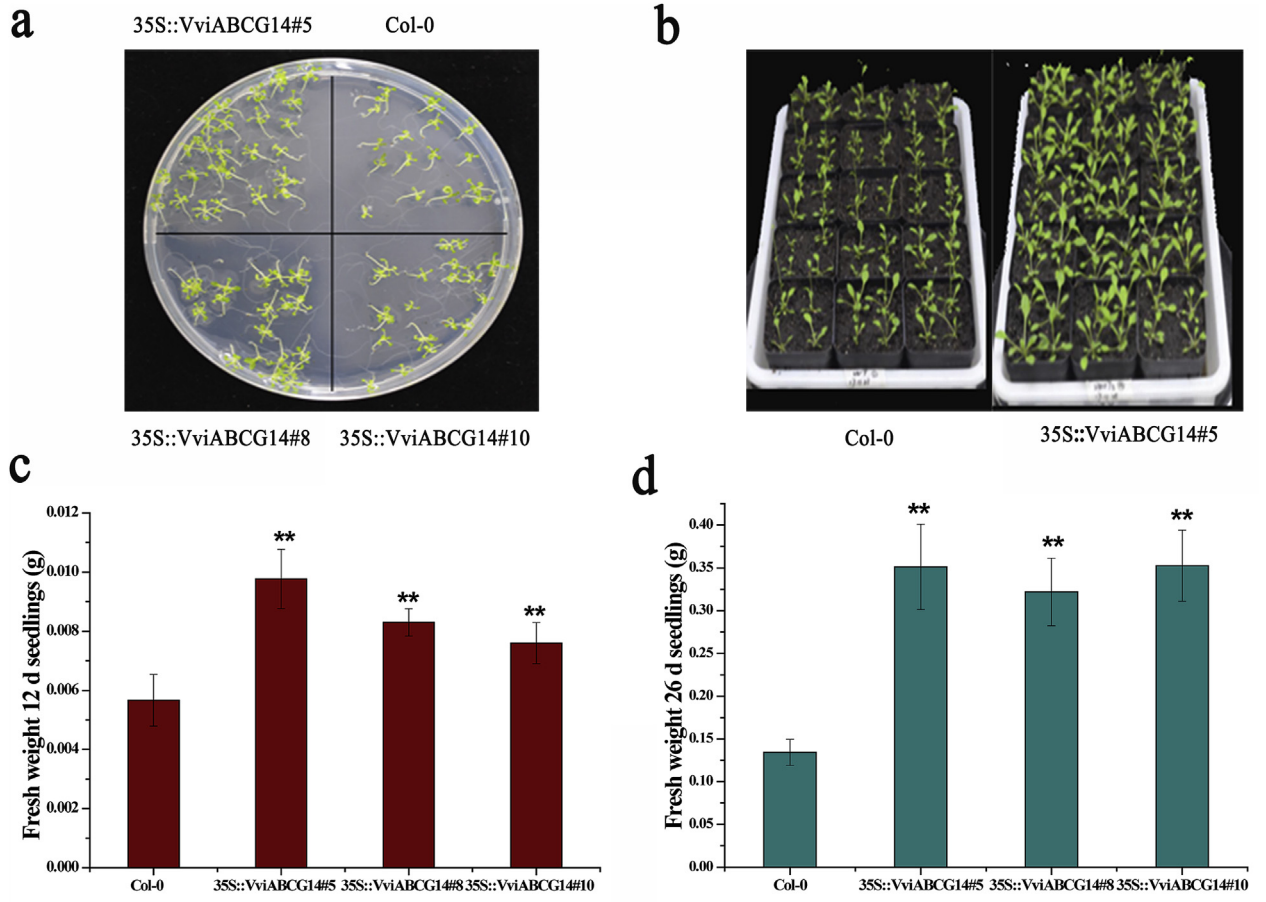

Fig. 4. Comparative analysis on growth status of WT and T3 generation *Arabidopsis* seedlings for 12 and 26 days. a Phenotypic analysis of WT and transgenic seedlings on MS for 12 days. The diameter of round Petri dish is 9 cm **b** Phenotypic analysis of Col-0 and T3 generation seedlings in the soil for 26 days. 60 seedlings each were used for bolting rate analysis. **c** Measurement of fresh weight and significance analysis of 12 days seedlings of WT and transgenic grown on MS. **d** Measurement of fresh weight and significance analysis of 26 days seedlings of WT and transgenic in the soil. 10 seedlings (12 days or 26 days) each used with three biological repeats and error bars with mean values ± SD. Significant differences (*t*-test) are indicated by asterisks (*P < 0.05, **P < 0.01).

cytokinin to above-ground, the content of *tZ* in the shoots was analysed by LC/MS. The *tZ* content of Col-0 was 69.99 pmol/g; the contents of strains 5, 8, and 10 were 206.39 pmol/g, 121.57 pmol/g and 91.08 pmol/g, respectively, and there were significant differences. The results show the total content of *tZ* in the transgenic lines shoot was significantly higher than in the wild-type (Fig. 6a).

To further determine the relationship between *VviABCG14* and cytokinin, we analysed the expression level of nine cytokinin response marker gene *ARRs,* type-A *Arabidopsis* response regulators respond to early cytokinin-inducible (Fig. 6b). Among them, the expression levels of *ARR5*, *ARR8* and *ARR16* genes in the shoots of three transgenic lines were induced and significantly higher than that of Col-0 plant; the expression of *ARR3*, *ARR4*, *ARR7*, *ARR9* and *ARR16* was significantly higher in the shoots of Strins 5 than in Col-0 plant. The results indicate that the increase of *tZ* content in transgenic *Arabidopsis* causes an increase of *ARRs* gene expression.

3.5. Identification of VviABCG7 as a VviABCG14 interacting protein in split-ubiquitin yeast membrane system

Since the ABCG transporter is membrane protein, the split-ubiquitin yeast membrane system was used to validate the interacting proteins of VviABCG14. First of all, the autotoxicity and self-activation of VviABCG14 bait protein were studied. The positive control pTSU2-APP + pNubG-Fe65, negative control pTSU2-APP + pPR3-N, self-activated positive pDHB1-VviABCG14 + pOstI-NubI, self-activated negative pDHB1-VviABCG14 + pPR3-N co-transformed colonies were diluted and colour detection was carried out on SD/-Ade/-His/-Leu/-Trp + X-α-gal medium. The result indicated the bait protein was none toxic and self-activated (Fig. 7a).

The bait and prey protein combinations that may interact, were co-transformed into NMY51 yeast strain, and the colonies diluted and coated on SD/-Ade/-His/-Leu/-Trp + X-α-gal medium. Only pDHB1-VviABCG14 + pPR3-N-VviABCG7 co-transformed colonies grew normally and turned blue in SD/-Ade/-His/-Leu/-Trp + X-α-gal medium (Fig. 7b). It was suspected that VviABCG14 interacts with VviABCG7 proteins. However, no interaction results were observed after the exchange of the bait and prey vectors of the VviABCG7 and VviABCG14 proteins (the autotoxicity and self-activation of VviABCG7 bait protein were studied, Fig. 7a).

3.6. Verification of VviABCG14 interact with VviABCG7 using the BiFC assay

To verify the interaction of VviABCG14 and VviABCG7 *in vivo*, the *Agrobacterium*-mediated transient co-transformation of tobacco leaves method was used for BiFC assays. Significant YFP fluorescence was observed only when pSPYNE(R)173-VviABCG14 was co-expressed with pSPYCE(M)-VviABCG7 (Fig. 8), this indicated that VvABCG14 interacts with VvABCG7 to form a heterodimer in *vivo*.

3.7. Response patterns of VviABCG7 to exogenous hormones

Since VviABCG14 interacts with VviABCG7 to form a heterodimer,

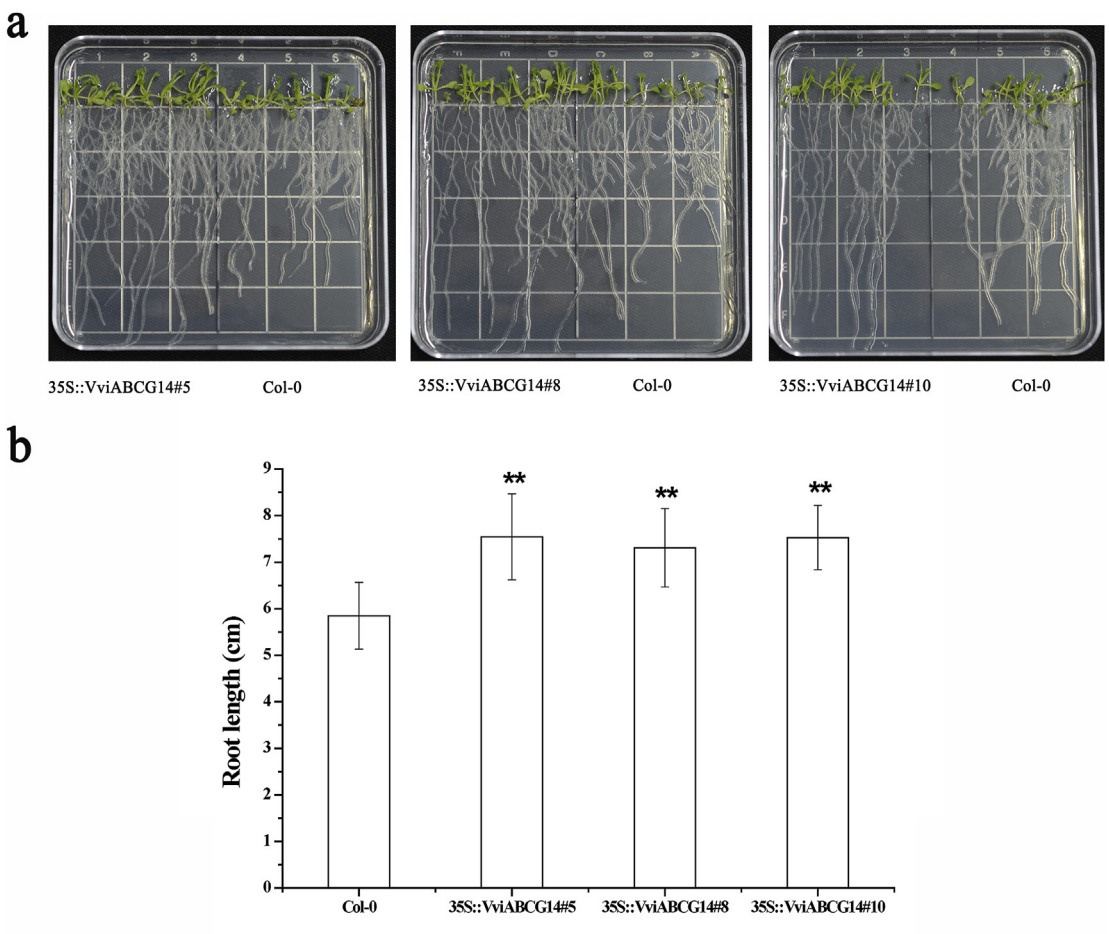

Fig. 5. The root growth of 12 days seedlings of T3 generation in *Arabidopsis thaliana*. a The primary root length comparisons of WT and transgenic seedlings on MS for 12 days. The length of square Petri dish is 10 cm **b** Measurement of primary root length of 12 days seedlings of WT and transgenic grown on MS. At least 15 seedlings each were measured and error bars with mean values ± SD. Significant differences (*t*-test) are indicated by asterisks (*P < 0.05, **P < 0.01).

the response of *VviABCG7* gene in the grape berries to exogenous cytokinins was also analysed by qRT-PCR (Fig. 9). The transcription levels of *VviABCG7* in the two grape cultivars Thompson seedless and Youngle increased significantly during 7 days period after CPPU and *tZ* treatment, although the response patterns were different. In Thompson seedless berries (Fig. 9a), the expression level of *VviABCG7* increased continuously for 12 h during CPPU treatment, then decreased, and finally reached the highest at 7 days of treatment. After *tZ* treatment, the patterns of gene expression was the same as treatment with CPPU, however the expression level reached the highest at 12 h. In Youngle (Fig. 9b), the expression level of *VviABCG7* was highest at 48h after treatment with the two exogenous hormones.

4. Discussion

In a previous study, tissue specific analysis was carried out on ABCG (WBC) transporters and it was found *VviABCG14* gene was expressed in all tissues of grapevine. Especially, in the vascular tissues such as in young roots, young stems and young tendrils (Tang et al., 2018). The *AtABCG14* gene was also expressed mainly in the pericycle, middle column cells and vascular bundle of the root (Zhang et al., 2014). Based on the similarity of patterns of tissue expression, it was speculated that *VviABCG14* gene may participate in the transport of cytokinins in grapevines, similar to the function of *AtABCG14* gene. In this study, we analysed the effects of exogenous cytokinins CPPU and *tZ* on the expression of *VviABCG14* in grape berries. In Thompson seedless and Youngle, *VviABCG14* responds inconsistently to the same hormone. This may be related to the specificity of the cultivars. Even in the same species, the gene's response to the same exogenous hormone is affected by differences between cultivars. The *VviABCG14* had a smaller response to CPPU and *tZ* in Youngle, which may be due to the larger fruit and the shorter treatment time of the exogenous hormone. Meanwhile, the response of the *VviABCG14* gene to exogenous hormones requires a certain reaction time, perhaps because with *VviABCG14* these must accumulate in the berries to play a role. In combination with the *AtABCG14* gene in *Arabidopsis* mediating cytokinin transport to extracellular, we first hypothesised that *VviABCG14* may have a similar effect.

The *atabcg14* mutant of *Arabidopsis* suffers a phenotypic cytokinin defect. Under normal growth conditions, mutant plants grow only weakly, inflorescences are small, stems are thin and primary roots are short, compared with the wild-type (Ko et al., 2014; Zhang et al., 2014). In our experiments, *VviABCG14* over-expression in *Arabidopsis* at 12 days after sowing had a significant different phenotypic type in seedlings. In over-expressing lines, the aerial parts grew faster than the wild-type, the growth rate of the root was also faster and root length greater. The transgenic and the wild-type lines showed obvious differences in vegetative growth and reproductive growth after 26 days of culture. That is, the over-expression of *VviABCG14* caused the enlargement of the rosettes and the prolongation of vegetative growth in *Arabidopsis*. So, we hypothesised that *VviABCG14* may increase the movement of cytokinin from the roots to the aerial parts in *Arabidopsis*. This in turn led to an increase in cytokinin in the aerial parts and so promoted their growth.

In our study, the *tZ* content in the shoots of *VviABCG14* over-expressing overexpressing transgenic *Arabidopsis* was determined, and the

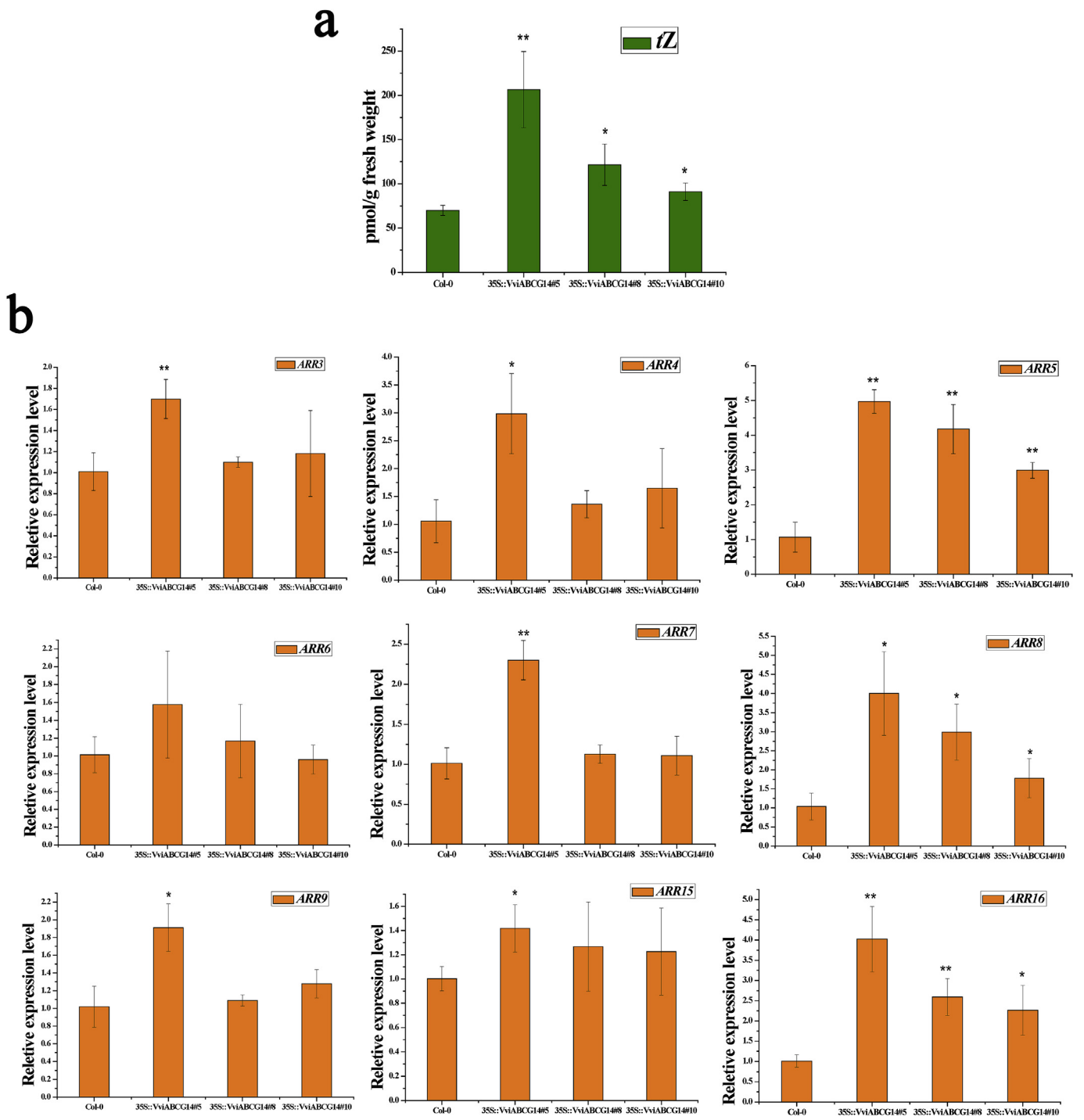

Fig. 6. Analysis of content of *trans*-zeatin (*tZ*) and cytokinin response marker genes (type-A *ARRs*) expression in *Arabidopsis thaliana* shoots. a Content of *tZ* in the shoots of wild-type and transgenic lines determined by HPLC/MS. The content of *tZ* in 100 mg fresh sample derived from the shoots of 10 days *Arabidopsis* seedling grown on MS-agar medium. The content of *tZ* was calculated by external standard method and carried out three biological replicates. *Trans*-Zeatin standards (HPLC ≥ 98%) were used as external standards. **b** Expression analysis of type-A *ARRs* in *Arabidopsis* shoots. The data were from three transgenic lines and wild-type seedlings for 12 days, each with three biological replicates. Values are means ± SD. Significant differences (*t*-test) are indicated by asterisks (*P < 0.05, **P < 0.01).

results showed that in 35S:VviABCG14#5, the *tZ* content was higher than in other two transgenic lines. This may be due to the higher expression of *VviABCG14* gene in 35S:VviABCG14#5, so more cytokinin was transported from the roots to the shoots. At the same time, the expression of *ARR* gene in *Arabidopsis* transgenic lines was higher than that in wild-type and the expression was higher in 35S:VviABCG14#5 than in the other two transgenic lines; and only the expression levels of all *ARR* genes (except for *ARR6*) in 35S:VviABCG14#5 were significantly higher than in WT, indicating that the *ARR* gene in 35S:VviABCG14#5 was more significantly induced by cytokinin. So we suggested that the higher response of *ARR* gene in 35S:VviABCG14#5 may be caused by the higher cytokinin content in the shoots. So the cytokinin concentration and related signal activity in the shoots of overexpressing transgenic plants have changed. Combined with the

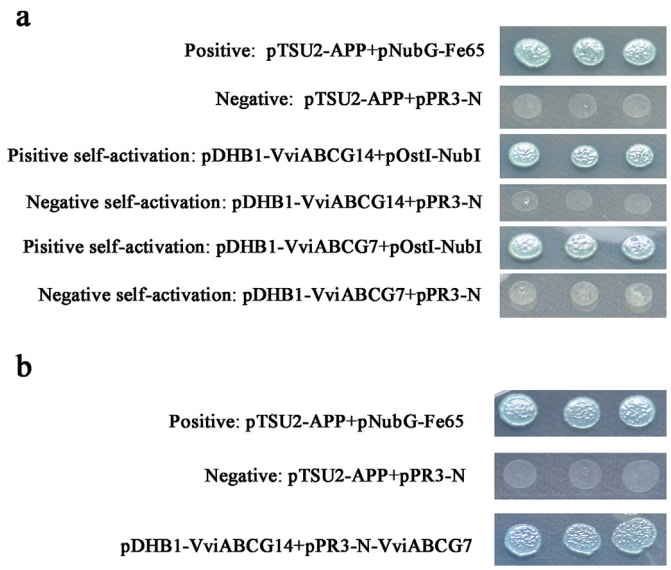

Fig. 7. Verification of VviABCG14 interacting protein using the split-ubiquitin yeast membrane system. a Self-activation and toxicity testing. **b** Yeast two-hybrid assay of membrane proteins.

determination of the phenotype, cytokinin content and the expression of *ARR* genes (Fig. 9) in the aerial parts of transgenic and wild-type plants, it was further suggested that VviABCG14 is also involved in long-distance transport of cytokinin from root to shoot.

In *Arabidopsis*, AtABCG14 is involved in the transport of *t*Z-type cytokinin, it contains *t*Z and its natural derivatives such as the riboside and O/N-glycoside, so the exact transporting form of *t*Z-type cytokinins mediated by AtABCG14 remains undetermined. In our study, we measured the *t*Z content in shoots of wild-type and transgenic *Arabidopsis* and found that its content is higher in the transgenic lines, but this is probably because the transport of *t*Z riboside (*t*ZR) by VviABCG14 from roots to shoots followed by the conversion of *t*ZR to *t*Z. Therefore, we also cannot determine the exact form of VviABCG14 involved in the transport of cytokinin.

To further verify the characteristics of VviABCG14, the subcellular localization of VviABCG14 was analysed. As shown in Supplementary Fig. S4, it is preliminary indicates that VviABCG14 localized on the plasma membrane, and was consistent with the localization of AtABCG14 (Ko et al., 2014; Zhang et al., 2014). Therefore, we used the split-ubiquitin yeast membrane system to validate the VviABCG14 interacting proteins. Our yeast two-hybrid results show significant interactions for pDHB1-VviABCG14 + pPR3-N-VviABCG7 co-transformed yeast. But no interaction when the bait and prey carriers were exchanged. This may be related to the uncertainty of protein folding and modification in the yeast system. It further showed that VviABCG14 and VviABCG7 can form heterodimer using the BiFC assay. At the same time, we found VviABCG7 was localized in the cytoplasmic membrane.

The ABCG transporter can form a dimer with different semi-transporters in various tissues to transport a range of substrates. For example, AtABCG11 interacts with many half-size transporters to carry out a number of different functions. AtABCG11 forms a specialized heterodimer with AtABCG12 in stem epidermal cells and participates in the export of epidermal wax (Bird et al., 2007). AtABCG14 and AtABCG11 form heterogeneous dimer, while AtABCG11 and AtABCG9 can form heterogeneous dimer and homologous dimer; AtABCG9, AtABCG11 and AtABCG14 transporters are involved in the development of vascular systems and sterols/lipid homeostasis in *Arabidopsis* (Le Hir et al., 2013). At the same time, the absence of AtABCG11 also causes many phenotypic changes. In *Arabidopsis*, AtABCG14 interacts with AtABCG11, while our research only found that VviABCG14 and VviABCG7 could form heterodimer; this may be due to the specificities between species, and also proved that ABCG14 transporter can form dimers with different half-size transporters, then play different transport functions. Our result provides a new evidence for the study of ABCG transporters that can form dimer with different half-size ABCGs. Theoretically, according to the structural characteristics of ABCG members (Velamakanni et al., 2007), interactions of half-size ABCGs should occur frequently. More interaction proteins VviABCG14 and other ABCG transporter proteins should be studied in greater depth. The interaction of VviABCG14 with VviABCG7 and the significant increase in transcription levels of these two *VviABCG* genes after grape berries are treated with exogenous CPPU and *t*Z together, indicates VviABCG7 may be also involved in the transport of cytokinins. Nevertheless, further research is required. It is unclear if interactions of VviABCG14 and VviABCG7 play a combined role in cytokinin transport.

5. Conclusion

Taken together, based on our results, overexpressing *VviABCG14* in *Arabidopsis* resulted in significant differences in phenotype, compared with wild-type. The growth of the aboveground and belowground parts

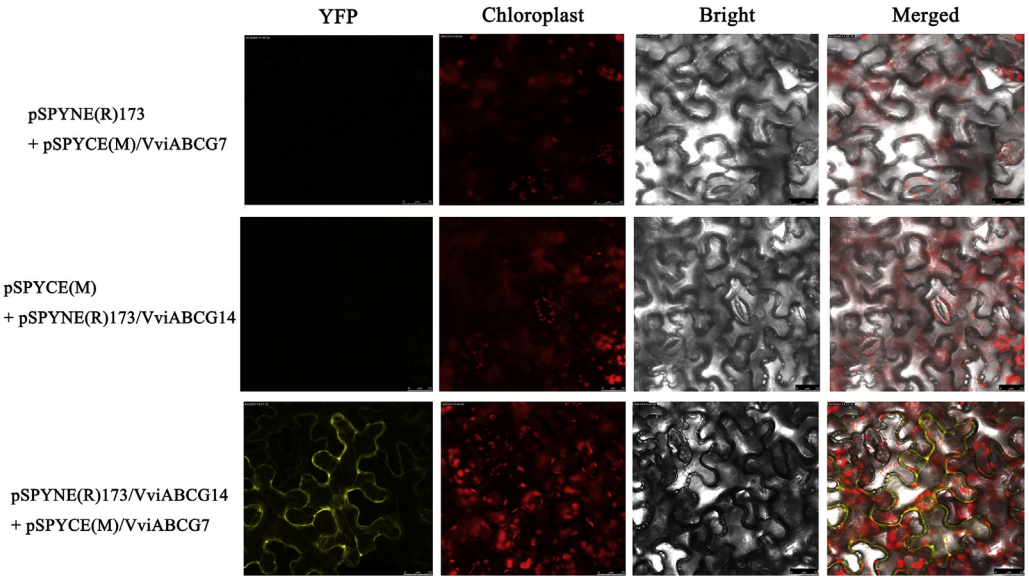

Fig. 8. BiFC assay confirms that VviABCG14 interacted with VviABCG7. The different plasmid combinations were co-transformed into tobacco leaf. Yellow fluorescent protein (YFP) fluorescence was observed when pSPYNE(R) 173-VviABCG14 was co-expressed with pSPYCE(M)-VviABCG7. YFP fluorescence was observed after transformation for 72 h. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

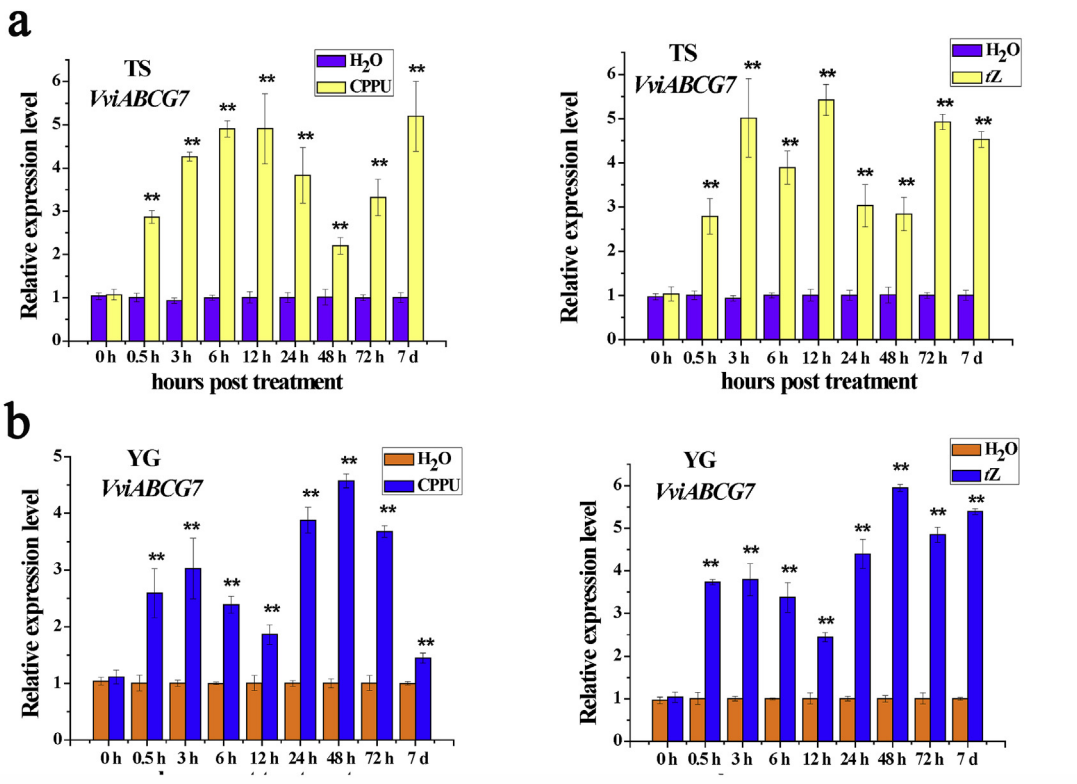

Fig. 9. Expression analysis of *VviABCG7* to exogenous cytokinins. a The expression of *VviABCG7* in Thompson seedless berries 7 days after treatment with 100 μM CPPU and *trans*-zeatin (*tZ*). **b** The expression of *VviABCG7* in Youngle berries 7 days after treatment with 100 μM CPPU and *tZ*. For each time, the expression is shown relative to the control (water treatment). Values shown are means ± SD from three biological independent experiments. Significant differences (*t*-test) are indicated by asterisks (*P < 0.05, **P < 0.01). TS, Thompson seedless; YG, Youngle.

of transgenic plants was significantly stronger than of wild-type plants. The high level of *tZ* in transgenic shoots indicates VviABCG14 is involved in the transport of cytokinin. VviABCG14 membrane protein transporter can form heterodimer with VviABCG7. Meanwhile, *VviBCG14* and *VviABCG7* transporter genes were significantly induced by exogenous CPPU and *tZ*.

CRediT authorship contribution statement

Ling Wang: Formal analysis. **Jingyi Xue:** Formal analysis. **Jing Yan:** Formal analysis. **Meng Liu:** Formal analysis. **Yujing Tang:** Formal analysis. **Yuejin Wang:** Formal analysis. **Chaohong Zhang:** Formal analysis.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by the National Key R&D Program of China (Grant No.2019YFD1001405), the earmarked fund for the China Agriculture Research System (CARS-30-y-7) and Key Technologies R & D Program of Xi'an city (201806114 YF02NC10-4).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2020.05.011.

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