PCP
PLANT & CELL PHYSIOLOGY

Regular Paper

# The Potato ERF Transcription Factor StERF3 Negatively Regulates Resistance to *Phytophthora infestans* and Salt Tolerance in Potato

Zhendong Tian[1,*], Qin He[1], Haixia Wang[1], Ying Liu[1,2], Ying Zhang[1,3], Fang Shao[1,4] and Conghua Xie[1]

[1]Key Laboratory of Horticultural Plant Biology (HAU), Ministry of Education, National Center for Vegetable Improvement (Central China), Huazhong Agricultural University, Wuhan, Hubei, 430070, China
[2]Present address: Science and Technology School of Shiyan City, Danjiangkou, Shiyan City, Hubei Province, 442701, China.
[3]Present address: Shanghai ChemPartner Co., LTD., Shanghai, 201203, China.
[4]Present address: Agricultural Bureau of the Laiwu City, Shandong Province, 271100, China.
*Corresponding author: E-mail, tianzhd@mail.hzau.edu.cn; Fax, +86-027-87286939.
(Received November 9, 2014; Accepted February 7, 2015)

**Ethylene response factors (ERFs) are unique to the plant kingdom and play crucial roles in plant response to various biotic and abiotic stresses. We show here that a potato StERF3, which contains an ERF-associated amphiphilic repression (EAR) motif in its C-terminal region, negatively regulates resistance to *Phytophthora infestans* and salt tolerance in potato. The *StERF3* promoter responds to induction by salicylic acid, ABA ethylene and NaCl, as well as *P. infestans*, the causal agent of potato late blight disease. StERF3 could bind to the GCC box element of the *HIS3* promoter and activate transcription of *HIS3* in yeast cells. Importantly, silencing of *StERF3* in potato produced an enhanced foliage resistance to *P. infestans* and elevated plant tolerance to NaCl stress accompanied by the activation of defense-related genes (*PR1*, *NPR1* and *WRKY1*). In contrast, *StERF3*-overexpressing plants showed reduced expression of these defense-related genes and enhanced susceptibility to *P. infestans*, suggesting that *StERF3* functions as a negative regulator of downstream defense- and/or stress-related genes in potato. StERF3 is localized to the nucleus. Interestingly, yeast two-hybrid assay and a bimolecular fluorescence complementation (BiFC) test clarified that StERF3 could interact with other proteins in the cytoplasm which may lead to its re-localization between the nucleus and cytoplasm, revealing a novel means of StERF3 regulation. Taken together, these data provide new insights into the mechanism underlying how StERF3 negatively regulates late blight resistance and abiotic tolerance in potato and may have a potential use in engineering late blight resistance in potato.**

**Keywords:** EAR motif • Late blight resistance • Negative regulation • Potato • Salt tolerance • StERF3.

**Abbreviations:** ANOVA, analysis of variance; 3-AT, 3-amino-1,2,4-triazole; BiFC, bimolecular fluorescence complementation; CaMV, *Cauliflower mosaic virus*; DAPI, 4′,6-diamidino-2-phenylindole; dpi, days post-inoculation; EAR, ERF-associated amphiphilic repression; ERF, ethylene response factor; ET, ethylene; GFP, green fluorescent protein; GUS, β-glucuronidase; JA, jasmonic acid; MS, Murashige and Skoog; OE, overexpressing; ORF, open reading frame; PR, pathogenesis-related; qPCR, quantitative PCR; RNAi, RNA interference; SA, salicylic acid; TF, transcription factor; YFP, yellow fluorescent protein; Y2H, yeast two-hybrid.

The nucleotide sequence of *StERF3* reported in this paper has been submitted to the NCBI with GenBank accession number EF091875.

## Introduction

Plants have evolved a wide variety of mechanisms to regulate the expression of defense-related genes upon pathogen attack. A number of transcription factor (TF) families are involved in a complicated regulation network of plant defense responses (van Verk et al. 2009). Among these transcription activators, ERF (ethylene response factor) TFs have been shown to play a crucial role in regulating a wide range of plant defense- and stress-related genes that are associated with a variety of biological processes of plants, such as metabolism, growth development and responses to environmental stimuli (Mizoi et al. 2012, Núñez-Pastrana et al. 2013).

ERFs comprise a large family of plant-specific stress-responsive TFs, which are characterized by a conserved 58–59 amino acid DNA-binding domain (designated as the AP2/ERF domain) that can specifically bind to GCC *cis*-elements and related elements in the promoter of the target genes to modulate their expression (Fujimoto et al. 2000, Nakano et al. 2006, Sharma et al. 2010). Most of them are pathogenesis-related (PR) genes, such as the defensin gene *PDF1.2*, the basic chitinase gene *ChiB*, the thionin gene *Thi2.1* as well as *PR1b* and *PR2*, which are inducible by jasmonic acid (JA)/ethylene (ET) to activate their expression in plants upon infection by a range of pathogens, both necrotrophic and biotrophic (McGrath et al. 2005, Oñate-Sánchez et al. 2007, Liang et al. 2008).

Among the members of the ERF proteins, most ERFs act as activators that positively regulate the transcript levels of their target genes. Overexpression of Arabidopsis *ERF1* and tomato *Ptis* activates expression of defense-related genes

*Plant Cell Physiol.* 56(5): 992–1005 (2015) doi:10.1093/pcp/pcv025, Advance Access publication on 13 February 2015, available online at www.pcp.oxfordjournals.org

(Solano et al. 1998, Gu et al. 2002). Overexpression of *NtERF2*, *NtERF4*, *AtERF1*, *AtERF2* and *AtERF5* led to enhanced pathogen, drought or cold tolerances in plants (Ohta et al. 2000, Guo et al. 2004). In contrast to the ERF activator, a subclass of the AP2/ERF protein family is classified as class II ERFs that function as dominant repressors of gene expression (Fujimoto et al. 2000, Ohta et al. 2001). They contain a conserved (L/F)DLN(L/F)xP motif structure, also called the ERF-associated amphiphilic repression (EAR) motif, in their C-terminal regions (Ohta et al. 2001). For example, McGrath et al. (2005) reported that AtERF4 acts as a negative regulator of JA-responsive defense gene expression and resistance to the necrotrophic fungal pathogen *Fusarium oxysporum*. Overexpressing peach EAR-type *PpERF3b* in tobacco increased disease symptom response to *Pseudomonas syringae* pv. *tabaci* (Sherif et al. 2013). *OsERF922*-overexpressing lines were more susceptible to rice blast fungus *Magnaporthe oryzae* (Liu et al. 2012). In addition to being involved in defense, some EAR-type ERFs also negatively regulate plant response to abiotic stresses, such as SlERF3 for salt stress (Pan et al. 2010), RAP2.1 for cold and drought (Dong and Liu 2010) and OsERF3 for drought tolerance (Zhang et al. 2013). Taken together, different type of ERFs may have specific regulatory functions to co-ordinate the innate response of plants upon diverse stimuli. However, the mechanism underlying ERFs acting as activators or repressors still remains to be elucidated.

Late blight, caused by the oomycete *Phytophthora infestans*, is a destructive disease of potato. It has historical significance as the cause of 'the Irish Potato Famine' during the 1840s (Fisher et al. 2012). Previously, a potato *ERF* expressed sequence tag (EST) was identified that could be induced by *P. infestans* infection (Wang et al. 2005), and the full-length *StERF3* was isolated (GenBank No. EF091875). Here, we further elucidate the functions and possible mechanisms of StERF3 in regulating potato defense and stress responses. We demonstrate that EAR-type StERF3 responds to biotic and abiotic induction and could interact with the GCC box in vitro. Stable silencing by RNA interference (RNAi) of *StERF3* in potato leads to reduced *P. infestans* colonization and enhanced salt tolerance, while transgenic potato plants overexpressing *StERF3* show enhanced colonization and reduced salt tolerance, confirming that StERF3 acts as a negative regulator in defense and stress response in potato. Furthermore, we demonstrate that StERF3 could interact with other proteins, which might lead to its relocalization between the cytoplasm and nucleus. This shift in localization may affect the fate of StERF3 in regulating plant response. The present research enriches our knowledge of ERFs in regulating plant response to biotic and abiotic stresses and in particular provides a novel clue towards potato improvement of late blight resistance and salt tolerance.

## Results

### Characterization of *StERF3*

*StERF3* (GenBank No. EF091875), the potato ERF transcription factor gene, was isolated by the rapid amplification of cDNA ends (RACE) method. *StERF3* encodes a deduced protein of 223 amino acids which shows highly similarity (85%) to tomato SlERF3 (GenBank No. AAO34705), and shares 46.5, 37.9 and 36.9% identity to NtERF3 (protein ID BAJ72664), OsERF3 (BAB03248) and AtERF4 (AAM98171), respectively. The deduced protein has a highly conserved DNA-binding domain AP2/ERF (Nakano et al. 2006), which consists of 50–60 amino acids (**Fig. 1**). Additionally, the StERF3 protein also contains a conserved EAR motif in the C-terminal region characterizing StERF3 as a member of the class II ERF TFs which have been shown to act as repressors of transcription (Ohta et al. 2001), suggesting that StERF3 might negatively regulate defense in potato.

### *StERF3* promoter responses to *P. infestans*, SA, ABA and NaCl induction

The promoter region of *StERF3* was cloned to investigate its expression pattern (**Supplementary Fig. S1**). Promoter *cis*-element analysis showed that the 1,067 bp promoter fragment of *StERF3* contains several inducible *cis*-acting regulatory elements which are considered to respond to signal molecules as well as biotic and abiotic stimuli, such as ABRE (abscisic acid response), MBS (MYB binding site involved in drought response), LTR (low-temperature response) and TC-rich repeats (involved in defense and stress response), implicating that *StERF3* may play roles in potato response to diverse stresses.

To test the inducible pattern of the cloned *StERF3* promoter fragment, the binary vector pBI-pStERF3-*GUS* was stably transformed into *Nicotiana benthamiana* and treated with *P. infestans*, salicylic acid (SA), ethephon, ABA and NaCl separately. The results showed that β-glucuronidase (GUS) was expressed around the region of the inoculation site after infection of *P. infestans* (**Fig. 2B**), and could be induced by NaCl (**Fig. 2C**), ABA (**Fig. 2D**), ethephon (**Fig. 2E**) and SA (**Fig. 2F**). These findings suggests that StERF3, as a TF, might functions through different signaling pathways in potato.

### StERF3 interacts with the GCC box in yeast cells

Plant ERF proteins contain a highly conserved DNA-binding domain which could specifically bind to the GCC box present in the promoter of many functional genes and manipulate their expression (Hao et al. 1998). To examine the interaction of StERF3 with the GCC box in vivo, a synthesized DNA fragment harboring five GCC tandem copies was inserted upstream of the *HIS3* reporter gene in the pHIS2.1 vector (pHIS-5×GCC) for yeast one-hybrid assay. If StERF3 could bind to the GCC box on the pHIS-5×GCC vector, it will induce *HIS3* reporter gene expression and accordingly complement the histidine requirement of the host yeast strain (Y187). As show in **Fig. 3**, yeast carrying pHis2.1-5×GCC and pGADT7-Rec2-StERF3 grew well on minimal medium lacking histidine and containing 3-amino-1,2,4-triazole (3-AT). The positive control that contained p53His2 and pGAD-Rec2-53 plasmids showed a vigorous growth, while the negative control carrying pHis2.1 + pGAD-Rec2-StERF3 grew weakly (**Fig. 3**). These results demonstrate that StERF3 could bind to the GCC box, as is the case with other ERFs.

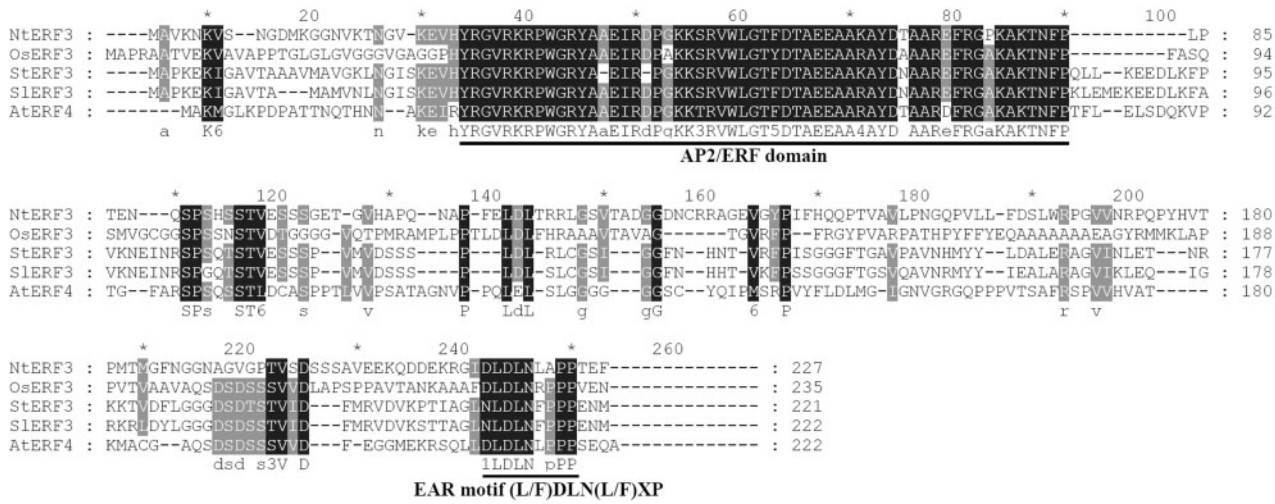


**Fig. 1** Structure and conservation of the StERF3 protein sequence in alignment with four class II ERF transcription factors. The AP2/ERF domain and EAR motif are underlined. Amino acid residues that are conserved in at least four of the five sequences are shaded in gray, while amino acids identical in all five proteins are shown in black. Dashes show gaps in the amino acid sequences introduced to optimize alignment. NtERF3 (protein ID BAJ72664) is derived from *N. benthamiana*, OsERF3 (BAB03248) is derived from *O. sativa*, *SlERF3* is derived from *S. lycopersicum* (AAO34705), StERF3 (ABK96798) is derived from *S. tuberosum* and *AtERF4* (AAM98171) is derived from *A. thaliana*.

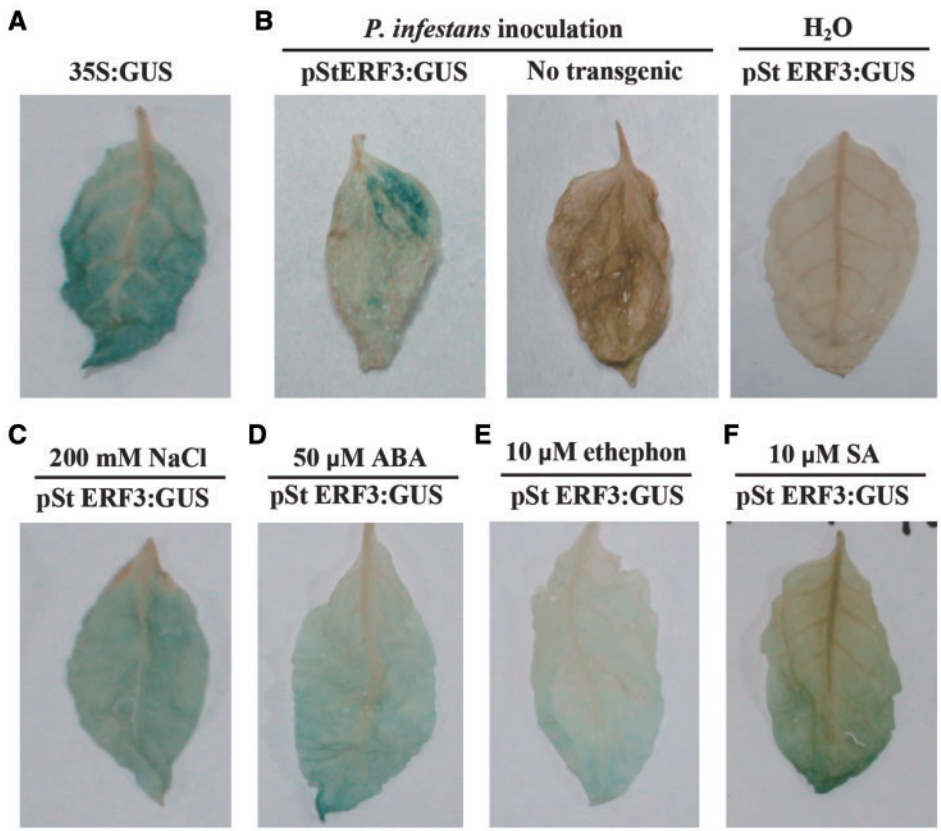


**Fig. 2** Responses of the *StERF3* promoter to various biotic and abiotic stimuli. Histochemical analysis of *pStERF3:GUS* gene activities in *N. benthamiana* leaves. Leaves of 5-week-old transgenic and control *N. benthamiana* in vitro plantlets were detached for various treatments. (A) Positive control (*35S:GUS*). (B) A leaf 5 d post-inoculation with *P. infestans*. (C) A leaf treated with 200 mM NaCl solution for 12 h. (D–F) Leaves treated for 5 h with 50 μM ABA, 10 μM ethephon and 10 μM salicylic acid (SA), respectively. The leaves were stained 5 h after treatments. All treated and control leaves were incubated in GUS staining solution for 12 h. Three repeats showed similar results.

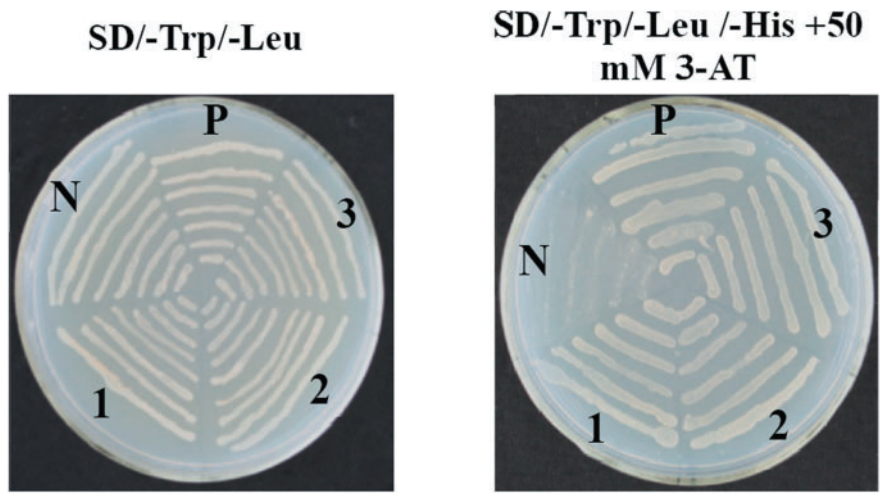


**Fig. 3** StERF3 binds to the GCC box in yeast. N, negative control (pHis2.1 + pGADT7-Rec2-ERF3); P, positive control (p53His2 + pGAD-Rec2-53); 1–3, three different colonies containing pHis2.1-5×GCC and pGADT7-Rec2-StERF3. The 5×GCC motif was used as bait. The reporter and effector plasmids were introduced into yeast strain Y187 and cultured on SD/−Trp/−Leu medium and SD/−Trp/−Leu/−His medium containing 50 mM 3-AT. The yeast were grown for 3 d at 28°C.

## *StERF3* negatively regulates disease resistance in potato

Since *StERF3* could be induced by *P. infestans* (**Fig. 2**), we extended our research to test whether *StERF3* is involved in defense against this late blight pathogen. Two Chinese potato cultivars, 'E-potato-3' and 'Zhuanxinwu', were transformed to overexpress and silence *StERF3* via *Agrobacterium*-mediated transformation. More than 20 independent OE (overexpressing) or RNAi lines were obtained for each cultivar. Two 'Zhuanxinwu' RNAi lines (ZXW-Ri-1 and ZXW-Ri-2) and three 'E-potato-3' RNAi lines (E3-Ri-2, E3-Ri-3 and E3-Ri-16) were selected for further analysis. Each revealed approximately 65–84% reduction in *StERF3* transcript accumulation (**Supplementary Fig. S2A**, **B**). The detached leaves of transgenic plants were inoculated with two virulent pathogen *P. infestans* isolates separately. As illustrated in **Fig. 4A** and **B**, the sizes of the lesions formed by pathogen infection at 4 dpi (days post-inoculation by *P. infestans*) were remarkably decreased in the StERF3-RNAi lines compared with their corresponding control. To confirm the possible negative role of StERF3 in basal immunity to *P. infestans*, two 'Zhuanxinwu' and two 'E-potato-3' OE lines, showing elevated transcript accumulation (5- to 8-fold) (**Supplementary Fig. S2A**, **B**), were selected for further pathogen inoculation. Each OE line of both cultivars displayed an increase in lesion size relative to the control (**Fig. 4A, B**).

To assess further the differences in late blight resistance, we determined mean lesion areas at 5 dpi. As shown in **Fig. 4C** and **D**, significantly smaller lesions were observed on the RNAi lines of both cultivars in comparison with controls. At the same time, the OE lines of both cultivars show increased lesion areas compared with controls. Quantitative PCR (qPCR) was used to monitor the relative biomass of *P. infestans* according to Llorente et al. (2010). As illustrated in **Fig. 4E**, the *P. infestans* biomass was decreased in E3-RNAi lines and enhanced in the E3-OE lines compared with controls. The *P. infestans* growth pattern in infected leaves was consistent with the lesion expansion of the transgenic lines. The resistance levels of both RNAi and OE lines are consistent with a role for *StERF3* in negatively regulating potato defense to *P. infestans*.

## Silencing *StERF3* enhances potato salt tolerance in vitro

*StERF3* can be induced by NaCl and ABA treatments (**Fig. 2C, D**), suggesting that *StERF3* may be involved in potato stress tolerance. Therefore, nodal cuttings of the 'Zhuanxinwu' and 'E-potato-3' transgenic lines and untransformed controls were cultured on Murashige and Skoog (MS) medium with or without 150 mM NaCl to test salt tolerance. After 4 weeks, the plantlets on the MS medium without NaCl grew well, while the growth of both transgenic lines and the controls of the two cultivars was seriously suppressed on the medium containing 150 mM NaCl (**Fig. 5A, B**). However, it is obvious that 'Zhuanxinwu' RNAi lines showed an elevated level of tolerance to NaCl compared with the OE lines and the control, indicated by root regeneration in the RNAi lines. Even though few roots were regenerated in both transformed and control 'E-potato-3', a greater plant height and higher fresh weight were observed in RNAi lines grown on the medium containing 150 mM NaCl (**Fig. 5C, D**). These results indicate that silencing of *StERF3* enhances potato salt tolerance in vitro.

## *StERF3* expression affects the transcripts of defense-related genes

To gain insight into how StERF3 could regulate plant stress responses, expression of several potato defense marker genes was quantified by qRT-PCR in *P. infestans*-inoculated leaves (48 h post-inoculation) of the transgenic lines and controls. As shown in **Fig. 6**, differences in transcript levels were found between the RNAi and OE lines of both cultivars. The SA-mediated defense marker gene *PR1* (pathogen-related protein

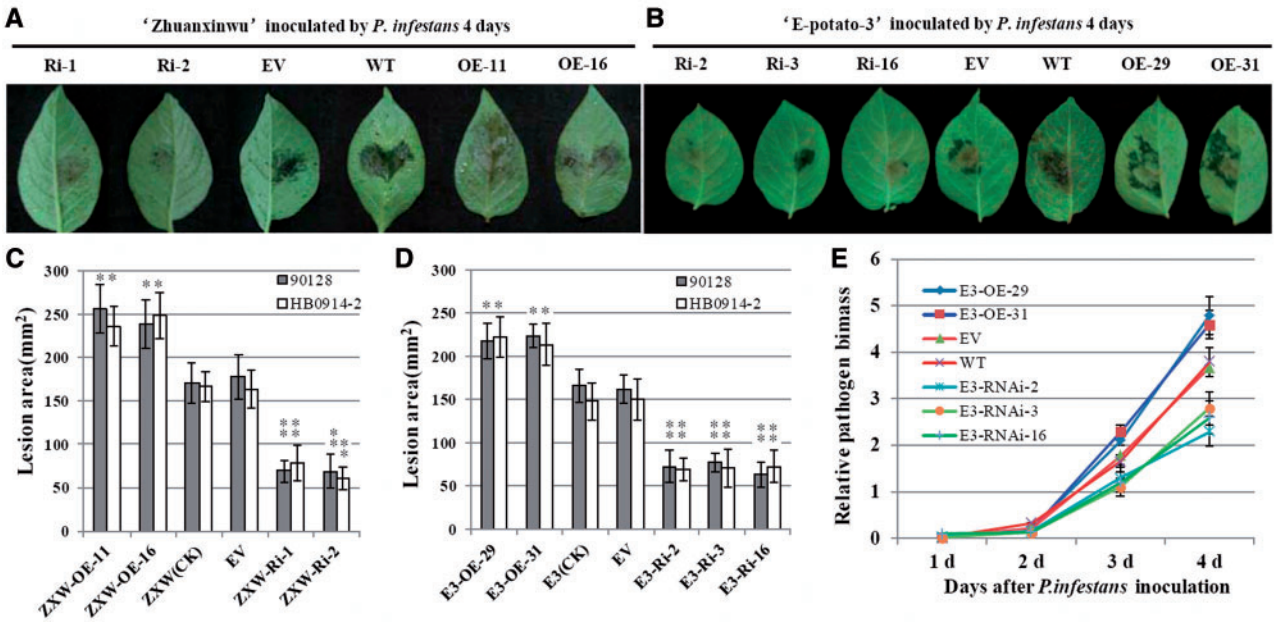


**Fig. 4** Overexpression and suppression of *StERF3* alter potato resistance to *Phytophthora infestans*. (A and B) Images of a test of representative detached leaves of 'E-potato-3' and 'Zhuanxinwu' transgenic plants showing resistance to *P. infestans* at 4 dpi. Leaves were detached from 6-week-old potato plants and inoculated with suspensions of sporangia of *P. infestans* isolates 90128 and HB09-14-2 ($10^5$ sporangia $ml^{-1}$) separately. Infection assays were repeated three times with similar results, and the results obtained from one representative experiment are shown. (C and D) Mean lesion areas at 5 dpi. Data are based on the area of lesions formed in at least 15 inoculation sites on 20 leaves from four plants of each line. Statistical analysis was carried out using ANOVA with pairwise comparisons performed with a Holm–Sidak test. Asterisks denote the *P*-value as follows $*P < 0.05$, $**P < 0.01$; error bars show the SD of three independent tests. Ri- and OE- represent RNA interference and overexpression lines, respectively. WT, untransgenic plant; EV, empty vector (*35S:GUS*) transformants. (E) Dynamic growth of the pathogen biomass around inoculated sites on leaves of 'E-potato-3' (E3) RNAi and OE potato lines. The graph shows *P. infestans* biomass calculated by qPCR on control (E3 and EV) and E3-RNAi and E3-OE potato lines from 1 to 4 dpi of *P. infestans* infection. Error bars represent the SEM. Three biological replicates were performed, each using five inoculation sites.

gene 1), which is known to contain a GCC box in the promoter, showed significantly increased expression in the *StERF3* RNAi lines of both cultivars, while it was remarkably decreased in the OE lines compared with the corresponding controls (**Fig. 6A**). Similar expression patterns were observed for the SA signaling regulatory genes *NPR1* and *WRKY1* which were up-regulated in the RNAi lines and repressed in the OE lines (**Fig. 6B, C**). In addition to the SA-mediated defense marker genes, genes encoding mitogen-activated protein kinase (*StMAPK3*-like), phenylalanine ammonia lyase (PAL) and osmotin protein, which have been reported to play roles in plant abiotic stress and pathogen defense (Mauch-Mani et al. 1996), were also tested. The results showed that only the OE lines showed significantly reduced transcripts of the *MAPK* gene compared with controls, whereas a significant difference was not detected in RNAi lines for *MAPK* (**Fig. 6D**). On the other hand, expression of *Osmotin* and *PAL* showed no differences in either OE or RNAi lines (data not show). These findings imply that the *StERF3* gene has impacts on the SA-dependent gene expression.

## Interaction proteins of StERF3

Our results showed that StERF3 may be involved in different pathways to execute its regulatory functions in plant biotic and abiotic stress responses. To provide evidence of the possible interaction network, StERF3 was used as bait to screen the interaction proteins from the potato yeast two-hybrid (Y2H) cDNA library. In total, six proteins, aconitate hydratase (ACN), putative cinnamyl alcohol dehydrogenase (CAD), SNF1-related protein kinase (KIN1), cyanate hydratase (CYN), CONSTANS interacting protein 3 (CIP3) and 60S ribosomal protein L19, were found putatively to interact with StERF3 (**Supplementary Table S2**). These proteins were reported to participate in several physiological processes including energy metabolism (ACN and KIN1) (Ghillebert et al. 2011), defense and stress response (CAD and CYN) (Trabucco et al. 2013), plant development (CIP3) (Ben-Naim et al. 2006) and protein synthesis (60S ribosomal protein L19).

To confirm the interactions further, three proteins (KIN1, CYN and CIP3) were subjected to yeast hybridization with StERF3 individually (gene sequences were put in **Supplementary Fig. S3**). The Y2H showed that the yeast cells co-transformed with pGBKT7-StERF3 + pGADT7-StKIN1, pGBKT7-StERF3 + pGADT7-StCYN and pGBKT7-StERF3 + pGADT7-StCIP developed well on synthetic dropout (SD)/−Trp/−Leu/−His/−Ade plates and were positive in the X-α-Gal assay (**Fig. 7A**), indicating that StERF3 can physically interact with StKIN1, StCYN and StCIP3.

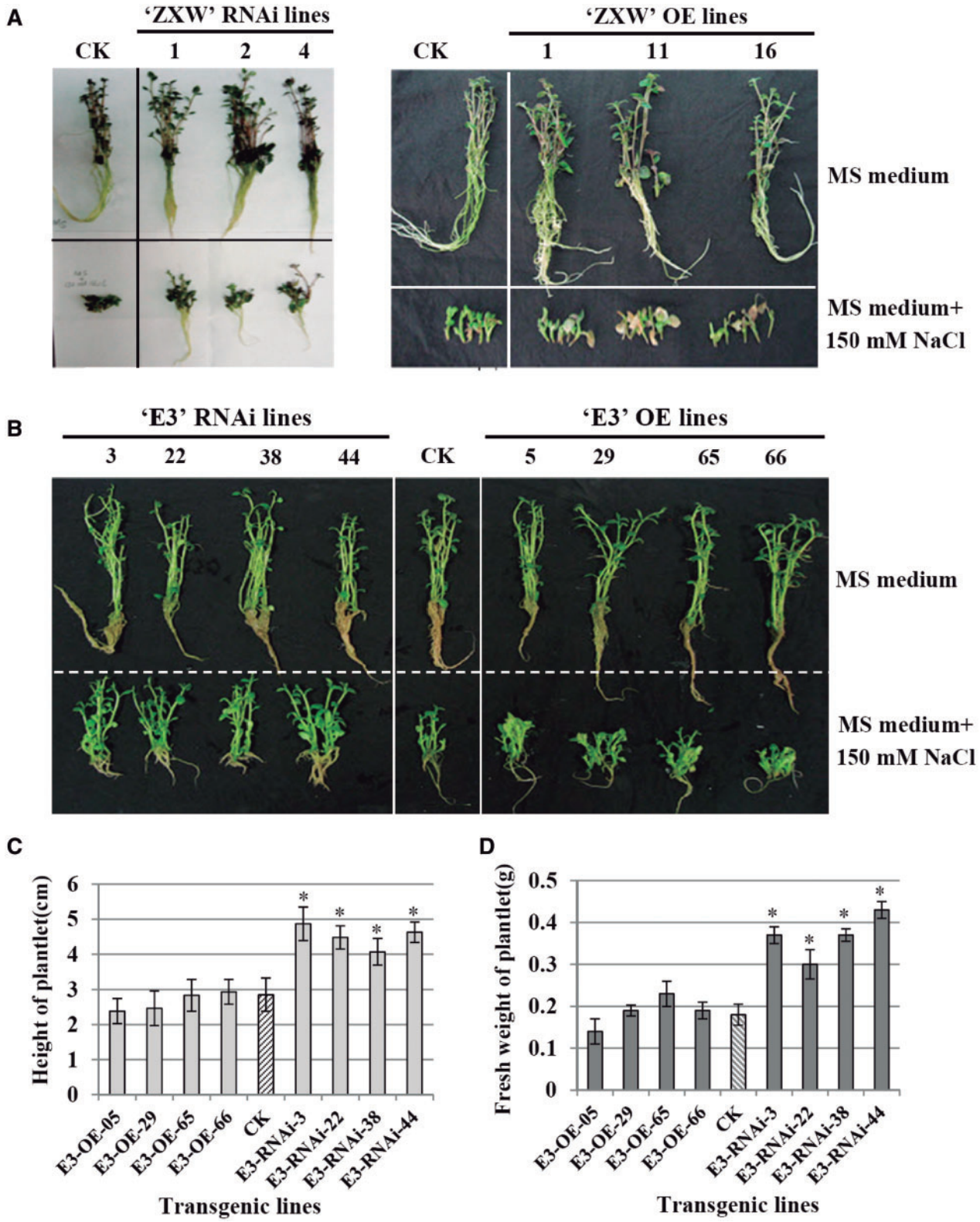


**Fig. 5** Performance of *StERF3* transgenic potato lines grown on MS medium without or supplemented with 150 mM NaCl. In vitro plantlets were cultured on MS medium or medium supplemented with 150 mM NaCl for 4 weeks. (A) Growth performance of RNAi and OE lines of potato cultivar 'Zhuanxinwu' (ZXW). (B) Growth performance of potato cultivar 'E-potato-3' (E3) transgenic OE and RNAi plantlets. 'CK' represents a non-transgenic plant. (C) Plantlet height of 'E-potato-3' transgenic lines cultured on MS medium + 150 mM NaCl. (D) Plantlet fresh weight of 'E-potato-3' transgenic lines grown on MS medium + 150 mM NaCl. Salt tolerance assays were repeated three times with similar results, and the results obtained from one representative experiment are shown. Error bars represent the SEM, and significant differences (*$P = 0.05$) in plantlet fresh weight and height compared with the control were determined by one-way ANOVA. Data are based on at least 36 plantlets per transgenic line.

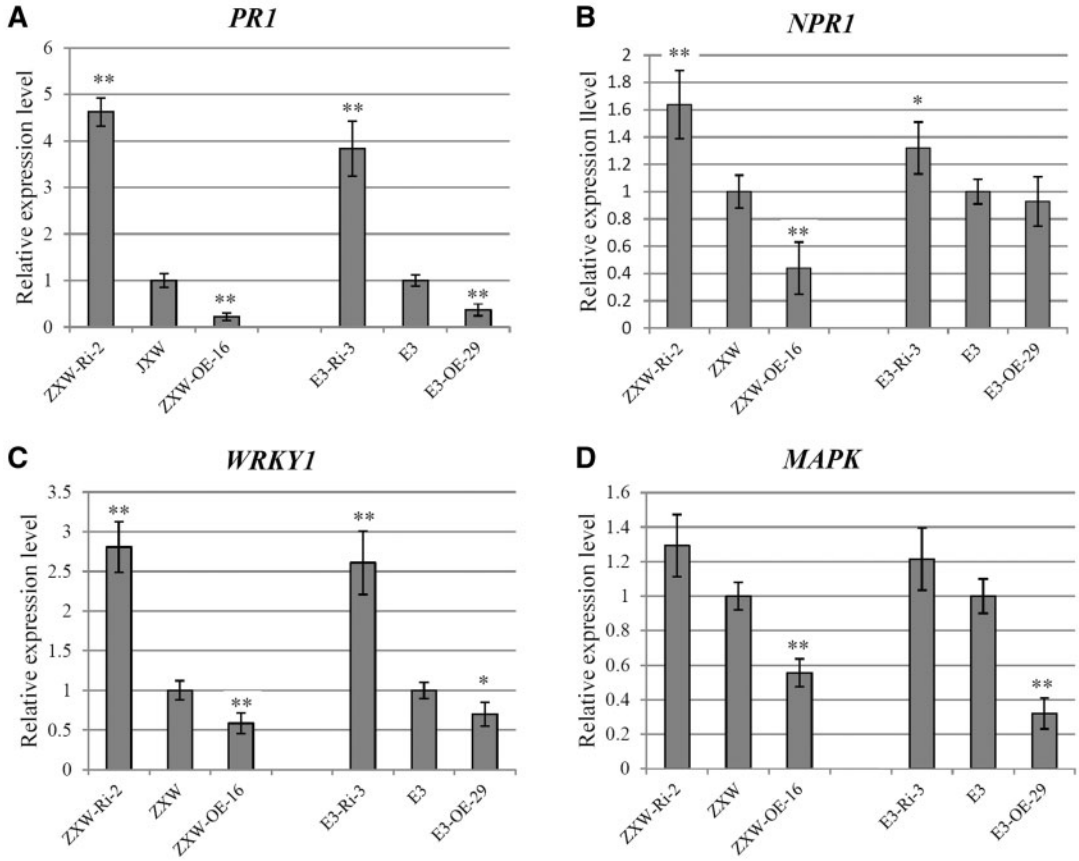


**Fig. 6** Expression of *PR1*, *NPR1*, *WRKY1* and *MAPK* genes in *StERF3* RNAi and OE potato lines after *P. infestans* inoculation. Total RNA was extracted from the leaves of *StERF3* RNAi and OE lines after *P. infestans* inoculation for 48 h. 'ZXW', cv. 'Zhuanxinwu'. 'E3', cv. 'E-potato-3'. RNAi and OE, RNA interference and overexpression transgenic lines, respectively. (A, B, C and D) The relative expression level of *PR1*, *NPR1*, *WRKY1* and *MAPK*, respectively. Quantitative analysis was performed using qRT-PCR. Amplification of the *Ef-1α* gene was used as an internal control to normalize the data. Significant differences between transgenic lines and control were analyzed based on three biological repeats (*t*-test: $^{*}P < 0.05$; $^{**}P < 0.01$). Error bars indicate the SD.

To confirm further the potential interaction between StERF3 and StKIN1, StCYN or StCIP3 in planta, a bimolecular fluorescence complementation (BiFC) assay was utilized. This assay demonstrated that tobacco BY2 cells co-transfected with StERF3:nYFP/StCYN:cYFP (with the N- and C-terminus of yellow fluorescent protein, respectively), StERF3:nYFP/StKIN:cYFP and StERF3:nYFP/StCIP:cYFP displayed yellow fluorescence (**Fig. 7B**), showing that they are in close proximity in vivo, consistent with their interaction in yeast cells.

## Subcellular localization of StERF3 and its interacting proteins

To elucidate the biological and physiological role of StERF3 in vivo, we investigated the subcellular localization of StERF3. The constructs *35S: StERF3:GFP* (green fluorescent protein) and *35S:GFP* were transiently introduced into *N. benthamiana* epidermal cells by agroinfiltration. Fluorescence analysis indicated that 35S:StERF3:GFP was expressed only in the nuclei, while 35S:GFP was detected in both the nuclei and the cytoplasm (**Fig. 8**). These results indicate that StERF3 is exclusively localized to the nucleus. Transient expression showed that 35S:StKIN1:GFP is localized to the cytoplasm, whereas 35S:StCIP3:GFP and 35S:StCYN:GFP were observed in the nucleus and cytoplasm (**Fig. 8**). Interestingly, yellow fluorescence was observed to be mainly in the cytoplasm when StERF3:nYFP was co-expressed with StKIN:cYFP, StCYN:cYFP or StCIP:cYFP (**Fig. 7B**). Typically, StERF3:nYFP was expressed with cytoplasm-localized StKIN1, implicating that protein interaction might lead to re-localization of StERF3. These observations imply that the subcellular re-localization of StERF3 caused by the interaction with its target proteins may be important for a subtle manipulation of StERF3. However, the biological significance of this will be worth investigating in the future.

## Discussion

ERFs have been reported to be responsive to biotic and abiotic stimuli and consequently modulate defense- and stress-related gene expression in plants (Núñez-Pastrana et al. 2013). Members of the ERF family can control defense genes positively or negatively. Usually, EAR-containing ERFs are involved in the repression mechanism (Ohta et al. 2001, McGrath et al. 2005).

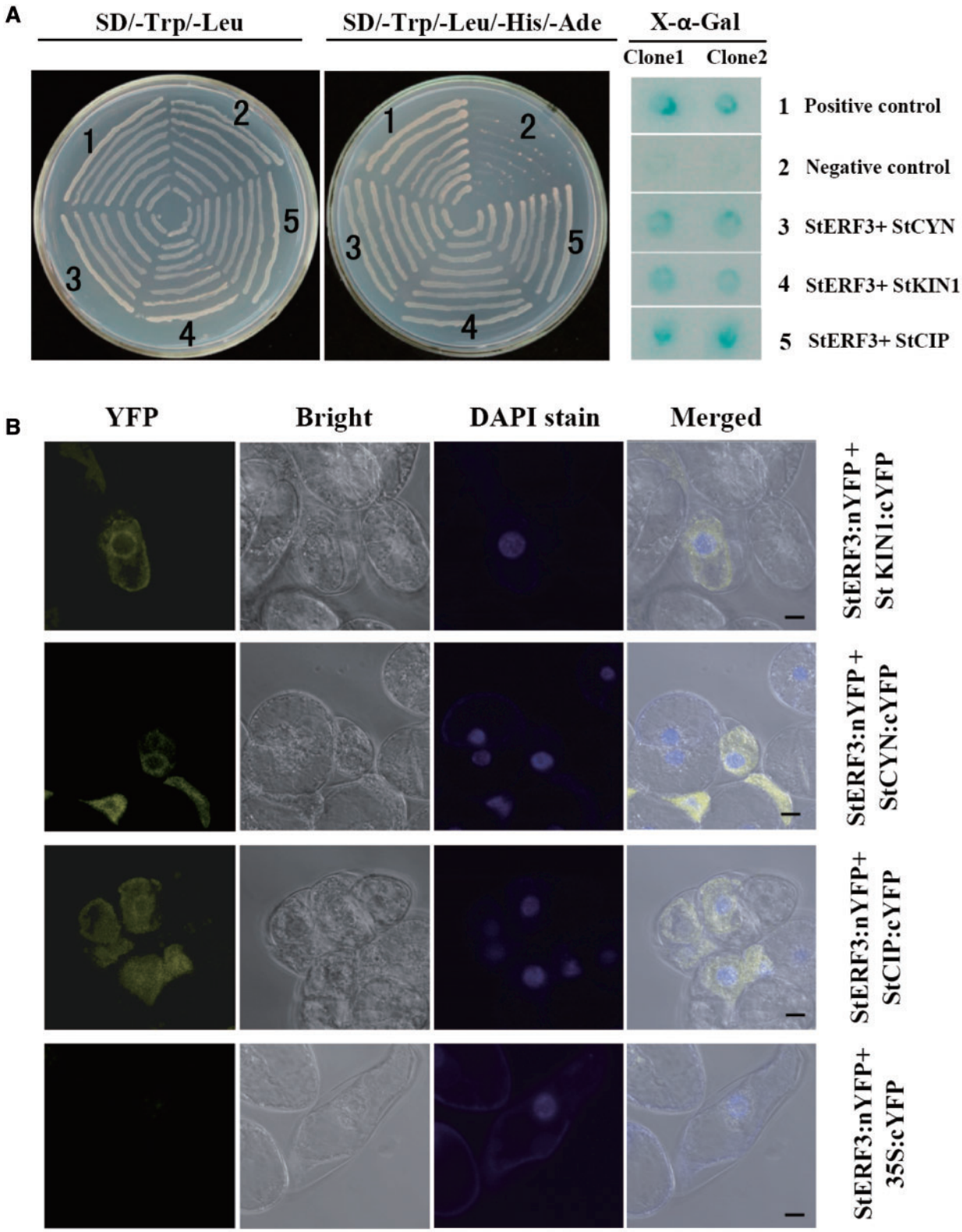


**Fig. 7** Physical interaction between StERF3 and StCYN, StKIN1 and StCIP detected in yeast two-hybrid assays and the BiFC system in tobacco BY2 cells. (A) Interactions between StERF3 and StKIN1, StCYN and StCIP in the Y2H assay. AH109 yeast strains were transformed with plasmids: 1, pGBKT7-53 + pGADT7-recT (positive control); 2, pGBKT7-Lam + pGADT7-recT (negative control); 3, pGBKT7-StERF3 + pGADT7-StKIN1; 4, pGBKT7-StERF3 + pGADT7-StCYN; and 5, pGBKT7-StERF3 + pGADT7-StCIP). Those yeast cells that grow well on SD/−Trp/−Leu/−His/−Ade plates and can be stained blue in X-α-Gal assay are considered positive for the interaction. (B) BiFC visualization of the interaction of StERF3 with StKIN1, StCYN and StCIP transiently co-expressed in tobacco BY2 cells. StERF3:nYFP, the N-terminus of YFP was fused to the C-terminus of StERF3; StKIN1:cYFP, StCYN:cYFP and StCIP:cYFP, the C-terminus of YFP was fused to the C-terminus of StKIN1, StCYN and StCIP, respectively. 35S:cYFP represents the empty vector. Tobacco BY2 cells were stained with DAPI to indicate nuclear localization. Scale bars = 10 μm.

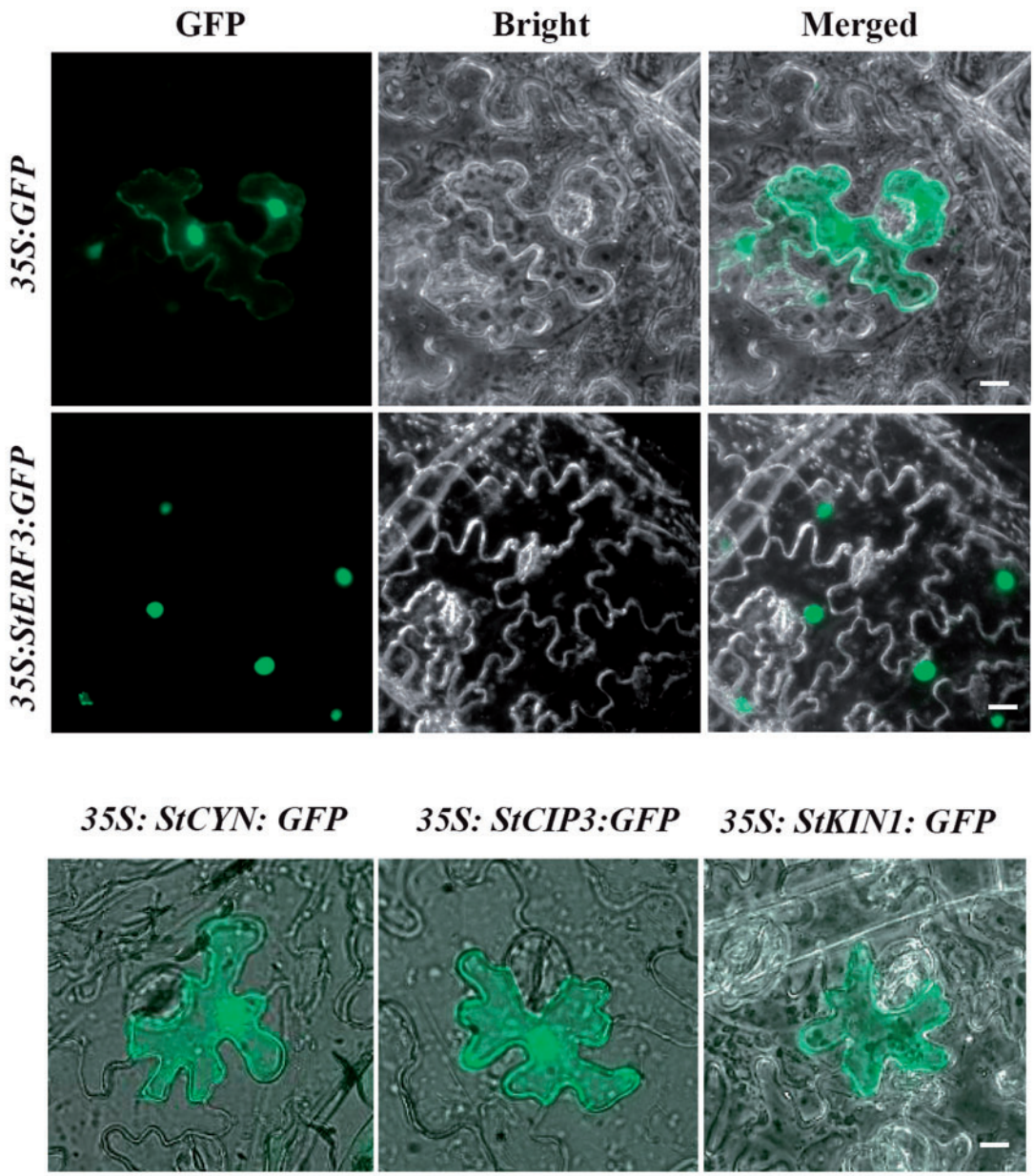


**Fig. 8** Nuclear localization of StERF3 and its interacting proteins in *N. benthamiana* epidermal cells. *GFP* was fused to the C-terminus of four genes under the control of the 35S promoter. *Nicotiana benthamiana* leaves were agroinfiltrated with *Agrobacterium* GV3101 containing *GFP* constructs individually. Cell fluorescence was observed using an inverted fluorescence microscope 2 d post-infiltration. GFP fluorescence (left), bright-field (middle) and the corresponding merged (right) images of cells are shown. Scale bars = 10 μm.

For example, AtERF4, AtERF9 and OsERF922 are known to regulate transcript levels of defense-related genes, and subsequent plant stress tolerance, negatively (McGrath et al. 2005, Liu et al. 2012, Maruyama et al. 2013). In this study, StERF3 is characterized as a member of the potato class II ERFs according its EAR motif in the C-terminus (**Fig. 1**). We first elucidate its functions in regulating potato response to *P. infestans*, the pathogen that causes the most important blight disease, and to salt stress. *StERF3* was shown to respond to diverse stimuli including *P. infestans*, SA, ABA, ethephon and salt stress through analysis of the responses of the StERF3 promoter to pathogen infection and signal molecules (**Fig. 2**). We speculate that there could be a cross-talk among different signaling pathways associated with StERF3. Silencing of the *StERF3* gene in potato produces increased resistance against *P. infestans* (**Fig. 4**) and enhanced salt tolerance (**Fig. 5**), suggesting that StERF3 functions as a repressor-type regulator that links multiple signaling networks in abiotic and biotic stress adaptation in potato. Pan et al. (2010) reported that ectopic expression of an EAR motif deletion mutant of SlERF3, a homolog of StERF3, enhances tolerance to salt stress and *Ralstonia solanacearum* in tomato. Our results are consistent with SlERF3 acting as a negative regulator manipulating tomato abiotic and biotic stress.

The ERF subfamily is mainly involved in response to biotic and abiotic stresses by recognizing the *cis*-elements (i.e. the

GCC box or DRE) in the promoters of target genes and then initiates a transcriptional cascade and leads to activation of downstream genes (Hao et al. 1998, Cheng et al. 2013). ERF TFs directly regulate PR gene expression by binding to DNA with a GCC box, such as in *PR1–PR5* (Ohme-Takagi and Shinshi 1995). Our results show that StERF3 confers the ability to bind to GCC box sequences and acts as a transcriptional activator in yeast (**Fig. 3**). Nevertheless, our study showed that overexpression of StERF3 leads to repression of *PR1*, suggesting that StERF3 might act as an active repressor. This is consistent with a previous report showing that overexpression of *SlERF3* leads to repression of GCC-mediated transcription of genes such as *PR1*, *PR2* and *PR5* (Pan et al. 2010). How GCC box binding to EAR-containing ERFs negatively regulates downstream target genes is an interesting question for future studies.

ERF genes have been proven to play key roles as regulators in ET, SA and JA defense signaling pathways (McGrath et al. 2005, Pre et al. 2008, Zarei et al. 2011, Liu et al. 2012). The present research showed that StERF3 RNAi potato lines activated the expression of the SA-mediated defense marker genes *PR1* and *WRKY1* while StERF3 OE transgenic lines suppressed their expression after *P. infestans* inoculation. Another key regulator gene of the SA signaling transduction pathway, *NPR1*, showed a similar expression pattern in the StERF3 RNAi and OE lines (**Fig. 6**). These results imply that the SA signaling transduction pathway is involved in StERF3-mediated negative regulation of potato late blight resistance. *PAL* plays important roles in plant pathogen defense. It is noticeable that *PAL* showed no differences in transcript levels between StERF3 RNAi and OE lines in the present study. The situation is the same as the case of overexpression of an ERF member of the EREBP/AP2 family gene *DREB2A* which did not result in significant induction of downstream genes in Arabidopsis (Umezawa et al. 2006). Perhaps some modification could be required for StERF3 to induce the expression of some downstream target genes.

Modifications including interactions with other TFs/proteins or phosphorylation could be required during or after transcription to induce the expression of the downstream target genes (Zhang et al. 2007). It has been reported that plant repressor-type ERF might recruit a co-repressor and interact with histone deacetylases to block transcriptional activation of the target genes (Song et al. 2005, Kagale and Rozwadowski 2011). Recently Kuang et al. (2012) reported that a histone deacetylase, HD2, may interact with longan DlERF1 to regulate fruit senescence-related gene expression. Wang et al. (2013) demonstrated that Arabidopsis ERF6 could interact physically with mitogen-activated protein kinase 6 (MPK6) and be phosphorylated by MPK6, which affected the dynamic alternation of the ERF6 protein and resulted in changes in reactive oxygen species (ROS)-responsive gene transcription. In Arabidopsis, phosphorylation of AtERF7 affects its DNA binding and/or repression activity (Song et al. 2005). Here, we found that StERF3 interacted physically with several proteins which engage in several physiological processes including energy metabolism, defense and stress response, plant development and protein synthesis (**Fig. 7**; **Supplementary Table S1**). Our studies indicate that post-transcriptional regulation such as protein–protein interaction is needed for StERF3 protein to perform its regulatory functions in potato.

Re-localization is a common mode of action for nuclear-localized TFs to regulate target gene expression in plant. For example, during *P. infestans* infection in potato, effectors secreted by *P. infestans* can prevent culture filtrate-triggered re-localization of the potato TFs StNTP1 and StNTP2 from the endoplasmic reticulum (ER) into the nucleus to activate defense-related genes (McLellan et al. 2013). StERF3–GFP localized to the nucleus (**Fig. 8**). However, we found that interactions of StERF3 with StKIN1, StCYN or StCIP were mainly observed in the cytoplasm (**Fig. 7B**), suggesting that protein interaction affects StERF3 re-localization from the nucleus to the cytoplasm. Wang et al. (2013) reported that phosphorylation of Arabidopsis ERF6 could change the nucleo-cytoplasmic shuttling of ERF6. Activated ERF6 was localized mainly in the nucleus, while mutation of the phosphorylation sites of ERF6 resulted in the accumulation of ERF6 in both the cytoplasm and the nucleus. Interestingly, StKIN1, one of the StERF3-interacting proteins, is an SNF1-related protein kinase (Ghillebert et al. 2011), suggesting that phosphorylation may be essential for StERF3 to regulate the potato defense. Since StERF3 is a negative regulator, it was hypothesized that protein interactions in the cytoplasm might prevent StERF3 from moving into the nucleus to perform its suppressive functions. Nevertheless, identification of more proteins interacting with StERF3 and further investigation is necessary to clarify these hypotheses.

Our results demonstrate that *StERF3* is induced by *P. infestans*. However, StERF3 OE lines were more susceptible to *P. infestans* (**Fig. 5**). The question is why the potato plant possesses this kind of negatively regulating gene in response to pathogen attack. One possibility is that *StERF3* might be activated by perception of *P. infestans* to suppress host defense and facilitate *P. infestans* invasion and propagation in the plant. Modulation of host immunity by plant pathogenic effectors is a key strategy for successful pathogens to grow and multiply (Boller and He 2009). *Xanthomonas oryzae* pv. *oryzae*, a causal agent of bacterial blight of rice, induces the expression of the host genes *Os8N3*, *OsTFX1* and *OsTFIIAγ1*, which results in increased host susceptibility to bacterial blight of rice (Yang et al. 2006, Sugio et al. 2007). It will be interesting to determine further whether any effectors or secreted proteins from *P. infestans* are involved in manipulating negative regulatory StERF3 to attenuate host defense responses. In addition, StERF3 could also play a role in preventing overactivation of defense reactions that may have an overall fitness cost. Overall, our study provides new information to dissect the poorly understood mechanism of the EAR-containing ERFs acting negatively on plant immune pathways.

## Materials and Methods

### Transformation vector construction

The promoter fragment of *StERF3* (*pStERF3*) was amplified from genomic DNA of a Chinese potato cultivar 'E-potato-3' using high efficiency thermal asymmetric interlaced PCR (hiTAIL-PCR) (Liu and Cheng 2007). The longest PCR fragment (1,067 bp) was used to analyze the pattern of induction of its

expression . The detailed *pStERF3* sequence and *cis*-elements predicted using the PlantCARE database (Lescot et al. 2002) are shown in **Supplementary Fig. S1**. For construction of the *pStERF3*-controlled *GUS* vector, the 1,067 bp promoter fragment was digested from pUC18-*pStERF3* using *Bam*HI and *Hin*dIII and inserted into the corresponding sites of pBI121 to replace the *Cauliflower mosaic virus* (CaMV) 35S promoter. For construction of the OE vector, full-length *StERF3* was amplified from pUC18-*StERF3* with primers *StERF3*-OE-F and *StERF3*-OE-R (**Supplementary Table S1**). The PCR fragments were cloned into the binary vector pBI121 through *Bam*HI and *Sac*I sites to produce OE vector pBI-*35S:StERF3*. To construct the RNAi vector, a 236 bp fragment located in a non-conserved region of *StERF3* (base pairs 335–571) was amplified by *StERF3*-RNAi-F and *StERF3*-RNAi-R primers (**Supplementary Table S1**). BP recombination enabled cloning into the binary vector pHGRV (pHELLSGATE2 modified by replacing the intron) (Chen et al. 2006) through the Gateway method (Invitrogen). Binary vectors were transformed into the *Agrobacterium tumefaciens* strain LBA4404 through electroporation and cultured on YEB medium containing appropriate antibiotics.

## Plant growth and transformation

Binary plasmid pBI-pStERF3-*GUS* was used to transform *N. benthamiana* leaf discs as described by Clemente (2006). Transformed clones were selected on MS medium containing 100 mg $l^{-1}$ kanamycin and 400 mg $l^{-1}$ cefotaxime. Transgenic *N. benthamiana* plantlets were propagated in vitro in growth chambers at 22°C and a 16 h photoperiod on MS medium.

Two Chinese potato cultivars 'E-potato-3' and 'Zhuanxinwu' were used for transformation of the OE vector pBI-*35S:StERF3* and the RNAi vector pHGRV-*StERF3*. Transgenic potato plants were obtained by *Agrobacterium*-mediated microtuber disc transformation according to Si et al. (2003). Transgenic plants were selected on the MS medium containing kanamycin and confirmed by PCR and Southern hybridization. The plantlets were maintained and propagated by growing single nodes on MS medium in growth chambers at 22°C and a 16 h photoperiod. To obtain fresh plant material for *P. infestans* infection assays, 4-week-old in vitro plantlets were transferred to plastic pots filled with greenhouse complex and grown in the greenhouse under normal conditions.

For agroinfiltration (*A. tumefaciens* infiltration), *N. benthamiana* plants were grown and maintained at 22–25°C in a controlled greenhouse under a 16/8 h light–dark photoperiod.

## Histochemical analysis of pStERF3: *GUS* activities in *N. benthamiana* leaves

For clarification of the induction pattern of the *StERF3* promoter by biotic and abiotic factors, leaves of 5-week-old transgenic and control *N. benthamiana* in vitro plantlets were detached for the treatments. For induction of signaling compounds, the leaves were sprayed separately with 10 μM SA, 50 μM ABA and 10 μM ethephon, and then stained 5 h after treatments. For salt stress, leaves were immerged in 200 mM NaCl solution for 12 h. For biotic treatment, the leaves were drop inoculated with a 10 μl droplet of a freshly prepared suspension of *P. infestans* sporangia ($1\times10^5$ sporangia $ml^{-1}$) onto the abaxial side of the leaf and kept in Petri dishes on a wet paper disc. As a control, water was pipetted onto the leaves. At 5 dpi, the inoculated leaves were subjected to GUS staining. All treated and control leaves were incubated in GUS staining solution for 12 h according to Jefferson (1987). Three biological replicates were set up with 3–4 leaves for each.

## Construction of GFP fusion and transient expression

For construction of *StERF3:GFP*, *StKIN1:GFP*, *StCYN:GFP* and *StCIP:GFP* fusions, the open reading frame (ORF) of these genes without a stop codon was amplified by PCR from potato cDNA with gene-specific primers modified to contain the Gateway (Invitrogen) attB recombination sites. PCR products were recombined into pDONR201 (Invitrogen) to generate entry clones. Primer sequences are shown in **Supplementary Table S1**. C-terminal GFP fusions were made by recombining the entry clones with the 35S-driven constitutive overexpression plant expression vector pB7FWG2 using LR clonase (Invitrogen). Then vectors were transformed into *Agrobacterium* GV3101. *Agrobacterium tumefaciens* containing each construct was pressure infiltrated into leaves of 4-week-old *N. benthamiana* as described (Wang et al. 2014). The $OD_{600}$ of *A. tumefaciens* was adjusted to 0.1. Cells expressing fluorescent protein fusions were observed using a Carl Zeiss AXIO Observer A1 inverted fluorescence microscope 2 d post-infiltration.

## Yeast one-hybrid assay

The yeast one-hybrid assay was performed using a MATCHMAKER One-Hybrid Library Construction and Screening Kit (Clontech) to examine the ability of StERF3 to bind to the GCC box. A synthesized DNA fragment harboring five GCC tandem copies (AAGAATTCGCCGCCGCCGCCGCCACTAGTAA) was ligated into the *Eco*RI and *Spe*I sites of the pHIS2.1 vector, upstream of the *HIS3* minimal promoter, to generate the reporter plasmid (pHIS2.1-5×GCC). The ORF of *StERF3* was fused in-frame with the GAL4 activation domain in a pGADT7 vector to generate the effector plasmid (pGADT7-Rec2-*StERF3*). Pairs of these reporter and effector plasmids, pHis2.1 and pGADT7-Rec2-*StERF3* (negative control), p53His2 and pGAD-Rec2-53 (positive control) and pHis2.1-5×GCC and pGADT7-Rec2-*StERF3* were introduced into yeast strain Y187 and the transformants were selected on SD medium lacking Leu and Trp. Transformed colonies were then streaked on SD/−His/−Leu/−Trp medium with 50 mm 3-AT and cultured at 28°C for 3 d according to the manufacturer's manual.

## Yeast two-hybrid (Y2H) screening

For screening the proteins that interact with StERF3, a potato cDNA library was constructed using BD Matchmaker™ LibraryConstruction & Screening Kits (Clontech) according to the User Manual. For construction of a DNA-BD fusion, the *StERF3* coding region was amplified by PCR using primers StERF3-BD-F and StERF3-BD-R (**Supplementary Table S1**) and then subcloned into the *Bam*HI and *Pst*I sites of pGBKT7 to generate the pGBKT7-*StERF3* vector as the bait. After confirmation by sequencing, pGBKT7-*StERF3* was transformed into yeast strain Y187. The transformants were assayed for transcriptional activation by selecting on high stringency plates: SD/−Ade/−His/-Leu/−Trp/X-α-gal. Then approximately $3\times10^6$ transformants were screened. Yeast colonies were assayed for X-β-gal activity using a colony-lift filter as follows: colonies were transferred to 3 MM filter paper, permeated by brief immersion in liquid nitrogen and incubated on filter paper saturated with Z-buffer containing 1 mg $l^{-1}$ X-β-gal at 30°C for 0.5–8 h. Positive clones were then subjected to sequencing.

To confirm the interactions between StERF3 and three selected positive clones, genes encoding the potential target proteins were cloned into pGADT7 (the sequences are available in **Supplementary Fig. S3**). Primers used for the vector construction are presented in **Supplementary Table S1**. All the constructs were verified by sequencing. pGBKT7-*StERF3* and pGADT7 with target genes were co-transformed into strain AH109. The transformants were assayed for MEL1 activation by selection on high stringency plates: SD/−Trp/−Leu/−His/−Ade/X-α-gal. Positive and negative controls were performed in parallel. Yeast colonies were assayed for α-gal activity using a colony-lift filter as described above.

## Bimolecular fluorescence complementation (BiFC) assay

pUC-SPYNE and pUC-SPYCE vectors (Walter et al. 2004) were used in the BiFC assay. Target gene fusions to the YFP N- or C-terminus were controlled by the CaMV35S promoter in both of the pUC-SPY vectors. Coding sequences of *StERF3* were fused with the N- terminus of YFP to form a *35S: StERF3:nYFP* construct. StKIN1, StCYN and StCIP were fused with the C-terminus of YFP individually. Primers used for plasmid construction are showed in **Supplementary Table S1**. Constructs *35S:StERF3:nYFP/35S: StKIN1:cYFP*, *35S:StERF3:nYFP/35S: StCYN:cYFP* and *35S:StERF3:nYFP/35S:StCIP:cYFP* were transiently co-expressed in tobacco BY2 cells using the particle bombardment method according to Juranič et al. (2012). After incubating for 16 h at room temperature, YFP fluorescence was observed with a confocal laser-scanning microscope (MRC-1024, Bio-Rad). For staining the nuclei, 10 mg $ml^{-1}$ 4′,6-diamidino-2-phenylindole (DAPI) was dropped on BY2 cells 10 min before observation. All experiments were repeated at least three times.

## Pathogen inoculum preparation and inoculation

Two *P. infestans* isolates, HB09-14-2 (race 1.2.3.4.5.6.7.8.9.10.11) (Wang et al 2014) and 90128 (A2 mating type, race 1.3.4.7.8.9.10.11) (Liu et al. 2005), were used for inoculation. *Phytophthora infestans* isolates were routinely grown on rye agar medium supplemented with 2% sucrose at 18°C in the dark. *Phytophthora infestans* sporangia were collected as described by Champouret et al. (2009). The concentrations of *P. infestans* inoculum were adjusted before leaf inoculation to $1\times10^5$ sporangia $ml^{-1}$.

Foliar resistance of transgenic potato was tested by detached leaf assays using the leaves of 7-week-old greenhouse-grown plants. Inoculation with *P. infestans* was performed by dropping 10 µl of freshly prepared sporangia suspension onto the abaxial side of the leaves. Inoculated leaves were incubated on wet filter paper in sealed boxes under controlled environmental conditions (20 ± 2°C, 16 h of light and 8 h of dark) and for the first 24 h kept in the dark. The disease lesion dimensions were measured at 3, 4 and 5 dpi; lesion areas were measured as described (Vleeshouwers et al. 1999). The inoculation experiment was repeated three times. At least 20 leaves from four plants were used in each repeat. Statistical analysis was carried out using analysis of variance (ANOVA).

For dynamically testing growth of the pathogen biomass around inoculation sites, leaf discs were cut around the inoculation sites from the first to the fourth day after *P. infestans* inoculation and DNA was extracted for qPCR to monitor the growth of *P. infestans* as described by Llorente et al. (2010). The pathogen biomass was quantified from five infected leaves per time point by normalizing the *PiO8* values with the corresponding *Ef-1α* values for each individual sample. qPCR was performed using the SYBR® Green Realtime PCR Master Mix (Toyobo) according to the manufacturer's protocol. Gene expression levels were calculated by a comparative Ct method as described by Cikos et al. (2007). Three biological replicates were performed, each using five inoculation sites.

## Salt tolerance assay of in vitro plantlets

In vitro transgenic and control plantlets of 'E-potato-3' and 'Zhuanxinwu' were used for the salt tolerance assay. Single-node cuttings were cultured on MS medium or MS medium containing 150 mM NaCl. Shoot length, root length and fresh weight of the plants were measured after 4 weeks. The salt tolerance assays were carried out in triplicat and each replication contained at least 36 plantlets (4 boxes×9 plantlets). Significant difference ($^*P = 0.05$) in plantlet fresh weight and height compared with the control was determined by one-way ANOVA.

## Expression analysis by qRT-PCR

Total RNA was isolated from potato leaves using a Plant total RNA isolate Mini Kit (Sangon Biotech, Co., Ltd.). Synthesis of cDNA was conducted using an oligo(dT) primer and an M-MLV reverse transcriptase kit (TAKARA) according to the manufacturer's instructions. qRT-PCR was performed on an ABI7300 PCR machine (Applied Biosystems) using the SYBR® Green Realtime PCR Master 282 Mix (Toyobo) according to the manufacturer's protocol. Six genes, *StPR1*, *StNPR1*, *StWRKY1*, the Osmotin-like protein gene, phenylalanine ammonia lyase (*StPAL*) and MAP kinase (*StMAPK3*-like), were selected. Gene expression levels were determined using appropriate primers (**Supplementary Table S1**) and normalized with respect to the *Ef-1α* gene. Relative expression levels were calculated by a comparative Ct method as described by Cikos et al. (2007).

## Supplementary data

**Supplementary data** are available at PCP online.

## Funding

This work was supported by the National High Technology Research and Development Program of China [grant No. 2013AA102603 to Z.-D.T.]; the National Natural Science Foundation of China [grant Nos. 31171603 and 31471550 to Z.-D.T.]; Research Fund for the Doctoral Program of Higher Education of China [grant No. 20110146110019 to Z.-D.T.].

## Acknowledgements

We are grateful to Yuan Lin for help with the BiFC assay.

## Disclosures

The authors have no conflicts of interest to declare.

## References

Ben-Naim, O., Eshed., R., Parnis, A., Teper-Bamnolker, P., Shalit, A., Coupland, G. et al. (2006) The CCAAT binding factor can mediate interactions between CONSTANS-like proteins and DNA. *Plant J.* 46: 462–476.

Boller, T. and He, S.Y. (2009) Innate immunity in plants: an arms race between pattern recognition receptors in plants and effectors in microbial pathogens. *Science* 324: 742–744.

Champouret, N., Bouwmeester, K., Rietman, H., van der Lee, T., Maliepaard, C.A., Heupink, A. et al. (2009) *Phytophthora infestans* isolates lacking class I *ipiO* variants are virulent on *Rpi-blb1* potato. *Mol. Plant Microbe Interact* 22: 1535–1545.

Chen, R.G., Zhang, L.Y., Zhang, J.H., Zhang, W., Wang, X., Ouyang, B. et al. (2006) Functional characterization of *Mi*, a root-knot nematode resistance gene from Tomato (*Lycopersicon esculentum* L.). *J. Integr. Plant Biol.* 48: 1458–1465.

Cheng, M.C., Liao, P.M., Kuo, W.W. and Lin, T.P. (2013) The *Arabidopsis* ETHYLENE-RESPONSE-FACTOR1 regulates abiotic-stress-responsive gene expression by binding to different cis-acting elements in response to different stress signals. *Plant Physiol.* 166: 1566–1582.

Cikos, S., Bukovska, A. and Koppel, J. (2007) Relative quantification of mRNA: comparison of methods currently used for real-time PCR data analysis. *BMC Mol. Biol.* 8: 113.

Clemente, T. (2006) Nicotiana (Nicotiana tobaccum, Nicotiana benthamiana). *Methods Mol. Biol.* 343: 143–154.

Dong, C.J. and Liu, J.Y. (2010) The Arabidopsis EAR-motif-containing protein RAP2.1 functions as an active transcriptional repressor to keep stress responses under tight control. *BMC Plant Biol.* 10: 47.

Fisher, M.C., Henk, D.A., Briggs, C.J., Brownstein, J.S., Madoff, L.C., McCraw, S.L. et al. (2012) Emerging fungal threats to animal, plant and ecosystem health. *Nature* 484: 186–194.

Fujimoto, S.Y., Ohta, M., Usui, A., Shinshi, H. and Ohme-Takagi, M. (2000) Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. *Plant Cell* 12: 393–404.

Ghillebert, R., Swinnen, E., Wen, J., Vandesteene, L., Ramon, M., Norga, K. et al. (2011) The AMPK/SNF1/SnRK1 fuel gauge and energy regulator: structure, function and regulation. *FEBS J.* 278: 3978–3990.

Gu, Y.Q., Wildermuth, M.C., Chakravarthy, S., Loh, Y.T., Yang, C., He, X. et al. (2002) Tomato transcription factors Pti4, Pti5, and Pti6 activate defense responses when expressed in Arabidopsis. *Plant Cell* 14: 817–831.

Guo, Z., Chen, X., Wu, X., Ling, J. and Xu, P. (2004) Overexpression of the AP2/EREBP transcription factor OPBP1 enhances disease resistance and salt tolerance in tobacco. *Plant Mol. Biol.* 55: 607–618.

Hao, D.Y., Ohme-Takagi, M. and Sarai, A. (1998) Unique mode of GCC box recognition by the DNA-binding domain of ethylene-responsive

element-binding factor (ERF domain) in plant. *J. Biol. Chem.* 273: 26857–26861.

Jefferson, R.A. (1987) Assaying chimeric genes in plants: the *GUS* gene fusion system. *Plant Mol. Biol. Rep.* 5: 387–405.

Juranič, M., Srilunchang, K.O., Krohn, N.G., Leljak-Levanic, D., Sprunck, S. and Dresselhaus, T. (2012) Germline-specific MATH-BTB substrate adaptor MAB1 regulates spindle length and nuclei identity in maize. *Plant Cell* 24: 4974–4991.

Kagale, S. and Rozwadowski, K. (2011) EAR motif mediated transcriptional repression in plants. *Epigenetics* 6: 141–146.

Kuang, J.F., Chen, J.Y., Luo, M., Wu, K.Q., Sun, W., Jiang, Y.M. et al. (2012) Histone deacetylase HD2 interacts with ERF1 and is involved in longan fruit senescence. *J. Exp. Bot.* 63: 441–454.

Lescot, M., Déhais, P., Thijs, G., Marchal, K., Moreau, Y., Van de Peer, Y. et al. (2002) PlantCARE: a database of plant cis-acting regulatory elements and a portal to tools for *in silico* analysis of promoter sequences. *Nucleic Acids Res.* 30: 325–327.

Liang, H., Lu, Y., Liu, H., Wang, F., Xin, Z. and Zhang, Z. (2008) A novel activator-type ERF of *Thinopyrum intermedium*, TiERF1, positively regulates defense responses. *J. Exp. Bot.* 59: 3111–3120.

Liu, D., Chen, X., Liu, J., Ye, J. and Guo, Z. (2012) The rice ERF transcription factor OsERF922 negatively regulates resistance to *Magnaporthe oryzae* and salt tolerance. *J Exp. Bot.* 63: 3899–3911.

Liu, Y.G. and Chen, Y.L. (2007) High-efficiency thermal asymmetric interlaced PCR for amplification of unknown flanking sequences. *Bio Techniques* 43: 649–656.

Liu, Z., Bos, J.I., Armstrong, M., Whisson, S.C., da Cunha, L., Torto-Alalibo, T. et al. (2005) Patterns of diversifying selection in the phytotoxin-like *scr74* gene family of Phytophthora infestans. *Mol. Biol. Evol.* 22: 659–672.

Llorente, B., Bravo-Almonacid, F., Cvitanich, C., Orlowska, E., Torres, H.N., Flawia, M.M. et al. (2010) A quantitative real-time PCR method for in planta monitoring of *Phytophthora infestans* growth. *Lett. Appl. Microbiol.* 51: 603–610.

Maruyama, Y., Yamoto, N., Suzuki, Y., Chiba, Y., Yamazaki, K., Sato, T. et al. (2013) The *Arabidopsis* transcriptional repressor ERF9 participates in resistance against necrotrophic fungi. *Plant Sci.* 213: 79–87.

Mauch-Mani, B. and Slusarenko, A.J. (1996) Production of salicylic acid precursors is a major function of phenylalanine ammonialyase in the resistance of Arabidopsis to *Peronospora parasitica*. *Plant Cell* 8: 203–212.

McGrath, K.C., Dombrecht, B., Manners, J.M., Schenk, P.M., Edgar, C.I., Maclean, D.J. et al. (2005) Repressor- and activator-type ethylene response factors functioning in jasmonate signaling and disease resistance identified via a genome-wide screen of Arabidopsis transcription factor gene expression. *Plant Physiol.* 139: 949–959.

McLellan, H., Boevink, P.C., Armstrong, M.R., Pritchard, L., Gomez, S., Morales, J. et al. (2013) An RxLR effector from *Phytophthora infestans* prevents re-localisation of two plant NAC transcription factors from the endoplasmic reticulum to the nucleus. *PLoS Pathog.* 9: e1003670.

Mizoi, J., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2012) AP2/ERF family transcription factors in plant abiotic stress responses. *Biochim. Biophys. Acta* 1819: 86–96.

Nakano, T., Suzuki, K., Fujimura, T. and Shinshi, H. (2006) Genome-wide analysis of the ERF gene family in Arabidopsis and rice. *Plant Physiol.* 140: 411–432.

Núñez-Pastrana, R., Anderson, J. and Singh, K.B. (2013) Ethylene response factors and their role in plant defense. *CAB Rev.* 8 008: 1–12.

Ohta, M., Matsui, K., Hiratsu, K., Shinshi, H. and Ohme-Takagi, M. (2001) Repression domains of class II ERF transcriptional repressors share an essential motif for active repression. *Plant Cell* 13: 1959–1968.

Ohta, M.M., Ohme-Takagi, H. and Shinshi, H. (2000) Three ethylene-responsive transcription factors in tobacco with distinct transactivation functions. *Plant J.* 22: 29–38.

Ohme-Takagi, M. and Shinshi, H. (1995) Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. *Plant Cell* 7: 173–182.

Oñate-Sánchez, L., Anderson, J.P., Young, J. and Singh, K.B. (2007) AtERF14, a member of the ERF family of transcription factors, plays a nonredundant role in plant defense. *Plant Physiol.* 143: 400–409.

Pan, I.C., Li, C.W., Su, R.C., Cheng, C.P., Lin, C.S. and Chan, M.T. (2010) Ectopic expression of an EAR motif deletion mutant of *SlERF3* enhances tolerance to salt stress and *Ralstonia solanacearum* in tomato. *Planta* 232: 1075–1086.

Pre, M., Atallah, M., Champion, A., De Vos, M., Pieterse, C.M. and Memelink, J. (2008) The AP2/ERF domain transcription factor ORA59 integrates jasmonic acid and ethylene signals in plant defense. *Plant Physiol.* 147: 1347–1357.

Sharma, M.K., Kumar, R., Solanke, A.U., Sharma, R., Tyagi, A.K. and Sharma, A.K. (2010) Identification, phylogeny, and transcript profiling of ERF family genes during development and abiotic stress treatments in tomato. *Mol. Genet. Genomics* 284: 455–475.

Sherif, S., El-Sharkawy, I., Paliyath, G. and Jayasankar, S. (2013) PpERF3b, a transcriptional repressor from peach, contributes to disease susceptibility and side branching in EAR-dependent and -independent fashions. *Plant Cell Rep.* 32: 1111–1124.

Si, H.J., Xie, C.H. and Liu, J. (2003) An efficient protocol for *Agrobacterium* mediated Transformation with microtuber and the introduction of an antisense class I patatin gene into potato. *Acta Agronom. Sin.* 29: 801–805.

Solano, R., Stepanova, A., Chao, Q. and Ecker, J.R. (1998) Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. *Genes Dev.* 12: 3703–3714.

Song, C.P., Agarwal, M., Ohta, M., Guo, Y., Halfter, U., Wang, P. et al. (2005) Role of an Arabidopsis AP2/EREBP-type transcriptional repressor in abscisic acid and drought stress responses. *Plant Cell* 17: 2384–2396.

Sugio, A., Yang, B., Zhu, T. and White, F.F. (2007) Two type III effector genes of *Xanthomonas oryzae* pv. *oryzae* control the induction of the host genes *OsTFIIAγ1* and *OsTFX1* during bacterial blight of rice. *Proc. Natl Acad. Sci. USA* 104: 10720–10725.

Trabucco, G.M., Matos, D.A., Lee, S.J., Saathoff, A.J., Priest, H.D., Mockler, T.C. et al. (2013) Functional characterization of cinnamyl alcohol dehydrogenase and caffeic acid O-methyltransferase in *Brachypodium distachyon*. *BMC Biotechnol.* 13: 61.

Umezawa, T., Fujita, M., Fujita, Y., Yamaguchi-Shinozaki, K. and Shinozaki, K. (2006) Engineering drought tolerance in plants: discovering and tailoring genes to unlock the future. *Curr. Opin. Biotechnol.* 17: 113–122.

Van Verk, M.C., Gatz, C. and Linthorst, H.J.M. (2009) Transcriptional regulation of plant defense responses. *Adv. Bot. Res.* 51: 397–438.

Vleeshouwers, V.G.A.A., van Dooijeweert, W., Keizer, L.C.P., Sijpkes, L., Govers, F. and Colon, L.T. (1999) A laboratory assay for *Phytophthora infestans* resistance in various *Solanum* species reflects the field situation. *Eur. J. Plant Pathol.* 105: 241–250.

Walter, M., Chaban, C., Schütze, K., Batistic, O., Weckermann, K., Näke, C. et al. (2004) Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. *Plant J* 40: 428–438.

Wang, B.L., Liu, J., Tian, Z.D., Song, B.T. and Xie, C.H. (2005) Monitoring the expression profiles of genes associated with quantitative resistance to late blight in R-gene-free potato by cDNA microarray analysis. *Plant Sci.* 169: 1155–1167.

Wang, H.Y., Sun, C.L., Jiang, R., He, Q., Yang, Y., Tian, Z.J. et al. (2014) The dihydrolipoyl acyltransferase gene BCE2 participates in basal resistance against *Phytophthora infestans* in potato and *Nicotiana benthamiana*. *J. Plant Physiol.* 171: 907–914.

Wang, P., Du, Y., Zhao, X., Miao, Y. and Song, C.P. (2013) The MPK6–ERF6–ROS-responsive cis-acting element7/GCC box complex modulates oxidative gene transcription and the oxidative response in Arabidopsis. *Plant Physiol.* 161: 1392–408.

Yang, B., Sugio, A. and White, F.F. (2006) *Os8N3* is a host disease susceptibility gene for bacterial blight of rice. *Proc. Natl Acad. Sci. USA* 103: 10503–10508.

Zarei, A., Korbes, A.P., Younessi, P., Montiel, G., Champion, A. and Memelink, J. (2011) Two GCC boxes and AP2/ERF-domain transcription factor ORA59 in jasmonate/ethylene-mediated activation of the *PDF1.2* promoter in Arabidopsis. *Plant Mol. Biol.* 75: 321–331.

Zhang, H., Li, W., Chen, J., Yang, Y., Zhang, Z., Zhang, H. et al. (2007) Transcriptional activator TSRF1 reversely regulates pathogen resistance and osmotic stress tolerance in tobacco. *Plant Mol. Biol.* 63: 63–71.

Zhang, H., Zhang, J., Quan, R., Pan, X., Wan, L. and Huang, R. (2013) EAR motif mutation of rice OsERF3 alters the regulation of ethylene biosynthesis and drought tolerance. *Planta* 237: 1443–1451.