TCP transcription factors interact with ZED1-related kinases as components of the temperature-regulated immunity

Zhicai Wang[1†], Dayong Cui[1,2†], Cheng Liu[1,3], Jingbo Zhao[1], Jing Liu[1], Na Liu[4], Dingzhong Tang[4] & Yuxin Hu[1,5]

[1]Key Laboratory of Plant Molecular Physiology, CAS Center for Excellence in Molecular Plant Sciences, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China; [2]School of Life Sciences, Qilu Normal University, Jinan 250200, China; [3]University of Chinese Academy of Sciences, Beijing 100049, China; [4]Key Laboratory of Ministry of Education for Genetics, Breeding and Multiple Utilization of Crops, Plant Immunity Center, Fujian Agriculture and Forestry University, Fuzhou 350002, China; [5]National Center for Plant Gene Research, Beijing 100093, China; [†]These authors contributed equally to this work.

Correspondence

Yuxin Hu, Key Laboratory of Plant Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China

Email: huyuxin@ibcas.ac.cn

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ABSTRACT

The elevation of ambient temperature generally inhibits plant immunity, but the molecular regulations of immunity by ambient temperature in plants are largely elusive. We previously reported that the *Arabidopsis* HOPZ-ETI-DEFICIENT 1 (ZED1)-related kinases (ZRKs) mediate the temperature-sensitive immunity by inhibiting the transcription of *SUPPRESSOR OF NPR1-1*, *CONSTITUTIVE 1* (*SNC1*). Here, we further demonstrate that the nucleus-localized ZED1 and ZRKs facilitate such inhibitory role in associating with the TEOSINTE BRANCHED1, CYCLOIDEA AND PROLIFERATING CELL FACTOR (TCP) transcription factors. We show that some of TCP members could physically interact with ZRKs and are induced by elevated temperature. Disruption of TCPs leads to a mild autoimmune phenotype, while overexpression of the *TCP15* could suppress the autoimmunity activated by the overexpressed *SNC1* in the *snc1-2*. These findings demonstrate that the TCP transcription factors associate with nuclear ZRK as components of the temperature-regulated immunity, which discloses a possible molecular mechanism underlying the regulation of immunity by ambient temperature in plants.

SUMMARY STATEMENT

Elevation of ambient temperature generally inhibits plant immunity. This work reveals that the *Arabidopsis* TCP transcription factors associate with nuclear ZED1-related kinases to govern the immunity by inhibiting the SNC1 in response to temperature change, which discloses a possible molecular mechanism underlying the regulation of immunity by ambient temperature in plants.

KEYWORDS: immunity; ZED1-related kinase; TCP transcription factor; temperature; *Arabidopsis*.

INTRODUCITON

To survive and reproduce successfully, the sessile plants have to effectively sense and respond to various environmental stimuli to optimize their growth and fitness. Ambient temperature is one of major environmental factors that remarkably affect numerous processes of plant growth and development (Penfield, 2008). The elevation of ambient temperature generally promotes plant development and flowering transition, leading to the alteration of plant architecture with elongated hypocotyls, petioles, and hyponastic growth of leaves (Blazquez, Ahn & Weigel, 2003; Gray, Ostin, Sandberg, Romano & Estelle, 1998; Koini *et al.*, 2009; Sun, Qi, Li, Chu & Li, 2012; van Zanten, Voesenek, Peeters & Millenaar, 2009). Moreover, the ambient temperature is also found to participate in the regulation of plant immunity. For instances, the incompatible hybrids of *Arabidopsis* natural accessions and some autoimmune mutants, including *mapk/erk kinase kinase 1* (*mekk1*), *bonzai1-1* (*bon1-1*), *bon association protein 1-1* (*bap1-1*), *chilling-sensitive 2* (*chs2*), *chs3* and *suppressor of salicylic acid insensitivity of npr1-5* (*ssi4*), display a constitutive activation of immune system with retarded growth at the comparatively low temperature condition (Alcazar & Parker, 2011; Hua, 2013), however, such autoimmune phenotype was suppressed by the relatively high ambient temperature (Alcazar & Parker, 2011; Hua, 2013). These observations strongly suggest that ambient temperature has the prevalent roles in optimizing plant immunity and development.

Previous studies in *Arabidopsis* have begun to reveal the molecular basis of ambient temperature-regulated immunity. It has been reported that, in *Arabidopsis*, the elevation of ambient temperature could reduce the abundance of some components involved in salicylic

acid (SA) production, such as PHYTOALEXIN DEFICENT 4 (PAD4) and ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) (Scott, Clarke, Wood & Mur, 2004; Yang & Hua, 2004). However, the reduction of SA or increase of *EDS1* or *PAD4* expression could not change the temperature sensitivity of the *Arabidopsis*, suggesting the alteration of these regulators is not the primary response in the high temperature-inhibited immunity (Wang, Bao, Zhu & Hua, 2009). In contrast, some of Toll–interleukin-1 receptor–nucleotide binding–leucine-rich repeat (TIR-NB-LRR) resistance (R) proteins are found to play a role in the temperature-sensitive immunity. The activity of these R proteins is finely controlled to avoid the activation of immunity under normal growth condition, whereas the inappropriate activation of BALL (BAL)/SUPPRESSOR OF NPR1-1, CONSTITUTIVE 1 (SNC1) in *snc1-1* or *bal/snc1-2* results in a typical autoimmunity with dwarfism without pathogen attack, and such autoimmune phenotypes are strongly inhibited by the elevation of ambient temperature (Stokes, Kunkel & Richards, 2002; Yang & Hua, 2004). Further studies reveal that the elevation of ambient temperature could substantially reduce the nuclear accumulation of some R proteins, including SNC1 and RESISTANCE TO PSEUDOMONAS SYRINGAE 4 (RPS4), and that nuclear accumulation of some NB-LRR proteins was found to be critical for activation of the immunity (Mang *et al.*, 2012; Zhu, Qian & Hua, 2010). Therefore, the reduced abundance of some R proteins appears to be one of possible mechanisms behind the high temperature-inhibited immunity (Elmore, Lin & Coaker, 2011; Heidrich, Blanvillain-Baufume & Parker, 2012; Hua, 2013). Indeed, some of the identified *Arabidopsis* temperature-sensitive autoimmune mutants, including *bon1-1*, *bap1-1*, *bak1-interacting receptor-like kinase 1* (*bir1*), *suppressor of rps4-RLD 1* (*srfr1*), *constitutive expresser of PR*

genes 1 (*cpr1*), *map kinase phosphatase 1* (*mkp1*) and *hopz-ETI-deficient1-D* (*zed1-D*), were found to be SNC1-dependent (Bartels *et al.*, 2009; Gou *et al.*, 2012; Hua, Grisafi, Cheng & Fink, 2001; Kim *et al.*, 2010; Wang, Meng, Zhang, Ren & Yang, 2011b; Wang *et al.*, 2017b), supporting that the fine-tuned SNC1 level and/or activity, at least in part, contribute to the immunity regulated by ambient temperature.

The TCPs belong to a large family of plant-specific transcription factors named from three identified members, TEOSINTE BRANCHED 1 in maize, CYCLOIDEA in snapdragon, and PROLIFERATING CELL FACTOR in rice (Cubas, Lauter, Doebley & Coen, 1999). Extensive studies have demonstrated that the TCP family plays the diverse roles in plant growth and development (Lopez, Sun, Blair & Mukhtar, 2015). For examples, the *Arabidopsis* TCP14 and TCP15 function redundantly in the regulation of internode elongation and leaf development (Kieffer, Master, Waites & Davies, 2011), and are found to interact with SPINDLY (SPY) to promote cytokinin response (Steiner *et al.*, 2012). Intriguingly, recent studies in *Arabidopsis* also demonstrate that the TCPs play an important role in regulating effector-triggered immune (ETI) response, providing an evidence that these widely recognized developmental modulators also participate in the regulation of plant defense response (Lopez *et al.*, 2015; Wang, Cui & Hu, 2017a). Indeed, the multiple TCP members, including TCP13, TCP14, TCP15, TCP19 and TCP21 are found to be targeted by various effectors from diverse pathogen classes (Arabidopsis Interactome Mapping, 2011), and the six members of class I family TCPs (TCP8, TCP14, TCP15, TCP20, TCP22 and TCP23) are found to have interactions with SUPPRESSOR OF rps4-RLD 1 (SRFR1), a negative regulator specific for ETI response (Kim *et al.*, 2014). Consistent with this, the

tcp13, *tcp14* and *tcp19* mutants display an enhanced disease susceptibility (Mukhtar *et al.*, 2011), and the *tcp8 tcp14 tcp15* triple mutant exhibits a decreased ETI response (Kim *et al.*, 2014). A recent study also reveals that the TCP14 could interact with *Pseudomonas syringae* type III effector HopBB1, consequently influencing jasmonic acid (JA) signaling outputs and promoting pathogen virulence (Yang *et al.*, 2017). Moreover, the TCP8 and TCP9 are found to act as the regulators of *ISOCHORISMATE SYNTHASE 1* (*ICS1*), a gene encoding the key enzyme in salicylic acid (SA) biosynthesis, and thus contribute to plant resistance to pathogens (Wang *et al.*, 2015b).

We recently identified a dominant mutant in the *Arabidopsis HOPZ-ETI-DEFICIENT 1* (*ZED1*), *zed1-D*, which exhibits a high temperature-dependent autoimmune phenotype. We showed that the ZED1 cooperates with ZED1-related kinases (ZRKs), which belong to the receptor-like cytoplasmic kinases required for effector-specific ETI responses (Lehti-Shiu & Shiu, 2012; Lewis *et al.*, 2013; Seto *et al.*, 2017; Wang *et al.*, 2015a), to modulate the ambient temperature-regulated immunity by inhibiting the *SNC1* transcription (Wang *et al.*, 2017b). Here, we further demonstrate that the TCPs physically interact with nuclear ZED1 and ZRKs and thus inhibit the SNC1-activated immunity. Our results strongly suggest that the nuclear TCP-ZRKs are components of the temperature-regulated immunity in plants.

MATERIALS AND METHODS

Plant materials and growth conditions

The *Arabidopsis thaliana* accession Columbia-0 (Col-0) was used in this study. The *zed1-6* (SALK_018065), *tcp15-3* (SALK_011491) and *tcp23-1* (SAIL_443_F02) were obtained from

Arabidopsis Biological Resource Center (ABRC) and verified by PCR as previously described (Aguilar-Martinez & Sinha, 2013; Kieffer *et al.*, 2011; Lewis *et al.*, 2013). The *zed1-D*, *tcp14-4* and *bal/snc1-2* mutants were described previously (Kieffer *et al.*, 2011; Stokes *et al.*, 2002; Wang *et al.*, 2017b). The primers used for PCR analysis were listed in the Table S1. All plants were grown in a culture room or growth chamber with an illumination intensity of 80-90 μmol m^{-2} s^{-1} and a 16-h light/8-h dark photoperiod, as described previously (Jing *et al.*, 2009).

Plasmid construction

To generate *p35S::TCP15* construct, the coding sequence of *TCP15* were ligated into the pEasy-Blunt vector (TransGen, China), and cloned into the pVIP96 plasmid (Hu, Xie & Chua, 2003). A DNA fragment containing 1.8 kb *TCP15* promoter was fused with the *β-glucuronidase* (*GUS*) gene and cloned into the pBI101 plasmid to form the *pTCP15::GUS* construct. For generation of the *pTCP15::TCP15-GFP* construct, the *TCP15* promoter and its coding sequence were fused with *GFP* and cloned into the pCAMBIA1301 vector. To examine the interactions of ZED1 and ZED1-D with the TCPs in *N. benthamiana* leaves and transgenic *Arabidopsis* plants, the coding sequence of *ZED1* or *ZED1-D* was fused with *GFP* and cloned into the pH7WGF2.0 vector, while the *TCP1*, *TCP4*, *TCP5*, *TCP14*, *TCP15* and *TCP23* were fused with *MYC*-tagged sequence and cloned into the pVIP96myc vector, respectively. To generate *p35S::TCP15-SRDX* transgenic plants, the *TCP15* coding sequence fused with the *SRDX* transcriptional repression domain linker sequence was cloned into the pVIP96 vector (Guo *et al.*, 2010; Li, Li & Dong, 2012). For generation of the *p35S::ZED1/ZED1-D-NLS -GFP* and *p35S::ZED1/ZED1-D-NES-GFP* constructs, the coding

sequence of *ZED1* or *ZED1-D* was amplified with the primers containing the NLS or NES coding sequence (Kim *et al.*, 2014), and fused with *GFP*, and then cloned into the pEarleyGate104 vector. All of the primers used for plasmid construction are listed in Table S1.

Bimolecular fluorescence complementation (BiFC) assay and confocal fluorescence microscopy

BiFC assay was carried out as previously described (Walter *et al.*, 2004). The full-length CDSs of *TCP5* and *TCP15* were cloned into the pUC-SPYNE vector and the full-length CDS of *ZED1* was cloned into pUC-SPYCE vector, respectively. *Agrobacterium* carrying the plasmids was infiltrated into 4-wk-old *N. benthamiana* leaves. GFP signals were visualized after infiltration for 24-48 h. To determine the subcellular localization of ZED1 and ZED1-D, 2-wk-old transgenic plants harboring the corresponding *GFP*-fused construct were used for visualizing GFP signal. The fluorescence in the specific cells was excited at 488 nm and monitored at 505-550 nm with a pinhole setting at 1.5 a.u. under a confocal microscope (Leica TCS SP5, Leica, Germany).

Gene expression analysis

The total RNA was isolated using a guanidine thiocyanate extraction buffer according to the previously described protocol (Hu, Bao & Li, 2000). The cDNA was synthesized from total RNA using SuperScript III Reverse Transcriptase (Invitrogen, USA) and the real-time quantitative reverse transcription PCR (qRT-PCR) was performed with a Rotor-Gene 3000 thermocycler (Corbett Research, Australian) with a SYBR Premix Ex Taq II kit (Takara,

Japan). The relative expression level for each gene was normalized to that of *ACTIN2* and the data were collected from three biological replicates, as described previously (Cui *et al.*, 2013). For histochemical GUS staining, the seedlings or organs were fixed in cold 90% acetone for 20 min, and stained for 6-10 h at 37°C in 2 mM 5-bromo-4-chloro-3-indoxyl-β-D-glucuronide (X-gluc), 50 mM sodium phosphate, pH 7.0, 5 mM $K_3/K_4Fe(CN)_6$, 0.2% (w/v) Triton X-100. After staining, samples were bleached in 70% ethanol several times, and photographed with a digital camera (Jefferson, Kavanagh & Bevan, 1987). The primers used for gene expression analyses were listed in the Table S1.

Trypan blue (TB) and 3,3'-diaminozenzidine (DAB) staining assays

For TB staining assay, the rosette leaves were cut off with a razor blade, and then placed into the centrifuge tube containing TB staining solution (10 ml lactic acid, 10 ml glycerol, 10 g phenol, and 10 mg trypan blue dissolved in 10 ml distilled water). The samples were heated in a boiling water bath for 5 min, cleared in the chloral hydrated solution overnight, and observed under a microscope (van Wees, 2008). For DAB staining assay, the aerial organs of 4-wk-old plants were collected and then vacuumed in DAB solution (1 mg/mL, pH 3.8) for 5 min in a shaking incubator for 4 h at 100 rpm, and destained in 100% ethanol for 3 h (Clarke, 2009).

Yeast two-hybrid (Y2H) assay

The *ZED1* and *ZED1-D* were cloned into the pCR8/GW/TOPO vector and then fused to the GAL4-bining domain of the pDEST32 (pD32) bait vector. A cDNA prey library prepared from *Arabidopsis* inflorescence RNA was generated and the yeast two-hybrid screen was

carried out by a standard procedure (ProQuest Two-Hybrid System, Invitrogen). Plasmids were isolated from positive yeast colonies and sequenced. To investigate the possible interactions of ZED1, ZED1-D, ZRK1 or ZRK12 with TCPs, *ZRK1* and *ZRK12* were cloned into the pDEST32 vector, while the coding sequence of *TCP*s were cloned into the pDEST22 (pD22) vector, and specific yeast two-hybrid interactions were probed according to the ProQuest Two-Hybrid System protocol. The co-transformation of pDEST32-KREV1 and pDEST22-RalGDS-wt constructs was used as a positive control, while the pDEST32-KREV1 and pDEST22-RalGDS-m2, pDEST32-ZED1, pDEST32-ZED1-D, and empty pDEST22 were used as the negative controls (Serebriiskii, Khazak & Golemis, 1999).

Protein extraction and immunoblot assay

To detect the cellular accumulation of TCP15 in WT and *zed1-D* background, the nuclear-cytoplasmic fractionation was performed. The transgenic seedlings carrying the *pTCP15::TCP15-GFP* construct were harvested, and then ground in liquid nitrogen and suspended in a cell lysis buffer (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 20 mM KCl, 2.5 mM $MgCl_2$, 25% glycerol and protease inhibitor cocktail) on ice. The suspension was filtered through double layers of miracloth, and the effluent was centrifuged at 1500 g for 10 min. The supernatant was centrifuged at 10000 g for 10 min, and thus cytoplasmic fraction was isolated. The nuclear fraction was obtained from the pellet as previously described (Wang *et al.*, 2011a). After the protein separation with a 10% SDS-polyacrylamide gel, the proteins from cytoplasmic and nuclear extracts were detected using anti-GFP antibody (MBL, Japan) by immunoblot assay as describe previously (Liu *et al.*, 2010). Histone H3 and β-ACTIN2 were used as internal controls of nuclear and cytosolic marker protein, respectively.

Coimmunoprecipitation (CoIP) assay

CoIP assay was performed in both *N. benthamiana* and *Arabidopsis*. Total proteins were extracted with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM DTT, 10 mM EDTA, 1 mM NaF, 1 mM Na_2MoO_4, 1% polyvinylpyrrolidone, 10% glycerol and protease inhibitor cocktail) from agro-infiltrated *N. benthamiana* leaves or transgenic *Arabidopsis* plants. 50 μl anti-GFP antibodies conjugated in micro beads (MBL, Japan) were added to the extracts and incubated for 4 h at 4°C. The beads were then washed at least 4 times (Roux *et al.*, 2011). The proteins bound to the beads were fractionated in a 10% SDS-PAGE gel and examined with anti-GFP or anti-MYC antibody (MBL, Japan).

RESULTS

High temperature-dependent autoimmunity in *zed1-D* relies on the cytosolic localization of ZED1-D

We previously showed that the *Arabidopsis* ZED1 was induced by elevation of ambient temperature and dually localized into the nucleus and cytosol, and that the dominant mutation isoform of the cytosol-localized ZED1-D confers the *zed1-D* with a HOPZ-ACTIVATED RESISTANCE 1 (ZAR1)-dependent autoimmune phenotype at high temperature condition (Wang *et al.*, 2017b). Since cytosolic ZED1 forms a precomplex with ZAR1 and is required for the *P. syringae* effector HopZ1a-triggred ETI (Lewis *et al.*, 2013; Lewis, Wu, Guttman & Desveaux, 2010), we thus reasoned that the autoimmunity in *zed1-D* is due to the high temperature-induced cytosolic ZED1-D and its interaction with ZAR1, which mimics the ZAR1-activated immune signaling. To further verify that the cytosol-localized ZED1-D is

essential for autoimmunity in the *zed1-D*, we manipulated the ZED1-D by fusing it with a nuclear localization or export signal (NLS or NES) and GFP (Figure 1a). Careful visualization of GFP signals in the root of transgenic *Arabidopsis* plants overexpressing the ZED1-D-NLS or ZED1-D-NES showed that ZED1-D-NLS was targeted into the nuclei whereas ZED1-D-NES was accumulated in the cytosol (Figure 1b). Next, we overexpressed the ZED1-D-NLS or ZED1-D-NES in the *zed1-6* mutant, in which the transcription of *ZED1* is disrupted (Wang *et al.*, 2017b). As expected, the transgenic *zed1-6* plants overexpressing cytosolic ZED1-D exhibited the developmental retardation as observed in the *zed1-D*, while the over-accumulation of ZED1-D in the nuclei did not result in the obvious organogenic phenotype in *zed1-6* plants (Figure 1c,d). Further immune analyses of the reactive oxygen species (ROS) stained by diaminozenzidine (DAB) and cell death stained by trypan blue (TB) clearly demonstrated that the immune signaling was activated by cytosolic ZED1-D but not by the nuclear ZED1-D (Figure 1e). These observations, together with the finding that the autoimmunity of *zed1-D* is ZAR1-dependent (Wang *et al.*, 2017b), strengthen that cytosolic localization of ZED1-D and its interaction with ZAR1 are responsible for the high temperature-dependent autoimmunity in *zed1-D*.

Over-accumulation of nucleus-targeted ZED1 or ZED1-D inhibits the autoimmunity of *zed1-D*

Since ZED1 and ZRK are dully localized in both cytosol and nuclei, we were curious whether or not the nucleus-localized ZED1 and ZRKs have a role in regulating plant immunity. To test this, we further manipulated the ZED1 by fusing it with NLS or NES and GFP, and the GFP signals visualized in the transgenic *Arabidopsis* plants overexpressing ZED1-NLS or

ZED1-NES revealed that the ZED1-NLS was targeted in the nuclei whereas the ZED1-NES was in the cytosol, respectively (Figure 2a). Next, we introduced these two constructs individually into the *zed1-D*. As expected, overexpression of ZED1-NES could complement the developmental defect in *zed1-D* by competing with the ZED1-D for ZAR1 interaction (Figure 2b,c). Surprisingly, the over-accumulation of nuclear ZED1 also suppressed the developmental defect in the *zed1-D* (Figure 2b,c). Consistent with this, the elevated expression of two immune marker genes *PATHOGENESIS-RELATED 1* (*PR1*) and *PR2* in the *zed1-D* was inhibited by overexpression of either cytosolic or nuclear ZED1 (Figure 2d), and the elevated reactive oxygen species (ROS) and enhanced cell death in the *zed1-D* grown at 25ºC were also suppressed in both genotypes (Figure 2e). Because overexpression of ZED1-D-NLS in the *zed1-6* did not cause any obvious phenotype, we also overexpressed the ZED1-D-NLS in the *zed1-D* plants. Similarly, the over-accumulation of ZED1-D in the nuclei also inhibited the high temperature-dependent autoimmune phenotype of *zed1-D* (Figure 2f,g). These observations strongly suggest that over-accumulation of nucleus-targeted ZED1 or ZED1-D interferes the cytosolic ZED1-D-activated immune signaling.

ZED1 and ZRK physically interact with TCP transcription factors

Since the cytosolic ZED1-D and its interaction with ZAR1 is responsible for the high temperature-dependent autoimmunity in *zed1-D* (Wang *et al.*, 2017b) (Figure 1), it becomes interesting how over-accumulation of nuclear ZED1 or ZED1-D could suppress the cytosolic ZED1-D-activated autoimmunity. To explore molecular mechanism behind this, we carried out a yeast two-hybrid screen to identify the proteins that could interact with ZED1 or ZED1-D, which allowed us to identify the TCP14 and TCP4 that had interactions with ZED1

and ZED1-D in yeast, respectively (Figure S1a). Since TCPs belong to a large plant-specific transcription factor family, we further selected a few of representative TCP members and tested their interactions with ZED1 and ZED1-D. Yeast two-hybrid assays showed that the multiple TCP members, including TCP1, TCP15, TCP23 but not TCP5, TCP8 and TCP22, could interact with ZED1 and ZED1-D (Figure 3a). Coimmunoprecipitation (CoIP) assay performed in *N. benthamiana* validated that the TCP1, TCP4, TCP14, TCP15 and TCP23 but not the TCP5 had an interaction with ZED1 or ZED1-D (Figure S1b). To validate the physical interaction of ZED1 with TCPs *in planta*, we used the TCP15 as a representative and further examined its physical interaction with ZED1 *in planta*. Bimolecular fluorescence complementation (BiFC) assay performed in *N. benthamiana* leaves showed that ZED1 physically interacted with the TCP15 but not with the TCP5 in the nucleus (Figure 3b), and CoIP assay carried out with the transgenic *Arabidopsis* plants confirmed the interaction of ZED1 or ZED1-D with TCP15 (Figure 3c). Because multiple ZRK numbers have been found to cooperate with ZED1 in regulating the immunity (Wang *et al.*, 2017b) , we also used yeast two-hybrid and tested the interaction of TCP15 with ZRK1 and ZRK12, which act redundantly with ZED1 in modulating the immunity when plants were not infected by pathogen (Wang *et al.*, 2017b), and our results clearly showed that TCP15 had an interaction with ZRK1 and ZRK12 (Figure S1c). Taken together, we concluded that ZED1 and some ZRK members have a physically interaction with multiple TCP transcription factors.

Some of TCPs are induced by elevated temperature and ZED1-D disturbs TCP15 localization at high temperature condition

Interaction of TCPs with nuclear ZRKs suggests that TCPs may also mediate the temperature-sensitive immunity. To test this, we first examined the tissue-specific expression of *TCP15* and its responsiveness to ambient temperature. As shown in Figure S2a, the GUS staining assay performed in the transgenic plants harboring a *pTCP15::GUS* construct showed that *TCP15* was highly expressed in the roots, vasculature of rosette leaves, and inflorescence stems, which is largely overlapping with that of *ZED1* (Wang *et al.*, 2017b). In addition, the mutation of ZED1-D in the *zed1-D* did not appear to have an effect on the *TCP15* transcription (Figure S2a). However, the quantitative reverse transcription PCR (qRT-PCR) analyses showed that the temperature shift from 18 into 25ºC induced the transcription of *TCP15* (Figure 4a), and the TCP15-GFP signals in the transgenic plants carrying a *pTCP15::TCP15-GFP* construct revealed that TCP15 accumulation in nucleus was increased when ambient temperature elevated (Figure 4b). Moreover, we also observed that the transcription of *TCP4* and *TCP8* was induced by elevated temperature (Figure S2b), indicating that some of TCP members are responsive to ambient temperature change.

Next, we examined the TCP15 accumulation in transgenic *pTCP15::TCP15-GFP* plants in both WT and *zed1-D* background after being transferred from 18 into 25ºC, by nuclear-cytosolic protein fractionation. As expected, the elevation of temperature indeed caused an obvious increase of nuclear TCP15 in the WT background, confirming that TCP15 is induced by elevated temperature (Figure 4c). Interestingly, we observed that some of TCP15 proteins were detained in the cytosol of *zed1-D* after the plants were transferred from

18°C into 25°C (Figure 4c), suggesting that the increased accumulation of cytosolic ZED1-D induced by high temperature could disturb the TCP localization. These observations further support that TCPs have interactions with ZRKs and possibly function in the temperature-regulated immunity.

Disruption of some TCPs leads to a mild autoimmunity

Since the TCPs and ZRKs have an interaction and are responsive to ambient temperature, we reasoned that TCPs may also participate in governing plant immunity in response to ambient temperature when plants are not attacked by pathogen. To test this, we first closely examined the morphologic and immune phenotype in the *tcp* mutants. Consistent with previous observation, disruption of the *TCP15* in *tcp15-3* resulted in a slight growth retardation, and such phenotype was further enhanced in the *tcp15-3 tcp14-4* double mutant and the *tcp14-4 tcp15-3 tcp23-1* triple mutant (Kieffer *et al.*, 2011) (Figure 5a,b). Further immune assays revealed that, although the *tcp15-3* and *tcp15-3 tcp14-4* did not exhibit any distinguishable autoimmunity when compared to WT plants, the *tcp15-3 tcp14-4 tcp23-1* triple mutant clearly displayed an obvious autoimmune phenotype by the enhanced ROS accumulation, increased cell death, and elevated expression of *PR1* and *PR2* (Figure 5c,d), suggesting that TCPs act redundantly to inhibit immunity without pathogen attack. To verify this, we also generated the *p35S::TCP15-SRDX* transgenic plants in which a chimeric repressor by fusing TCP15 with a modified EAR motif repression domain SRDX, which dominantly suppresses the activities of endogenous TCPs that function redundantly with TCP15 (Li *et al.*, 2012). Similar to previous findings (Zhang *et al.*, 2018), the transgenic *p35S::TCP15-SRDX* plants exhibited a apparently retarded growth with autoimmune phenotype (Figure S3). These

findings demonstrate that TCPs play an inhibitory role in governing plant immunity possibly by associating with ZRKs.

TCPs govern the immunity by inhibiting *SNC1* transcription

Since overexpression of *ZED1* could suppress the autoimmunity of *bal*/*snc1-2* by inhibiting *SNC1* transcription (Wang *et al.*, 2017b), and some of the temperature-sensitive autoimmune mutants are dependent on the *SNC1* activation (Gou & Hua, 2012; Hua, 2013; Wang *et al.*, 2017b), we thus reasoned that *SNC1* is a downstream target of the TCPs and nuclear ZRKs. To test this, we first examined whether disruption of *TCP* or suppression of TCP function could affect the expression of *SNC1*. As expected, the transcription of *SNC1* was obviously elevated in the *tcp14-4 tcp15-3 tcp23-1* triple mutant and the *p35S::TCP15-SRDX* transgenic plants grown at 25ºC (Figure 6a). Next, we overexpressed the *TCP15* in the *bal*/*snc1-2*, in which the *SNC1* transcription is activated and thus plants display an autoimmune phenotype at 22ºC, and we found that the overexpression of *TCP15* fully suppressed the elevated *SNC1* and thus the developmental retardation and autoimmunity of *snc1-2* at 22ºC (Figure 6b-e). These observations strongly suggest that the abundance of TCP15 and possible other TCPs, in associating with nuclear ZRKs, plays an inhibitory role to govern immunity by inhibiting the *SNC1* transcription when plants are not attacked by pathogens.

DISCUSSION

Generally, the plant immunity is tightly controlled without pathogen attack. Once the immune signaling is inappropriately activated, plants have to reallocate resources towards immunity by antagonizing growth and development (Alcazar & Parker, 2011). Recent studies show that

such autoimmune phenotype observed in most of *Arabidopsis* mutants could be inhibited by the elevation of ambient temperature (Alcazar & Parker, 2011; Hua, 2013). Although some of nuclear R proteins including the *Arabidopsis* SNC1 have been reported to be involved in the temperature-sensitive autoimmunity, how ambient temperature executes such role in regulation of these R proteins remains largely elusive. We recently showed that the *Arabidopsis* ZED1 and ZRKs are responsive to elevated temperature and act cooperatively to govern the immunity by modulating the transcription of *SNC1* (Wang *et al.*, 2017b). Here, we further demonstrate that the nuclear ZRKs facilitate such inhibitory role possibly by associating with the TCP transcription factors. Although the detailed molecular regulation of *SNC1* by nuclear TCP-ZRK remains to be further defined, our findings strongly suggest that the nuclear TCP-ZRK-modulated SNC1 might be a possible molecular mechanism underlying ambient temperature-regulated immunity. It is likely that, at low ambient temperature condition, the comparatively low level of nucleus-accumulated ZRKs and TCPs are sufficient to maintain the basal immunity and to support development by fine-tuned SNC1 expression. When ambient temperature elevates, the high temperature-induced abundant TCPs and nuclear ZRKs might be necessary to dampen the immunity by inhibiting *SNC1* transcription (Figure S4).

The *Arabidopsis* ZRKs and TCPs have been demonstrated to play critical roles in the defense response against pathogen. The ZED1 and some of ZRKs are found to form the cytosolic complex with ZAR1 to recognize the specific pathogenic type III effector (T3E) and thus activating effector-triggered immunity (Seto *et al.*, 2017; Wang *et al.*, 2015a). For examples, ZED1 forms a precomplex with ZAR1 to specifically recognize *P. syringae*

effector HopZ1a (Lewis *et al.*, 2013; Lewis *et al.*, 2010), while the RESISTANCE RELATED KINASE 1 (RKS1/ZRK1)-ZAR1 and ZRK3-ZAR1 complexes are required for *X. campestris* effector AvrAC- and *P. syringae* effector HopF2a-triggered ETI, respectively (Seto *et al.*, 2017; Wang *et al.*, 2015a). Similarly, the TCPs are also involved in the regulation of plant defense response (Kim *et al.*, 2014; Mukhtar *et al.*, 2011; Wang *et al.*, 2015b), because the *tcp8 tcp14 tcp19* triple mutant displays the reduced ETI triggered by a diverse set of effectors including avrRps4 (Kim *et al.*, 2014), and the multiple TCP members are found to be targeted by various effectors from diverse pathogen classes (Arabidopsis Interactome Mapping, 2011; Mukhtar *et al.*, 2011; Wessling *et al.*, 2014; Yang *et al.*, 2017). Interestingly, our previous and current works demonstrate that nuclear ZRKs could interact with TCPs and both play an inhibitory role in governing the immunity by inhibiting *SNC1* transcription without pathogen attack, which discloses another layer of the function of ZRKs and TCPs in the regulation of plant immunity. Because some of TCPs and ZRKs are induced or responsive to ambient temperature (Wang *et al.*, 2017b) (Figure 4), and some of identified *Arabidopsis* temperature-dependent autoimmune mutants are *SNC1*-dependent (Hua, 2013), it is plausible that such inhibition of SNC1 by nuclear ZRK-TCP apparently contributes, at least in part, to the temperature-regulated immunity. Furthermore, since disruption of multiple ZRKs or TCPs only leads to the mild autoimmunity and overexpressed ZED1 or TCP15 could suppress the autoimmune phenotype of *snc1-2* (Wang *et al.*, 2017b) (Figure 6), it is obvious that multiple members of TCP or ZRK family act redundantly or cooperate regarding such inhibition of *SNC1* transcription by TCPs and nuclear ZRKs.

It becomes interesting that ZRKs and TCPs play different roles in pathogen defense and basal immunity, which appears to involve the positive and negative regulation of SNC1, respectively. This could be why the disruption of multiple TCPs leads to a mild autoimmunity but an enhanced susceptibility to pathogens (Kim *et al.*, 2014; Mukhtar *et al.*, 2011) (Figure 5). Moreover, the cytosolic ZED1-D-activated immunity is dependent on ZAR1 and likely a mimic of ZAR1-triggred ETI by activation of *SNC1* transcription (Wang *et al.*, 2017b), by contrast, the role of nuclear ZRKs and TCPs in governing immune system seems via a ZAR1-independent signaling to inhibit *SNC1* transcription. This could also explain why over-accumulation of nuclear ZED1 or ZED1-D could inhibit the high temperature-induced autoimmunity in the *zed1-D*. Therefore, it is likely that the regulations of *SNC1* by TCPs and/or ZRKs are complicated or at multiple layers in the absence and/or presence of pathogen, which possibly depends on the varied abundances of TCPs, ZRKs and their interacting partners under different conditions. Indeed, a recent work also reported that TCP15 could interact with MOS1 (MODIFIER OF SNC1) to promote the expression of *SNC1* in a MOS1-dependent manner under normal growth condition, though an elevated *PR1* expression was still observed in their *TCP15-SRDX* plants (Zhang *et al.*, 2018). Nevertheless, our findings that TCPs and ZRKs are responsive to ambient temperature and the autoimmune phenotype of *snc1-2* could be inhibited by overexpressed TCP15 or ZED1 strongly suggest that the nuclear TCP-ZRK-modulated immunity might represent a possible guarding mechanism in response to the ambient temperature and/or other abiotic stimuli. This is also supported by our observation that only high level of mis-localized cytosolic ZED1-D could cause the autoimmunity and disturb the subcellular localization of TCP15 under high

temperature condition, though how ZED1-D or ZRK affects the TCP localization and whether the TCP influences ZRK localization under different temperature conditions remain to be determined. Finally, although the mild autoimmunity in the multiple *zrk* or *tcp* mutant might be caused by activation of *SNC1* transcription, it is also possible that the SNC1 guards the nuclear TCP-ZRK complexes and the disruption of these complexes leads to the autoimmunity. Therefore, further works on the nuclear-cytosolic distributions of NB-LRRs and their co-factors including ZRKs and TCPs and the effects of ambient temperature will be necessary to understand the molecular regulations of immunity under varied biotic and abiotic conditions.

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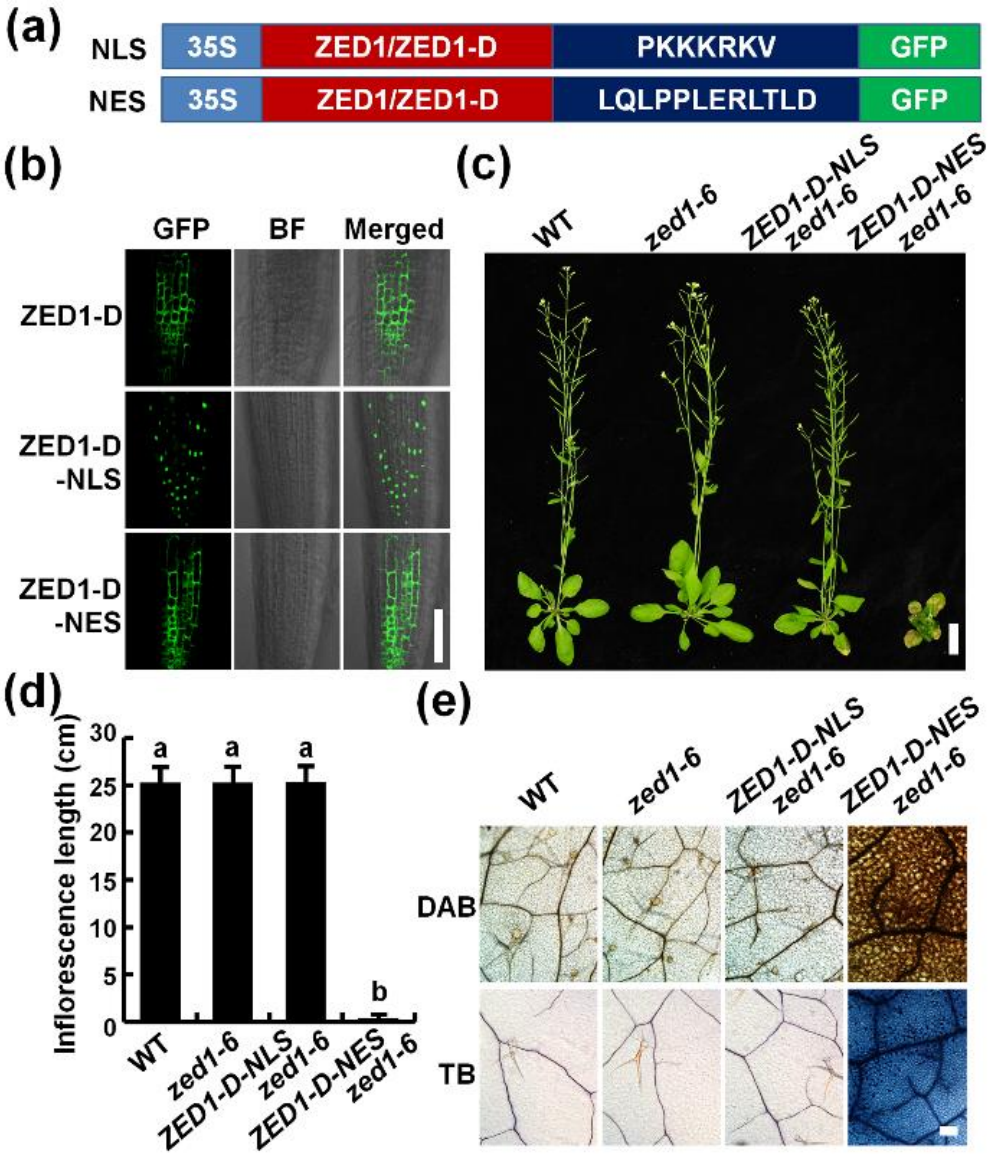

FIGURE 1 The cytosolic ZED1-D is responsible for high temperature-dependent autoimmunity in *zed1-D*. (a) Schematic illustration of the *p35S::ZED1/ZED1-D-NLS* and *p35S::ZED1/ZED1-D-NES* constructs. Nuclear localization signal (NLS) or nuclear export signal (NES) sequence was inserted between the ZED1 or ZED1-D and C-terminus-tagged GFP driven by the CaMV 35S promoter. (b) Subcellular localization of ZED1-D, ZED1-D-NLS and ZED1-D-NES. The GFP signals were visualized in the root tips of the transgenic *p35S::ZED1-D-GFP* (*ZED1-D*), *p35S::ZED1-D-NLS-GFP* (*ZED1-D-NLS*) and *p35S::ZED1-D-NES-GFP* (*ZED1-D-NES*) plants grown at 25ºC. At least 10 independent transgenic lines of each genotype were examined. Scale bar, 50 µm. (c,d) Morphology (c) and inflorescence stem length (d) of 6-wk-old wild-type (WT), *zed1-6*, *p35S::ZED1-D-NLS-GFP;zed1-6* (*ZED1-D-NLS zed1-6*) and *p35S::ZED1-D-NES-GFP;zed1-6* (*ZED1-D-NES zed1-6*) plants grown at 25ºC. Data are shown as means ± SD, and the letters (a, b) indicate the significance ($P<0.05$) according to one-way ANOVA tests. Scale bar, 2 cm. (e) Diaminozenzidine (DAB) (top panel) and trypan blue (TB) (bottom panel) stained leaves of 4-wk-old WT, *zed1-6*, *ZED1-D-NLS zed1-6* and *ZED1-D-NES zed1-6* plants grown at 25ºC. Scale bar, 100 µm.

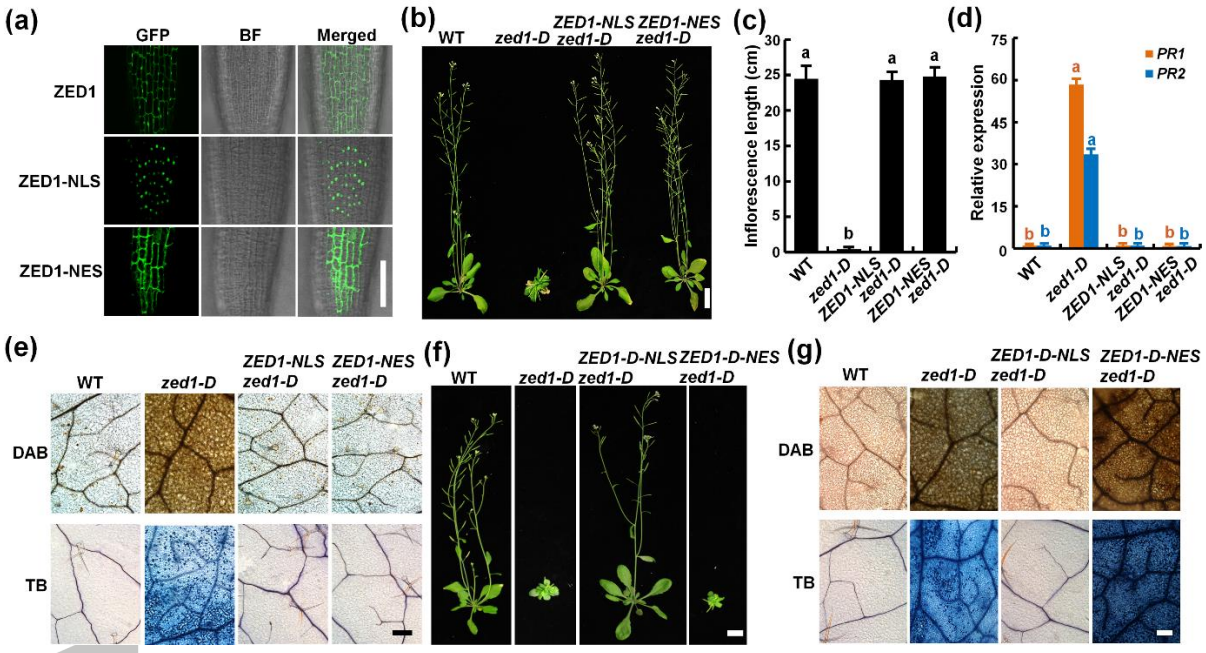

FIGURE 2 Over-accumulation of nuclear ZED1 or ZED1-D inhibits the autoimmunity of *zed1-D*. (a) Subcellular localization of ZED1, ZED1-NLS and ZED1-NES. The GFP signals were visualized in the root tips of transgenic *p35S::ZED1-GFP* (*ZED1*), *p35S::ZED1-NLS-GFP* (*ZED1-NLS*) and *p35S::ZED1-NES-GFP* (*ZED1-NES*) plants grown at 25ºC. At least 10 independent transgenic lines of each genotype were examined. Scale bar, 50 μm. (b,c) Morphology (b) and inflorescence stem length (c) of 6-wk-old WT, *zed1-D*, *p35S::ZED1-NLS-GFP;zed1-D* (*ZED1-NLS zed1-D*) and *p35S::ZED1-NES-GFP;zed1-D* (*ZED1-NES zed1-D*) plants grown at 25ºC. Data are shown as means ± SD, and the letters (a, b) indicate the significance ($P<0.05$) according to one-way ANOVA test. Scale bar, 2 cm. (d) Expression of the immune marker *PR1* and *PR2* genes in 4-wk-old WT, *zed1-D*, *ZED1-NLS zed1-D* and *ZED1-NES zed1-D* plants grown at 25ºC. Data are shown as means ± SD from three biological replicates, and the letters (a, b) indicate the significance ($P<0.05$) according to one-way ANOVA test. (e) DAB (top panel) and TB (bottom panel) staining of 4-wk-old WT, *zed1-D*, *ZED1-NLS zed1-D* and *ZED1-NES zed1-D* plants grown at 25ºC. Scale bar, 100 μm. (f) Morphology of 6-wk-old WT, *zed1-D*, *p35S::ZED1-D-NLS-GFP;zed1-D* (*ZED1-D-NLS zed1-D*) and *p35S::ZED1-D-NES-GFP;zed1-D* (*ZED1-D-NES zed1-D*) plants grown at 25ºC. Scale bar, 2 cm. (g) DAB (top panel) and TB (bottom panel) stained leaves of 4-wk-old WT, *zed1-D*, *ZED1-D-NLS zed1-D* and *ZED1-D-NES zed1-D* plants grown at 25ºC.

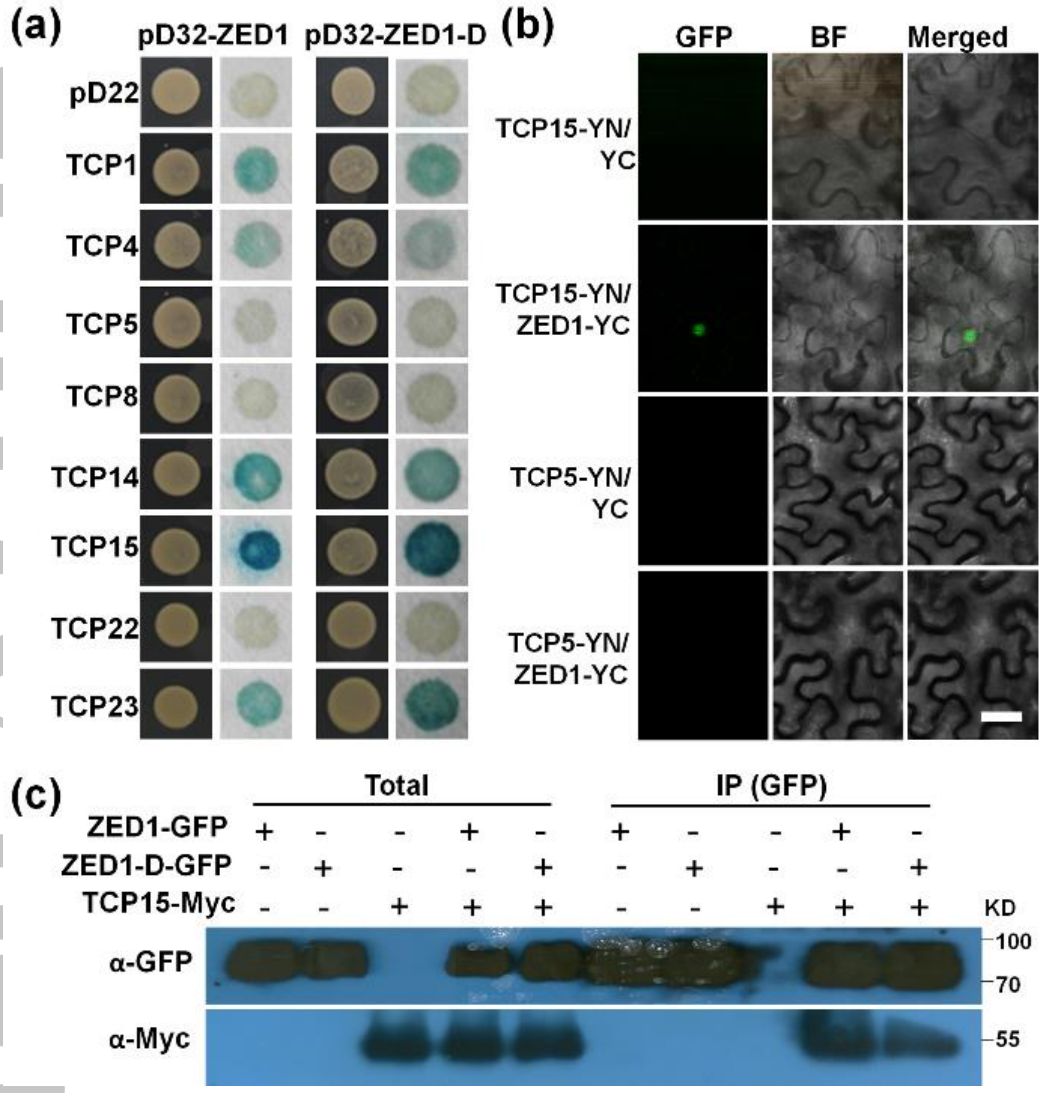

FIGURE 3 ZED1 and ZED1-D physically interact with TCPs. (a) Interaction of ZED1 or ZED1-D with TCPs in yeast. The representative TCP members were from those of TCP4, TCP14 and divergent clades. (b) Physical interaction of ZED1 with TCP15 assayed by BiFC. The BiFC assay was performed with transiently expressed proteins in *N. benthamiana*, and the TCP5 was used as a negative control. Scale bar, 20 μm. (c) Interaction of ZED1 or ZED1-D with TCP15 by CoIP assay in transgenic *Arabidopsis* plants carrying *p35S::ZED1-GFP* or *p35S::ZED1-D-GFP* and/or *p35S::TCP15-Myc* constructs.

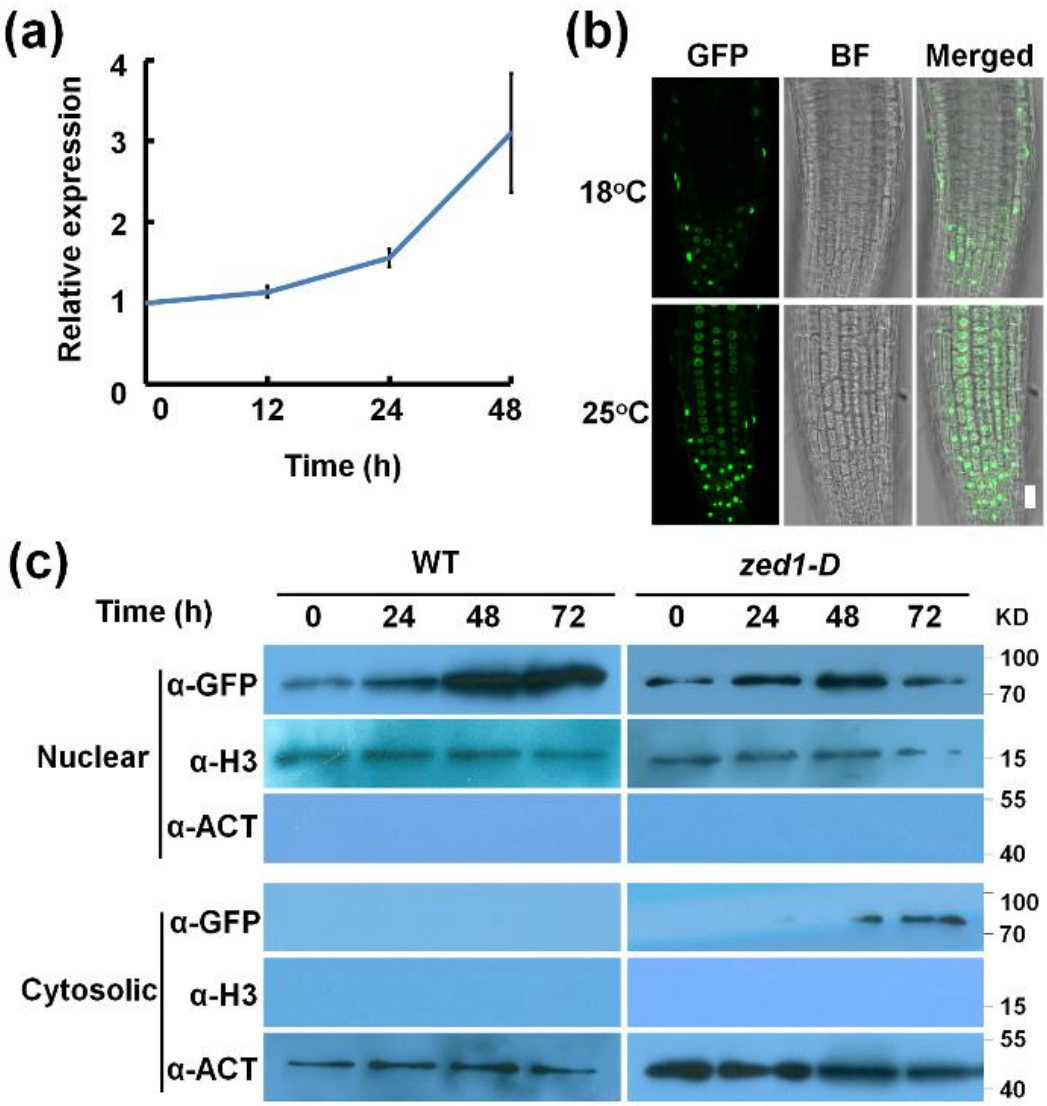

FIGURE 4 TCP15 is induced by elevated temperature and high temperature-induced ZED1-D disturbs TCP15 localization. (a) *TCP15* is induced by elevated temperature. The relative expression levels of *TCP15* were analyzed by qRT-PCR in the WT plants after being transferred from 18 to 25ºC for the indicated times, and the data from three biological replicates are shown as means ± SD. (b) TCP15 accumulation in the transgenic *pTCP15::TCP15-GFP* plants grown at 18 or 25ºC. GFP fluorescence signals were visualized in the primary roots of the transgenic plants grown at 18 or 25ºC. Scale bar, 50 μm. (c) Subcellular accumulation of TCP15 in the WT and *zed1-D* background. The fractionated nuclear and cytosolic proteins from transgenic *pTCP15::TCP15-GFP* and *pTCP15::TCP15-GFP;zed1-D* plants transferred from 18 into 25ºC for indicated times were immunoblotted, and the Histone H3 (H3) and β-ACTIN (ACT) were used as the nuclear and cytosolic internal controls, respectively.

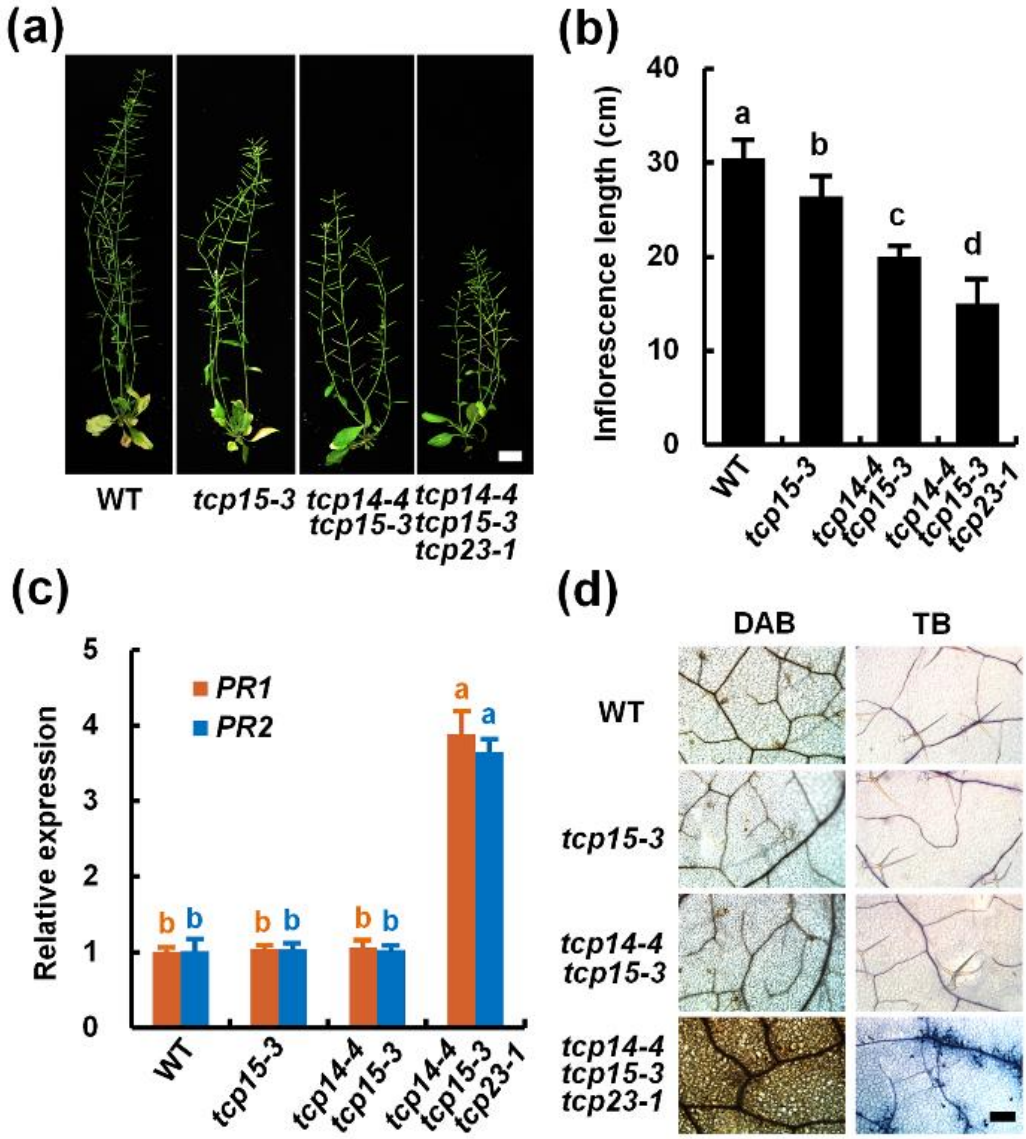

FIGURE 5 Disruption of TCPs leads to mild autoimmunity. (a,b) Morphology (a) and inflorescence stem length (b) of 6-wk-old WT, *tcp15-3*, *tcp14-4 tcp15-3* and *tcp14-4 tcp15-3 tcp23-1* plants grown at 25ºC. Data are shown as means ± SD, and the letters (a to d) indicate the significance (P<0.05) according to one-way ANOVA tests. Scale bar, 2 cm. (c) Expression of *PR1* and *PR2* genes in the 4-wk-old WT, *tcp15-3*, *tcp14-4 tcp15-3* and *tcp14-4 tcp15-3 tcp23-1* plants grown at 25ºC. Data are shown as means ± SD (n=3), and the letters (a, b) indicate the significance (P<0.05) according to one-way ANOVA test. (d) DAB (left panel) and TB (right panel) stained leaves of the plants described in (c). Scale bar, 100 µm.

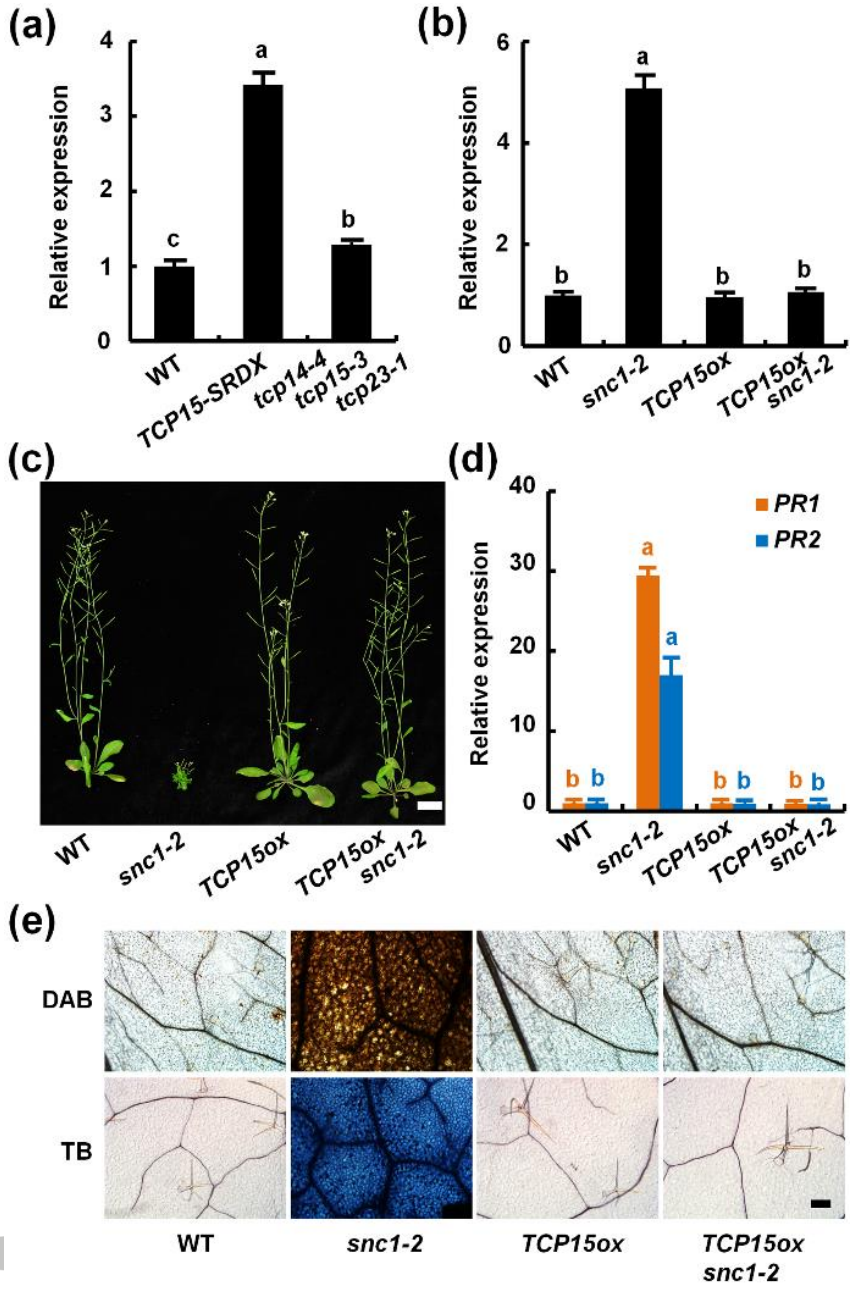

FIGURE 6 TCPs inhibit *SNC1* transcription. (a) Expression of *SNC1* in 4-wk-old WT, *p35S::TCP15-SRDX* (*TCP15-SRDX*) and *tcp14-4 tcp15-3 tcp23-1* plants grown at 25ºC. Data are shown as means ± SD (n=3), and the letters (a to c) indicate the significance (P<0.05) according to one-way ANOVA test. (b) Expression of *SNC1* in 4-wk-old WT, *snc1-2*, *p35S::TCP15* (*TCP15ox*) and *p35S::TCP15;snc1-2* (*TCP15ox snc1-2*) grown at 22ºC. Data are shown as means ± SD (n=3), and the letters (a, b) indicate the significance (P<0.05) according to one-way ANOVA tests. (c) Morphology of 6-wk-old WT, *snc1-2*, *p35S::TCP15* and *p35S::TCP15;snc1-2* plants grown at 22ºC. Scale bar, 2 cm. (d) Expression of *PR1* and *PR2* in 4-wk-old WT, *snc1-2*, *p35S::TCP15* and *p35S::TCP15;snc1-2* grown at 22ºC. Data are shown as means ± SD (n=3), and the letters (a, b) indicate the significance (P<0.05) according to one-way ANOVA test. (e) DAB (top panel) and TB (bottom panel) stained leaves of 4-wk-old WT, *snc1-2*, *p35S::TCP15* and *p35S::TCP15;snc1-2* grown at 22ºC. Scale bar, 100 μm.