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SlPti4 Affects Regulation of Fruit Ripening, Seed Germination and Stress Responses by Modulating ABA Signaling in Tomato

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Regular Paper

Although the role of the ethylene response factor (ERF) Pti4 in disease resistance has been demonstrated in higher plants, it is presently unknown whether the tomato SlPti4 protein plays a role in the regulation of fruit development and the stress response. Here, we show that *SlPti4* is involved in the regulation of fruit ripening, seed germination, and responses to drought and *Botrytis cinerea* infection through adjustments to ABA metabolism and signaling. *SlPti4* gene expression is very low early in fruit development, but increases rapidly during ripening and can be induced by exogenous ABA and 1-aminocyclopropane 1-carboxylate (ACC). RNA interference (RNAi)-induced silencing of *SlPti4* leads to an increase of ABA accumulation together with a decrease of ethylene release, which causes the high expression level of SlBcyc, and thus the transgenic fruit is orange instead of red as in wild-type fruit during ripening. *SlPti4*-RNAi seeds accumulate less ABA and mRNA for ABA receptor SlPYL genes, which causes insensitivity to ABA treatment. *SlPti4*-RNAi transgenic plants with low ABA levels and high ethylene release were more sensitive to drought stress. *SlPti4*-RNAi plants also showed weaker resistance to *B. cinerea* infection than the wild type. Thus, *SlPti4* is an important regulator of tomato fruit ripening, seed germination and abiotic/biotic stress responses. This study expands our knowledge on diverse plant physiologies which are regulated by ABA signaling and the function of SlPti4.

Keywords: ABA • Drought stress • Fruit ripening • Seed germination • SlBcyc • *SlPti4*-RNAi.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, ACC oxidase; ACS,ACC synthase; DAI, days after inoculation; ERF, ethylene response factor; GFP, green fluorescent protein; MS, Murashige and Skoog; PP2C, type 2C protein phosphatase; PYL, PYR/PYL/RCAR; qRT-PCR, real-time quantitative PCR; RNAi, RNA interference; RWC, relative water content; SnRK2, sucrose non-fermenting 1 (SNF1)-related protein kinase 2; WT, wild type.

Introduction

Fruits are classically divided into two groups, climacteric and non-climacteric, based on their patterns of respiration. In climacteric fruits, ethylene induces the transcription of many ripening-associated genes which results in specific features of fruit ripeness. In higher plants, ethylene is synthesized in a two-step reaction catalyzed by 1-aminocyclopropane-1-carboxylate synthases (ACSs) and ACC oxidases (ACOs) (Yang and Hoffman 1984, Bleecker and Kende 2000). The ethylene signal is transmitted via a linear signaling cascade (Guo and Ecker 2004) leading to the activation of the ethylene response factors (ERFs). ERFs belong to the AP2/ERF transcription factor superfamily that regulates the expression of ethylene-responsive genes by directly binding to their promoter regions (Fujimoto et al. 2000). Since a linear signaling cascade cannot account for the wide diversity of ethylene responses, differential responses to ethylene are dependent on the extensive expression of ERFs, which are encoded by a large family of plant transcription factor genes, and are therefore most suited to conferring such a large diversity of ethylene responses. Published studies indicate that alteration of most components of ethylene signaling and responses has an impact on the course of maturation (Liu et al. 2014). Most of the tomato *ERF* genes identified so far are ethylene inducible and show ripening-related expression (Pirrello et al. 2012, Fujisawa et al. 2013). The tomato transcription factor SlPti4 belongs to the ERF family and plays an important regulatory role in the expression of defense-related proteins whose activities are enhanced by phosphorylation (Gu et al. 2002, Mysore et al. 2002). Although the mechanisms by which SlPti4 regulates plant disease resistance have been studied extensively (Chakravarthy et al. 2003), little is known about the effects of SlPti4 on development and the responses to biotic or abiotic stresses in tomato fruits.

During fruit development, ethylene functions through interactions with other phytohormones (Yoo et al. 2009). Among these, ethylene interacts extensively with ABA (Benschop et al. 2007). ABA plays pivotal roles in plant development and adaptive stress responses. In higher plants the ABA biosynthetic pathway has been well characterized (Nambara and Marion-Poll 2005). The core components in the ABA signaling pathway consist of the ABA receptors PYR/PYL/RCAR, group A protein phosphatases 2C (PP2Cs) and SNF1-related protein kinase 2 (SnRK2) (Ma et al. 2009, Park et al. 2009). In the presence of ABA, ABA-bound receptors inhibit the activity of PP2C while allowing activation of SnRK2s and subsequent phosphorylation of ABA-responsive element-binding factors (ABFs) to activate ABA-responsive genes (Fujii et al. 2009, Cutler et al. 2010). PYR/PYL/RACR family proteins, as ABA receptors, the key

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proteins involved in ABA perception, have been discovered in many plant species such as tomato (González-Guzmán et al. 2014) and grape (Boneh et al. 2012).

Recently, functional interaction and synergism between ABA and ethylene during the early stages of fruit ripening has been reported (Boneh et al. 2012). These two hormones interact in both antagonistic and synergistic modes, which depend upon the developmental stage, organ/tissue and species (Böttcher et al. 2013). Although the integration between ethylene and ABA signaling has been elucidated in Arabidopsis (Linkies et al. 2009), it remains largely unclear in fleshy fruits. In this study, we used *SlPti4*-RNA interference (RNAi) transgenic tomato plants to explore the function of SlPti4 in fruits, and to provide direct evidence that SlPti4 is involved in the regulation of fruit ripening, seed germination and the response to drought stress by altering ABA metabolism and ABA signaling.

Results

Phylogenetic tree and gene expression of SlPti4

Previous studies have shown that SlPti4 belongs to Group IXa of the ERF transcription factor family (Nakano et al. 2006, Sharma et al. 2010). In order to study further the differences between SlPti4 and Group IXa ERFs from other plant species, we used the SlPti4 protein sequence together with 28 Group IXa ERFs from 14 different species to construct a Neighbor–Joining phylogenetic tree. As shown in Supplementary Fig. S1, tomato SlPti4 shows its closest phylogenetic relationship with potato StPti4, but SlPti4 is not in the same branch as AtERF1A (AT4G17500) and AtERF2 (AT5G47220) from Arabidopsis.

The *SlPti4* transcript levels were low during the immature and mature green stages of the fruit (**Fig. 1A**); however, mRNA levels increased rapidly from the breaker (B) stage onwards, and peaked 3 d later. The peak values of *SlPti4* mRNA at B3 and B5 suggest that it may play a role in triggering the onset of ripening. In addition, the spatio-temporal expression of *SlPti4* in the floral bud was detected through in situ hybridization. *SlPti4* was highly expressed in sporogenous cells in anther, pistil, stigma and ovules during flower development (**Fig. 1C**). We then investigated the response of *SlPti4* to application of ethylene and ABA at the mature green (MG) stage. Fruits attached to the plants were sprayed with 200 μM ABA or 500 μM 1-aminocyclopropane 1-carboxylate (ACC). The expression of *SlPti4* was induced by exogenous ACC and ABA treatments (**Fig. 1B**). These results indicate that *SlPti4* is likely to function in tomato fruit ripening.

Suppression of *SlPti4* expression alters the ripening phenotype of tomato fruits

To examine the potential function of *SlPti4* in tomato, we suppressed *SlPti4* expression by using RNAi (Supplementary Fig. S2B). Nine independent *SlPti4*-RNAi transgenic lines were generated, and all nine RNAi lines showed normal plant growth, like their wild-type (WT) counterparts. Among these, the two lines *SlPti4*-RNAi-21 and *SlPti4*-RNAi-68, in which *SlPti4* expression was significantly down-regulated (Supplementary Fig. S3A), were selected for further investigation. In addition, there was no significant difference in the expression levels of the other members of the tomato group IXa ERF family between WT and *SlPti4*-RNAi fruits (Supplementary Fig. S3B). As shown in **Fig. 1D**, the *SlPti4*-RNAi plants underwent accelerated onset of ripening of tomato fruit by 2–3 d compared with the WT fruits. The fruits of the transgenic lines were orange instead of the red of the WT fruits during full ripening. In addition, a 2- to 3-fold increase of β-carotene and a 1- to 2-fold decrease of lycopene were observed compared with the WT fruits (**Fig. 1G**). To determine whether *SlPti4* affected ABA levels, we examined the ABA accumulation in fruit pericarp. It was found that the concentration of ABA increased in transgenic fruits during fruit development and ripening, especially in the later period of ripening (**Fig. 1E**). In contrast, the ethylene release in transgenic fruits was down-regulated during B3 and B5 stages (**Fig. 1F**). However, there was no significant change in the fruit size, weight, soluble solid content and seeds per fruit between the WT and the transgenic lines (Supplementary Table S1).

Suppression of *SlPti4* alters the expression of genes regulating ethylene biosynthesis and ripening-related transcription factors

Since *SlPti4* is an ERF transcription factor gene, we measured the transcriptional level of genes involved in ethylene biosynthesis to investigate the regulatory mechanism of *SlPti4* on fruit ripening. Expression of ethylene biosynthesis pathway genes *ACS2*, *ACS4* and *ACO1* was down-regulated by silencing *SlPti4* during the ripening process (**Fig. 2**). The expression of *SlPSY1* and *SlPDS*, which encode phytoene synthetase and phytoene dehydrogenase, respectively, was slightly up-regulated during fruit ripening, compared with the WT (**Fig. 2**). In contrast, the expression of *SlBcyc*, which encodes lycopene β-cyclase, did not change from the MG to the B1 stage in transgenic fruits, while it was significantly up-regulated during B5–B10 stages compared with the WT fruits (**Fig. 2**). To determine whether the decreased *SlPti4* mRNA levels affected the expression of the other ripening-related transcription factor genes, we examined RIPENING INHIBITOR (RIN), NONRIPENING (NOR) and COLORLESS NONRIPENING (CNR). It was found that the expression of *RIN* was up-regulated while that of the other two genes was down-regulated in transgenic tomato fruits during ripening (**Fig. 2**). Taken together, these data show that expression of ethylene biosynthesis- and ripening-related genes was affected in *SlPti4*-RNAi fruits.

Suppression of *SlPti4* alters the expression of genes involved in ABA metabolism and signaling

Although the concentrations of ABA in the transgenic fruits were higher than in the WT fruits during development and ripening (**Fig. 1E**), the relative transcript level of *SlNCED1*, a main ABA biosynthetic enzyme gene, was only up-regulated at MG and B stages. In contrast, the transcription of *SlCYP707A2*, involved in ABA degradation, was down-regulated significantly in *SlPti4*-RNAi fruits throughout (**Fig. 3**). We next

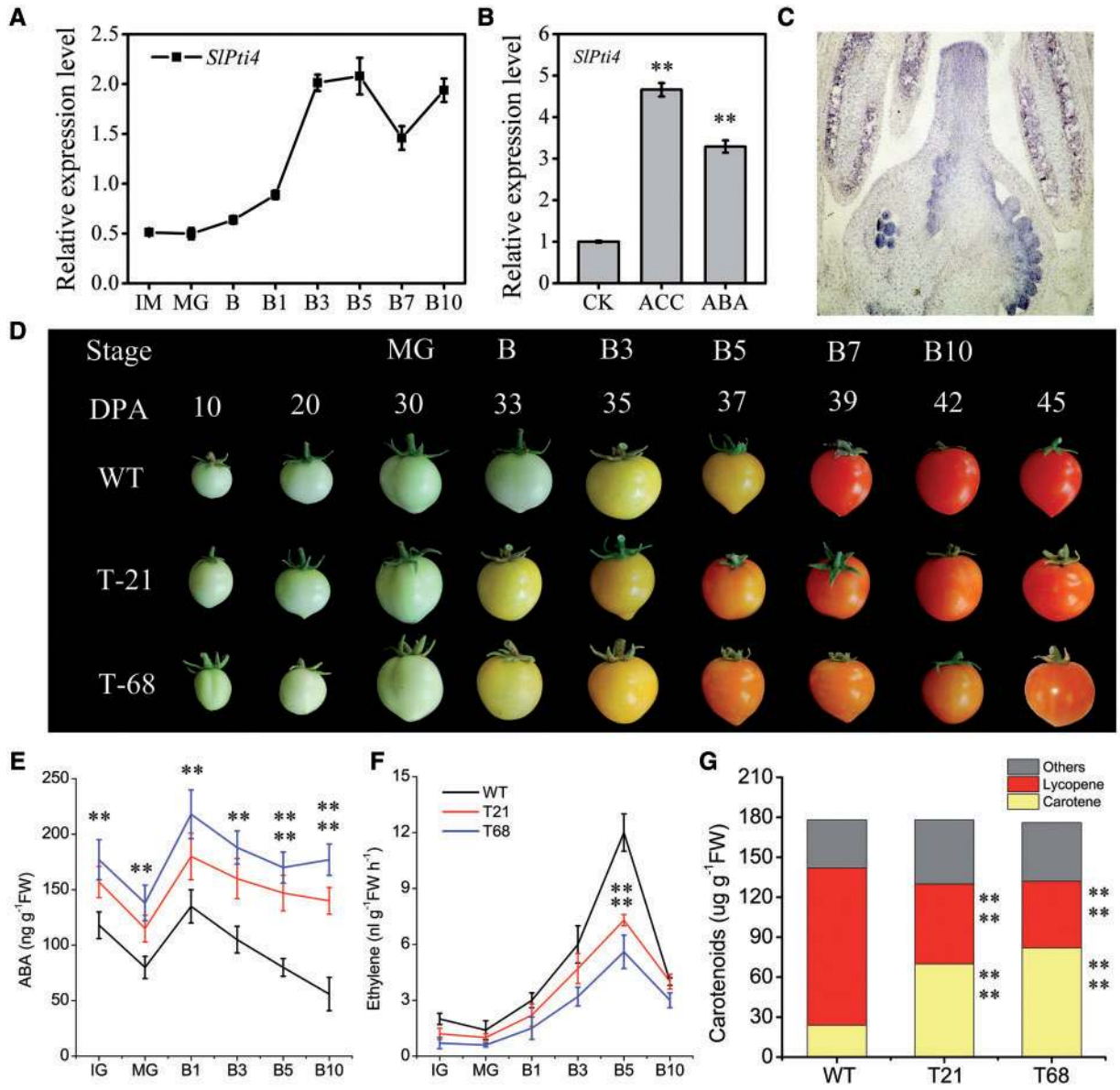

Fig. 1 Expression of *SlPti4* and phenotypes of the *SlPti4*-RNAi transgenic fruits. (A) Relative mRNA levels of *SlPti4* in pericarp during fruit development. (B) Effects of applications of ABA and ACC on the expression of *SlPti4*. MG fruits were treated with 100 μM ABA or 200 μM ACC, and the pericarp was sampled 3 d after treatments. (C) Expression of *SlPti4* in anther, pistil stigma and ovule in tomato flower under in situ hybridization detection. (D) Color phenotypes of *SlPti4*-RNAi lines and WT fruits. (E) ABA contents. (F) Ethylene released in fruits. (G) Carotenoid content of ripe fruits. $^{*}P < 0.05$; $^{**}P < 0.01$.

examined expression levels of the ABA receptor family *SlPYL* genes. Of these, the expression of *PYL1*, *PYL2*, *PYL5*, *PYL6* and *PYL9* (except at the B stage) was up-regulated, while the expression of *PYL4*, *PYL7* and *PYL10* was down-regulated in *SlPti4*-RNAi fruits during ripening (**Fig. 3**). These results showed that the expression patterns of the *SlPYL* genes in fruits were very different, suggesting a complicated regulation.

SlPti4-RNAi seeds accumulate less ABA and show reduced ABA sensitivity

To understand the role of *SlPti4* in seeds, we tested seed germination and the effects of exogenous ABA treatment. When the seeds were growth on 0.5× Murashige and Skoog (MS) medium (**Fig. 4**) or filter paper soaked with water (Supplementary Fig. S4), the germination rate of the *SlPti4*-RNAi seeds was significantly higher than that of WT seeds at 2 d after imbibition. This result suggested that the early germination of transgenic seeds may be attributed to the degree of dormancy which is mediated by ABA. Indeed, the ABA content and expression of *SlNCED1/2* in dry seeds of the transgenic lines were markedly lower than in WT seeds (**Fig. 4D–F**). We next examined whether ABA perception is altered in *SlPti4*-RNAi seeds. *SlPti4*-RNAi seeds germinated earlier than WT seeds after exogenous ABA treatments (**Fig. 4B, C**). These results indicate that ABA sensitivity was reduced by down-regulation of *SlPti4* in seeds. In addition, the response of root growth in

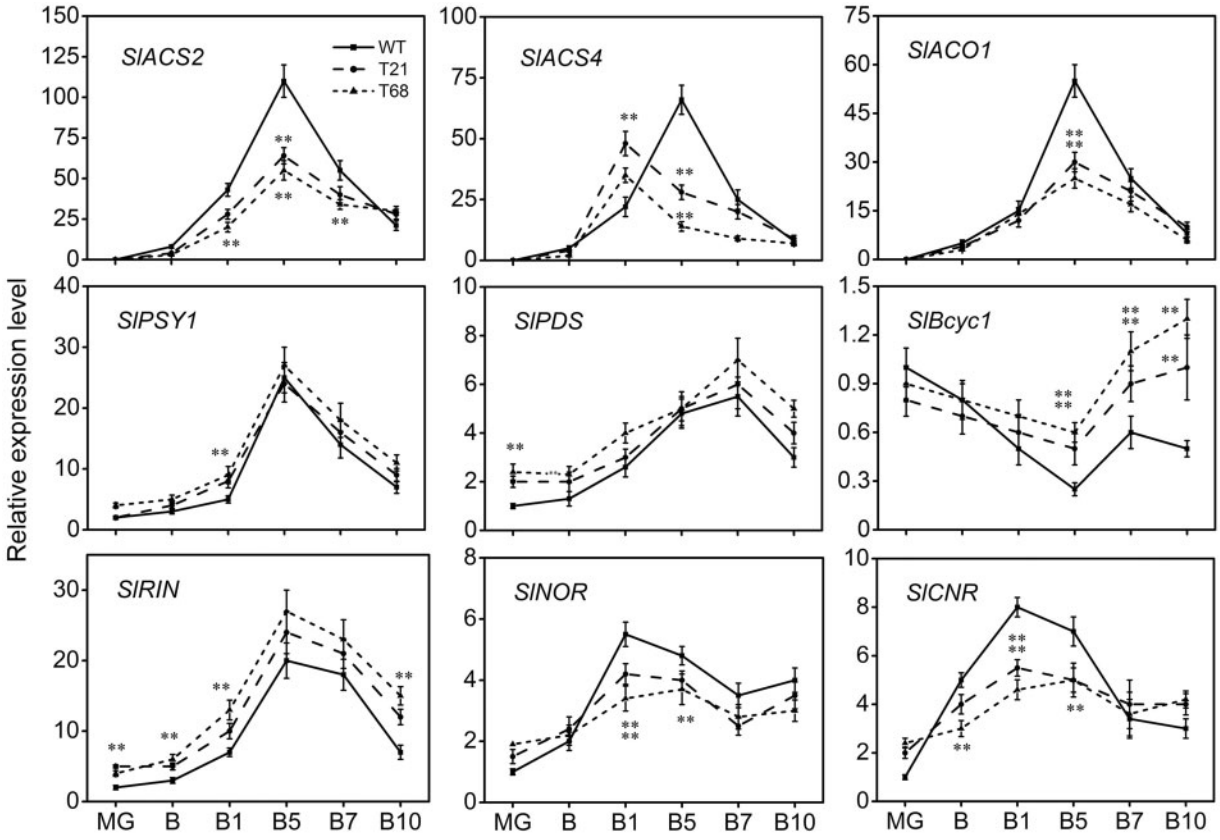

Fig. 2 Expression of genes involved in ethylene biosynthesis and fruit ripening in *SlPti4*-RNAi and WT fruits. (A–C) Expression of ethylene biosynthesis genes. (D and E) Expression of genes in the lycopene biosynthetic pathway. (F–I) Expression of transcription factor genes related to fruit ripening. *SlSAND* was used as the internal control. $^{*}P < 0.05$; $^{**}P < 0.01$.

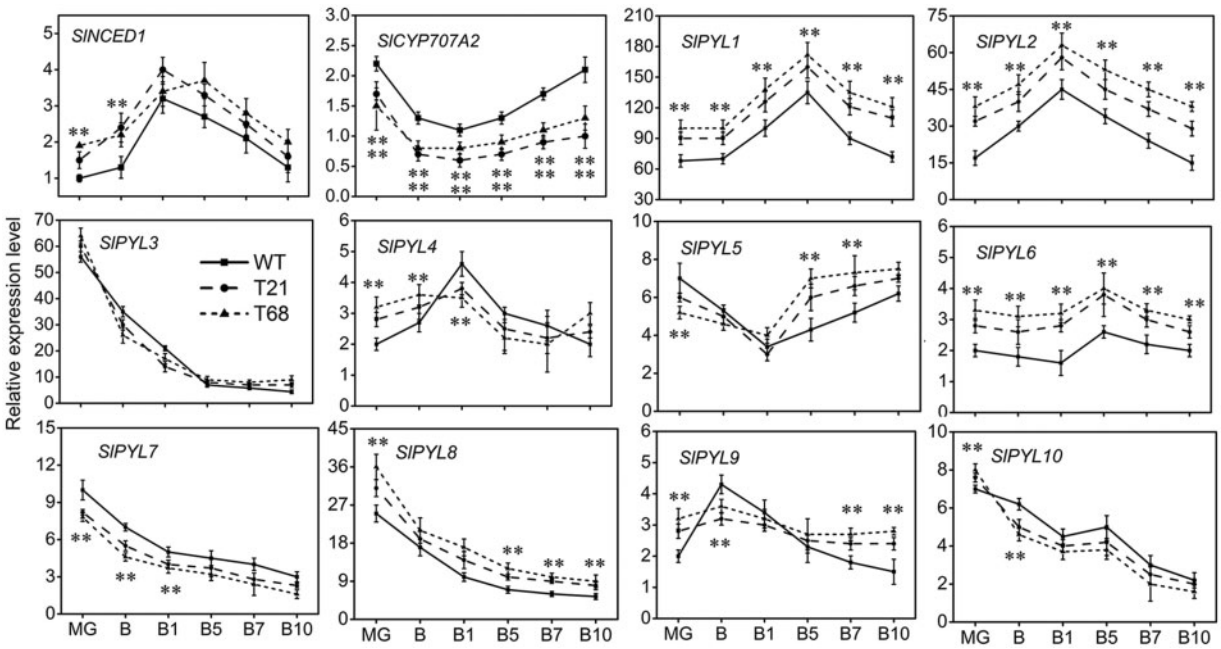

Fig. 3 Changes in expression of genes involved in ABA biosynthesis and catabolism, and ABA receptor SlPYL in *SlPti4*-RNAi and WT fruits during development and ripening. Pericarp in different stages was used for experimental analysis. *SlSAND* was used as the internal control. $^{*}P < 0.05$; $^{**}P < 0.01$.

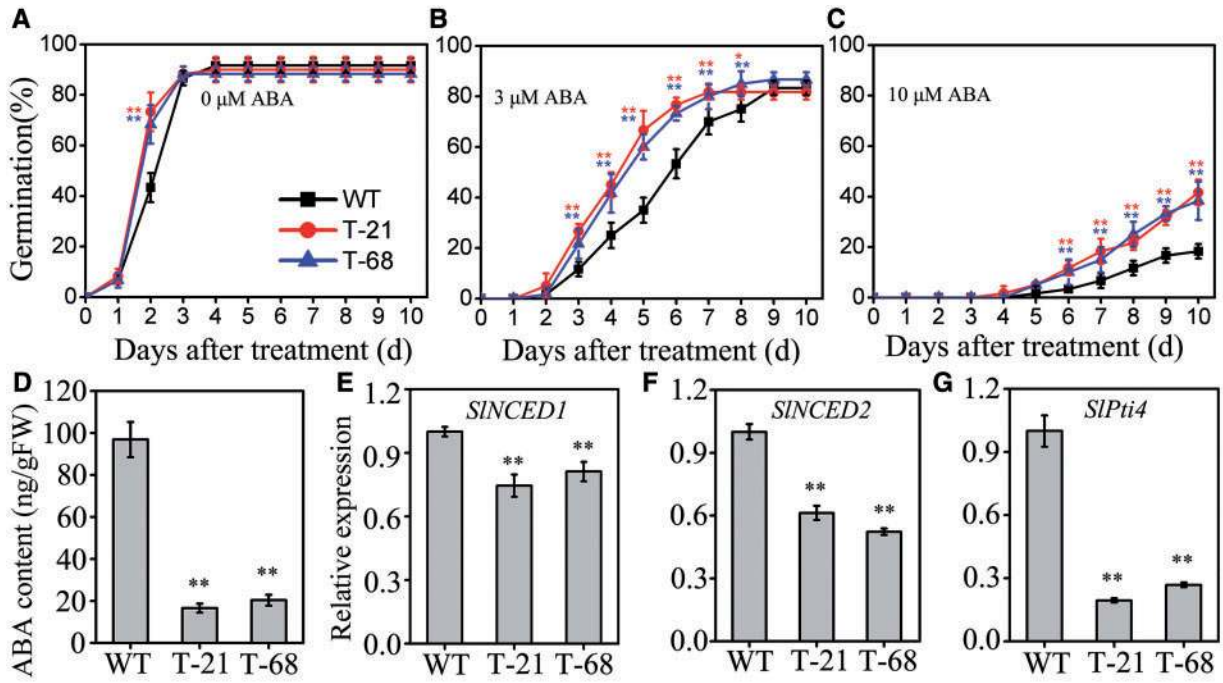

Fig. 4 Seed germination and the effects of exogenous ABA treatment in *SlPti4*-RNAi and WT seeds. (A–C) Effects of exogenous ABA treatment on seed germination. Seeds were sown on 0.5× MS medium without (A) or with 3 or 10 μM ABA (B, C), incubated in the dark at 25°C, and germination was monitored daily. (D) ABA content of dry seeds. (E–G) Expression of *SlNCED1/2* and *SlPti4* in dry seeds. $n = 3$. $^{*}P < 0.05$; $^{**}P < 0.01$.

transgenic lines under ACC treatment was detected. The root growth in transgenic plants was a bit faster than that of the WT plants under the same conditions (Supplementary Fig. S5).

To investigate further the internal mechanism of reduced ABA sensitivity in *SlPti4*-RNAi seeds, we examined the expression patterns of ABA receptor *SlPYL* genes in seeds (Supplementary Fig. S6). We found that the expression levels of *SlPYL3/4/6/9/10* in *SlPti4*-RNAi seeds were significantly lower than in the WT. Additionally, the expression of *SlABI3* and *SlABI5*, two ABA response genes, was down-regulated in transgenic seeds compared with the WT after ABA treatment, which is consistent with the reduced ABA sensitivity of the transgenic lines. These results show that the reduced ABA sensitivity found in *SlPti4*-RNAi seeds is at least partially attributable to the low expression levels of the *SlPYL* genes.

Silencing *SlPti4* reduced drought stress tolerance in tomato plants

In higher plants, ABA is an important hormone that is involved in the plant response to abiotic stresses. To study the role of *SlPti4* in the ABA-mediated drought response, we tested drought resistance of the WT and *SlPti4*-RNAi line plants. Two-month-old plants were divided into two groups: one group was subjected to drought treatment by withholding water for 18 d, and the other (control) group was watered normally. As shown in **Fig. 5A**, *SlPti4*-RNAi plants showed lower drought tolerance with visibly more wilted leaves than did WT plants 18 d after water was withheld. The relative water content (RWC) of leaves from transgenic plants was significantly lower than in WT leaves at 10 and 18 d after drought treatment (**Fig. 5B**). In addition, the water loss rate of detached leaves was measured. As shown in **Fig. 5E**, transgenic leaves lost water at a faster rate than WT leaves. These data indicated that the *SlPti4*-RNAi plants were more sensitive to drought than WT plants.

Consistent with the above results, we found that the ABA content (**Fig. 5C, F**) was markedly increased in WT plants but not in transgenic lines after drought stress or leaf dehydration. In contrast, the ethylene release in transgenic plants was higher than that of the WT plants (**Fig. 5D, G**). These results demonstrate that *SlPti4* may affect ABA biosynthesis and signaling under drought stress. We then analyzed the effects of *SlPti4*-RNAi on the expression of ABA receptor genes. Most of the ABA receptor *PYL* genes were down-regulated in WT leaves after drought treatment, and the expression levels of most *SlPYL* genes in the *SlPti4*-RNAi lines was less than in the WT (Supplementary Fig. S7). Overall, our data suggest that *SlPti4* may play a positive role in drought resistance through its regulation of ABA content and perception.

Susceptibility of *SlPti4*-RNAi plants to *Botrytis cinerea* infection

Both WT and *SlPti4*-RNAi plants were infected with *B. cinerea* at anthesis and then placed into an isolation room at 20°C and 95% relative humidity. Compared with WT plants (plants on the right side of the flowerpots), the transgenic plants (on the left) were more sensitive to *B. cinerea* at 15 days after inoculation (DAI) (**Fig. 6A, B**). The expression of all the genes related to

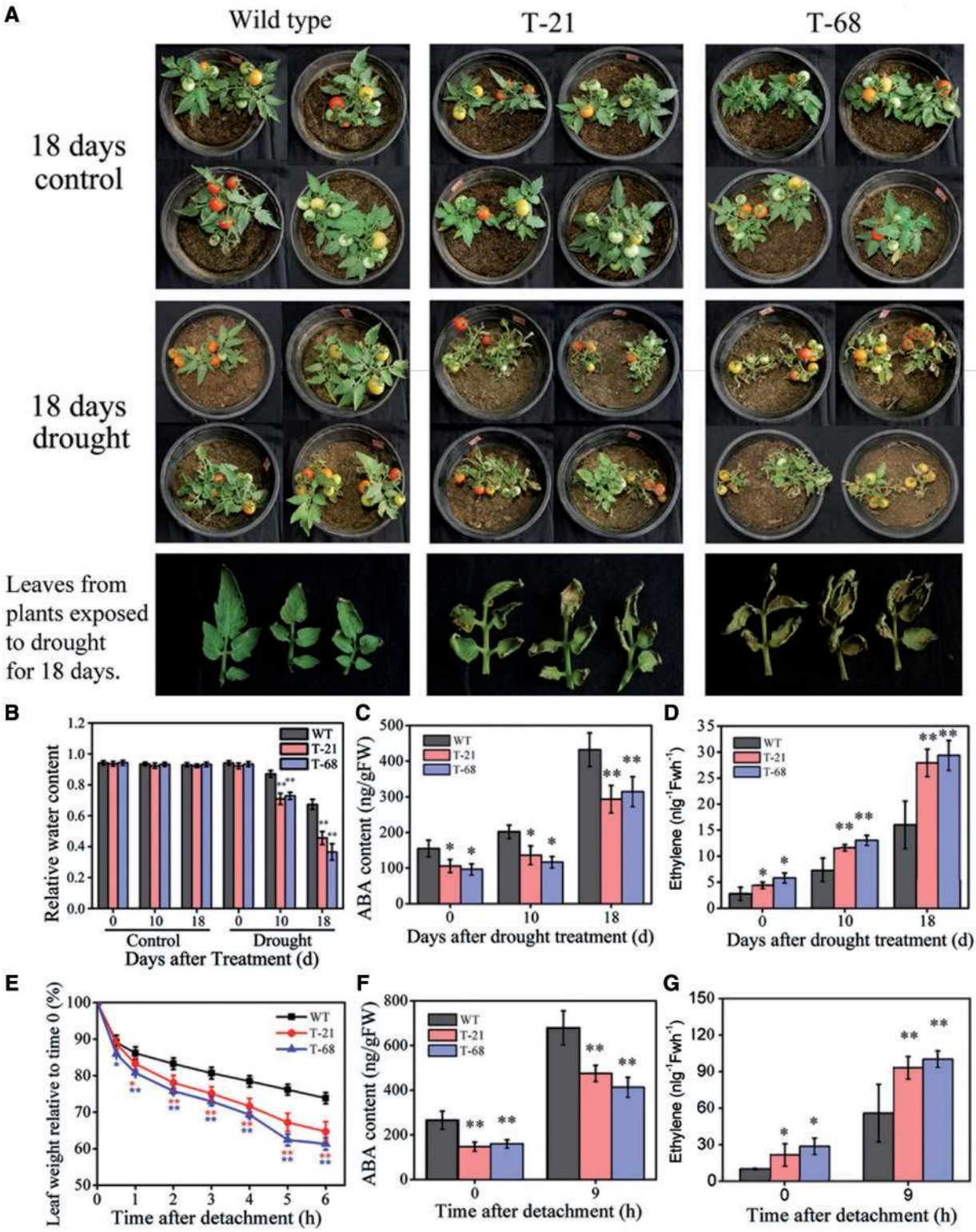

Fig. 5 Effects of RNAi-induced silencing of *SlPti4* expression on drought tolerance of tomato plants. The plants were divided into two groups; the first group was subjected to drought stress for 18 d, while the second group was watered normally as the control. (A) Phenotypes of WT and *SlPti4*-RNAi plants and leaves after drought treatment; photographs were taken at 18 d after drought treatment. (B) Relative water content (RWC) of leaves. (C) ABA content in leaves. (D) Release of ethylene in leaves. (E) Weight loss in detached leaves. (F) ABA content in detached leaves. (G) Release of ethylene in detached leaves. $n = 3$. $^{*}P < 0.05$; $^{**}P < 0.01$.

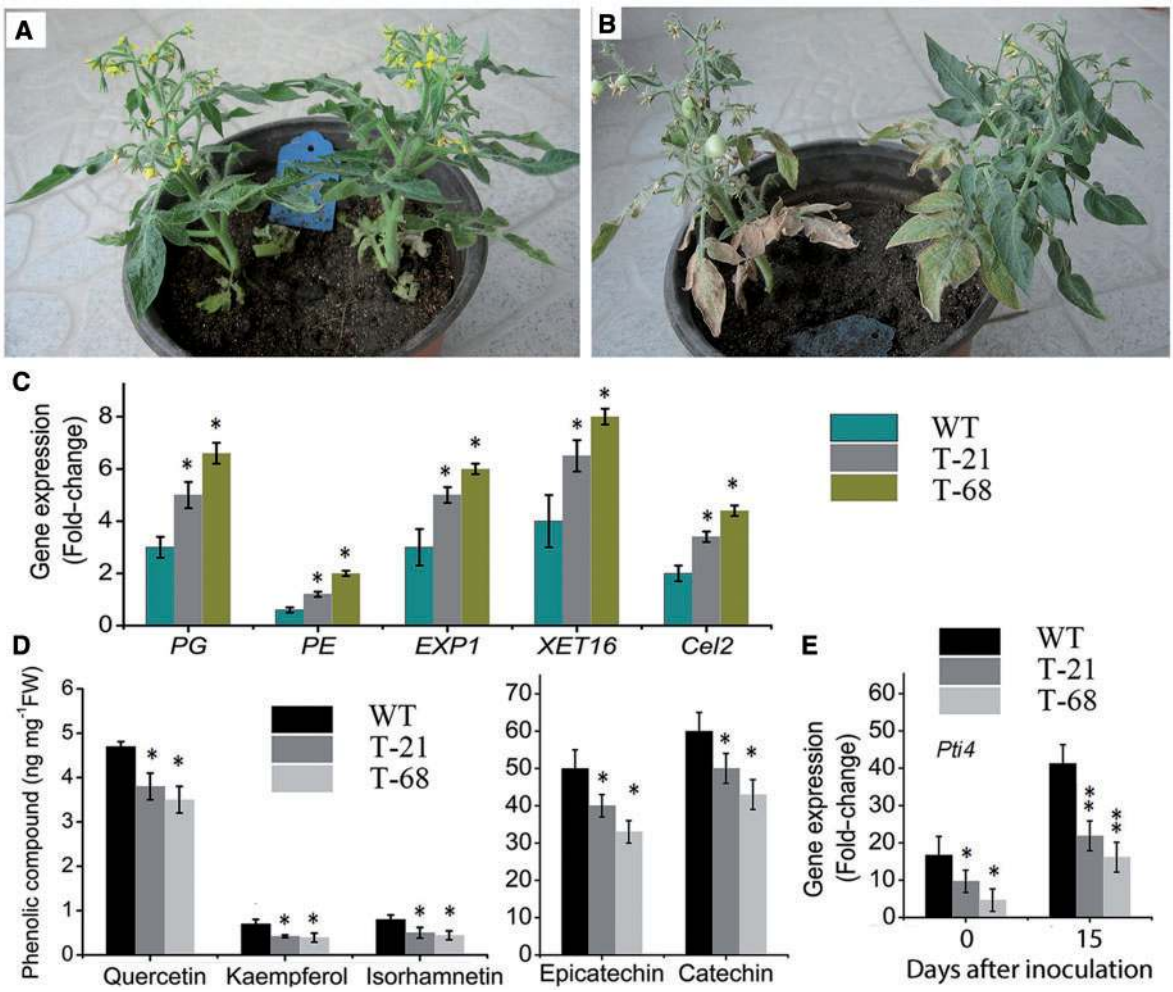

Fig. 6 Susceptibility of *SlPti4*-RNAi plants to *B. cinerea* infection in tomato. Both WT and *SlPti4*-RNAi plants were infected with *B. cinerea* at anthesis and then placed in an incubator at 25°C and 95% relative humidity. Plants were sampled at 0 and 15 d after inoculation with *B. cinerea*. (A) 0 d after inoculation. (B) 15 d after inoculation. WT plants are on the right and *SlPti4*-RNAi plants are on the left side of the flowerpots. (C) Expression of genes related to cell wall degradation in leaves 15 d after inoculation. (D) Phenolic compounds in leaves 15 d after inoculation. (E) Expression of *SlPti4*. $n = 3$. $^{*}P < 0.05$; $^{**}P < 0.01$.

cell wall degradation (PG, PE, EXP1, XET16 and Cel2) in the transgenic plants was up-regulated compared with the WT at 15 DAI (**Fig. 6C**). The contents of phenolic compounds, including quercetin, epicatechin, kaempferol, catechin and isorhamnetin, were lower in *SlPti4*-RNAi plants than in WT plants at 15 DAI (**Fig. 6D**). The *SlPti4* expression of *SlPti4*-RNAi plants was lower than in WT plants at 0 and 15 DAI with *B. cinerea* (**Fig. 6E**). These results show that the reduced *SlPti4* expression caused by RNAi is responsible for the increased sensitivity of transgenic plants to *B. cinerea*.

Discussion

SlPti4-RNAi affects the pigmentation and ripening of tomato fruit

To date, several studies have reported the expression patterns of *SlPti4* during fruit ripening and in response to stresses (Chen et al. 2008). The expression of *SlPti4* is regulated by ethylene in tomato fruit, which is supported by the *SlPti4*-specific mRNA levels in *Nr* (ethylene insensitive) mutant and T4B-11 (ACO1 suppressing line with low ethylene level) fruits, which were much lower than in the WT (Chen et al. 2008). In the present study, we found that, compared with WT fruits, *SlPti4*-RNAi results in increased ABA accumulation together with decreased ethylene release, which supports the negative cross-talk between the two hormones. It is worth noting that the pigmentation in *SlPti4*-RNAi fruits is orange instead of red as in the WT fruits (**Fig. 1D**). Coincidently, a greater β-carotene and lower lycopene content was observed in *SlPti4*-RNAi fruits (**Fig. 1G**). Carotenoids including lycopene, β-carotene and 9-*cis*-epoxyxanthophylls are metabolic precursors of ABA (Qin and Zeevaart 1999, Giuliano et al. 2003). An explanation for why the color of transgenic fruit is orange could be that when the transcriptional level of lycopene β-cyclase SlBcyc is significantly increased by an increased ABA level caused by *SlPti4*-RNAi, the metabolic material flux, which normally channels to linear lycopene during ripening, is partially transformed into the

cyclic carotene pathway in the RNAi lines, resulting in the increased β-carotene. In other words, the decreased lycopene is due to an increased activity of SlBcyc which channels fewer metabolites into the lycopene pathway or due to an inhibition of carbon flux to β-carotene and ABA. It is consistent with the previous reports in which it was shown that the reduced endogenous ABA levels in tomato fruits lead to an overproduction of ethylene and deep-red fruits with more lycopene (Galpaz et al. 2008, Sun et al. 2012a, Sun et al. 2012b).

SlPti4 is involved in ABA-mediated seed germination

Seed germination is regulated by multiple plant hormones. A decrease in ABA is thought to be the key step to initiate seed germination (Finkelstein et al. 2008). The *etr1* and *etr2* mutants of Arabidopsis show faster (*etr1*) or delayed (*etr2*) seed germination due to reduced and enhanced ABA sensitivity, respectively (Wilson et al. 2014). *LeERF2* affects seed germination by regulating the expression of *mannanase2* (Pirrello et al. 2006). However, there are presently no reports on the regulation of ABA metabolism and signaling in seeds by *ERF* genes. Our data show that seed germination was accelerated and the ABA sensitivity was reduced in *SlPti4*-RNAi seeds compared with the WT. In addition, we tested the ABA metabolism and signaling of *SlPti4*-RNAi seeds at the transcriptional level. The results demonstrate that reduced ABA biosynthesis and the expression of *SlPYL* genes contribute to the phenotype observed in *SlPti4*-RNAi seeds. This means that *SlPti4* plays a role in seed germination through its regulation of ABA content and signaling.

SlPti4-RNAi tomato plants exhibit reduced resistance to drought and *B. cinerea* infection

Previous studies have reported that *SlPti4* mRNA levels were significantly reduced in plants suffering from water stress (Chen et al. 2008), and overexpression of several tomato ERF genes such as *TERF1*, *TSRF1*, *JERF1* and *SlERF5* can improve plant drought tolerance (Pan et al. 2012). As a stress phytohormone, ABA and its signaling have pivotal regulatory functions in numerous stress responses (Pizzio et al. 2013). In this study, drought tolerance in *SlPti4*-RNAi plants was reduced when they were exposed to drought stress. Under normal conditions, leaves from *SlPti4*-RNAi lines accumulated less ABA and the expression of *SlPYL* genes was reduced compared with WT plants. Under drought treatment, the expression of *SlPYL* genes was up-regulated (PYL3) or down-regulated (SlPYLs except PYL3) in WT plants; however, the expression changes of *SlPYL* genes in transgenic leaves were less, with lower expression levels, compared with WT plants, which led to more rapid water loss, less growth and weak tolerance to drought stress. These results indicate that *SlPti4* positively regulates ABA accumulation and ABA signaling in response to drought stress. On the other hand, it is found that ethylene is overproduced in transgenic plants, and, consequently, RNAi lines show a weak phenotype to the drought stress (**Fig. 5**). This result supports the previous report showing that ethylene inhibits stomatal closure and debilitates the ABA action (Tanaka et al. 2005). However, many components in the signaling network under drought stress remain unknown. Further analysis is required to determine the exact functions and links between the components involved in the drought stress response.

At the same time, we found that suppressing the expression of *SlPti4* via RNAi increased sensitivity to *B. cinerea* infection in *SlPti4*-RNAi transgenic plants compared with WT plants, indicating that SlPti4 regulates the defense response to *B. cinerea* in tomato plants (**Fig. 6**). The plant cell wall may serve as an effective physical barrier to pathogens, but it is also a matrix where many proteins involved in pathogen perception are delivered. In this study, the expression of genes encoding cell wall hydrolases was up-regulated in *SlPti4*-RNAi plants infected with *B. cinerea*, indicating that SlPti4 is involved in the regulation of cell wall integrity (**Fig. 6**). In addition, we observed a decrease in phenolic compounds in the *SlPti4*-RNAi plants compared with WT plants. This could be due to the fact that *SlPti4*-RNAi weakens the flavonoid biosynthesis pathway, leading to decreases in the levels of phenolic compounds that have multiple biological activities, including antioxidant and antimicrobial effects (Canter and Ernst 2004). Our results indicate that SlPti4 plays a crucial role in the response to biotic (*B. cinerea* infection) stress.

SlPti4 differentially regulates ABA metabolism and signaling in various organs

According to our data, *SlPti4* plays a negative role in regulating the expression of ABA biosynthesis and receptor genes during tomato fruit ripening. In contrast, *SlPti4* has an opposite function during seed germination, and in the response to drought and *B. cinerea* infection. This can be explained by two hypotheses: first, fruit is a reproductive sink organ, while leaves or seeds are vegetative source organs, and the difference between organ types and/or functions may lead to different regulatory mechanisms. Secondly, ABA and ethylene regulate fruit ripening synergistically, while ethylene plays an antagonistic role against ABA during seed germination and in the drought response (Watkins et al. 2014). This discrepant interplay between ABA and ethylene may contribute to the regulation of *SlPti4* expression in different physiological processes. A similar regulation pattern was found in rice, in the *mhz4* and *mhz5* mutants, where the ethylene response was reduced in the roots but was enhanced in coleoptiles.

In conclusion, reduced expression of *SlPti4* via RNAi affects fruit coloring, reduces ABA sensitivity in seeds and decreases the resistance to drought and *B. cinerea* infection by altering ABA levels and the expression of *SlPYL* ABA receptor genes. *SlPti4* is an important regulator of fruit ripening, seed germination and the stress response in tomato.

Materials and Methods

Plant materials and growth conditions

Tomato plants (*Solanum lycopersicum* cv. 'Micro-Tom'), including WT and *SlPti4*-RNAi transgenic lines, were grown in a climate-controlled greenhouse at

25°C/18°C (day/night) with natural light. Transgenic tomato plants from the third generation that were homozygous for single-copy inserts were used in all experiments. The ripening stages of tomato fruits were divided according to days after flowering and fruit color. During development, fruits were sampled on the following days after anthesis: 10 d, 20 d (IG, immature green), 30 d (MG, mature green), 33d (B, breaker), 35 d (B3), 37 d (B5), 39 d, (B7) and 42 d (B10). Fifteen fruits were harvested randomly at each stage from each transgenic line and WT plants and were then divided into three groups (five for each) as three replicates. After harvest, each group of fruits was weighed and their ethylene production was measured. Fruit pulp was then sampled and immediately frozen in liquid nitrogen, powdered, mixed, and stored at −80°C until further use.

Generation of transgenic lines

For construction of the *SlPti4*-RNAi vector, a 444 bp fragment of *SlPti4* (Solyc05g052050) was amplified from 'Micro-Tom' tomato fruit cDNA and cloned into the binary vector pCambia1305.1 in both sense and antisense directions driven by the *Cauliflower mosaic virus* 35S promoter (Supplementary Fig. S2B). The plasmid was introduced into *Agrobacterium tumefaciens* LBA4404 and transformed into 'Micro-Tom' tomato.

Measurements of seed germination

For seed germination, WT and transgenic seeds, which were the homozygous third-generation transgenics, were harvested, air dried and stored for 3 weeks at 4°C. After surface sterilization with 10% sodium hypochlorite, seeds were sown on 0.5× MS medium containing 0, 3 or 10 μM ABA (approximately 100 seeds per line). Seeds were allowed to germinate in the dark at 25°C for 10 d. In order to score seed germination, radical emergence was analyzed 24 h after sowing. Radicle emergence >1 mm was considered to be seed germination.

Drought stress and water status measurement

Tomato plants were grown in 2 liter pots at 25°C with 35% relative humidity and normal watering conditions. Healthy 2-month-old or 30-day-old plants were used for the drought treatment assay, and 20 plants of the WT and each transgenic line were divided into two groups. The first group was subjected to drought stress by stopping irrigation for 18 d, while the second group was watered normally as the control. Leaves were sampled at 0, 10 and 18 d after drought treatment. The RWC of leaves was measured as described previously (Flexas et al. 2006), RWC = [(fresh weight − dry weight)/(turgid weight − dry weight)]. For the detached leaf water loss assay, 10 healthy adult leaves were removed from plants of each line, weighed and placed abaxial side up in an open plate at 25°C at 35% relative humidity, and weighed every hour. Water loss is expressed as relative leaf weight at a given time compared with time zero.

Infection with *Botrytis cinerea*

The ascomycete fungal pathogen *Botrytis cinerea* Pers. was cultured on tomato leaves at 25°C, 95% relative humidity. After 20 d, spores were washed from the leaves with distilled water containing 0.01% (v/v) Tween-20. After removing mycelial debris, the spores were counted and added to the inoculation solution at the proper concentration. Ten WT plants and 10 *SlPti4*-RNAi tomato plants were sprayed with the inoculation solution containing 10^5 or 10^7 spores μl^{-1}. The tomato plants were then placed in an isolation room at 25°C with 95% relative humidity. Twenty days after inoculation, *B. cinerea* infection was evaluated by counting the number of spreading lesions on each plant.

Real-time quantitative PCR (qRT-PCR) analysis

RNA was extracted from 1 g of sample through the SV Total RNA Isolation System (Promega), and the RNA was subsequently digested with DNase I (TAKARA). The extracted RNA was reverse transcribed to cDNA using a Takara RNA PCR Kit (TAKARA). qRT-PCR detection was performed using SYBR Premix ExTaq (Perfect Real Time; TAKARA BIO INC.) on a Rotor-Gene 3000 system (Corbett Research) to quantitate gene expression levels. *SAND* (SGN-U316474) was employed as an internal control. The expression level of each gene was calculated using Rotor-Gene 6.1.81 software with two standard curve methods. The primer sequences are shown in Supplementary Table S4.

Determination of ABA content

A 3 g aliquot of pericarp tissues was extracted twice in 40 ml of 80% (v/v) methanol and then centrifuged at 10,000×*g* for 20 min. The supernatants were dried under vacuum, and the residue was dissolved in 10 ml of 0.02 M phosphate assay buffer (pH 8.0) and extracted three times with 10 ml of petroleum ether. The organic phase was removed, and the pH of the aqueous phase was adjusted to 8.0, followed by the addition of 0.2 g of insoluble PVPP (polyvinylpolypyrrolidone). After stirring for 15 min at 0°C, PVPP was removed through vacuum filtration. The mixed liquid was adjusted to pH 3.0 and was subsequently extracted three times with ethyl acetate. The ethyl acetate phase was dried under vacuum and was dissolved in 1 ml of 50% methanol (v/v). The ABA content was determined via HPLC (Agilent Technologies, 1200) through a 4.8×150 mm C18 column (Agilent Technologies) at a flow rate of 0.8 ml min^{-1}. Elution was performed using both solvent A [0.8% (v/v) glacial acetic acid] and solvent B (100% methanol). (±)-Abscisic acid (Sigma) was employed as a standard for ABA content determination at 260 nm.

Determination of ethylene production

Three fruits were enclosed in a 50 ml airtight container for 2 h at 20°C, and then 1 ml of the headspace gas was withdrawn and injected into a gas chromatograph (Agilent model 6890N) with a flame ionization detector and an activated alumina column for ethylene measurement. Fresh tissues of each fruit were frozen in liquid nitrogen and stored at −80°C until further use.

Statistical treatment of data

Data were statistically analyzed by SPSS software using one-way analysis of variance and Duncan's test of significance. *P and **P denote significance for t-tests with P-values <0.05 and <0.01, respectively.

Phenolic compound analysis

Phenolic compounds in tomato plants were analyzed at 15 d after *B. cinerea* infection. Both WT and *SlPti4*-RNAi plants were sampled at 15 d after infection. (The complete protocol is provided in the Supplementary Data section of phenolic compound analysis.)

Determination of lycopene and β-carotene

Tomato fruits from both WT and RNAi plants were harvested at the full ripe stage. About 0.5 g of pericarp was put into 50 ml of petroleum benzene and acetone (1:0.5, v/v) until they were colorless. The extracts were centrifuged at 10,000×*g* for 20 min. The supernatant liquid was stored at −20°C for later use (the complete protocol is provided in Sun et al. 2012b).

Supplementary Data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflict of interest to declare.

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