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

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

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Abbreviations: TF, transcription factor; ERFs, Ethylene response factors; EAR, ERF-associated amphiphilic repression; CaMV, cauliflower mosaic virus; GFP, green fluorescent protein; YFP, yellow fluorescent protein; BiFC, bimolecular fluorescence complementation; Y2H, Yeast two-hybrid; SA, salicylic acid; RNAi, RNA interference; ABA, abscisic acid; ET, ethylene; 3-AT, 3-amino-1, 2, 4-triazole; DAPI, 4', 6-diamidino-2-phenylindole ; BY2 cells, cultured cells of tobacco BY2.

The nucleotide *StERF3* sequence reported in this paper has been submitted to [NCBI] with accession numbers [GenBank No. EF091875].

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Abstract

Ethylene response factors (ERFs) are unique to the plant kingdom that plays crucial roles in plant response to various biotic and abiotic stresses. We show here that a potato StERF3, which contains ERF-associated amphiphilic repression (EAR) motif in C-terminal region, negatively regulates resistance to *Phytophthora infestans* and salt tolerance in potato. Promoter of the *StERF3* responses to induction of salicylic acid (SA), abscisic acid (ABA), ethylene (ET) 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 *StERF3* in potato showed 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 localised to the nucleus. Interestingly, Yeast two-hybrid assay and bimolecular fluorescence complementation (BiFC) test clarified that the StERF3 could interact with other proteins in 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 in engineering late blight resistance in potato.

Key words: EAR motif • late blight resistance • negative regulation • Potato • salt tolerance • StERF3

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 complicated regulation network of plant defense responses (van Verk et al. 2009). Among these transcription activators, ERF (ethylene responsive factor) transcription factors 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 (Núñez-Pastrana et al. 2013; Mizoi et al. 2012).

ERFs compose of a large family of plant-specific stress-responsive TFs, which are characterized by a conserved 58-59 amino acids 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 (Sharma et al. 2010; Nakano et al. 2006; Fujimoto et al. 2000). Most of them are JA/ET inducible, pathogenesis-related (PR) genes, such as the defensin gene *PDF1.2*, basic chitinase gene *ChiB*, thionin gene *Thi2.1*, as well as *PR1b* and *PR2*, to activate their expression in plants upon infection of a range of pathogens, both necrotrophic and biotrophic (Liang et al. 2008; Oñate-Sánchez et al. 2007; McGrath et al. 2005).

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* activate expression of defense-related genes (Solano et al. 1998; Gu et al. 2002). Overexpression of *NtERF2*, *NtERF4*, *AtERF1*, *AtERF2* and *AtERF5* lead 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 the class II ERFs that functions as dominant repressors of gene expression (Ohta et al. 2001; Fujimoto et al. 2000). 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 symptoms 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). Besides involved in defense, some EAR type ERFs also negatively regulate plants 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 coordinate the innate response of plants upon diverse stimuli. However, the mechanism underlying ERFs acting as activators or repressors still remains to elucidate.

Late blight, caused by the oomycete *Phytophthora infestans*, is 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* 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). In this paper, we further elucidate the functions and possible mechanisms of StERF3 on regulating potato defense and stress responses. We demonstrate that EAR type StERF3 responses to biotic and abiotic induction and could interact with GCC box *in vitro*. Stable silencing by RNAi of *StERF3* in potato leads to reduced *P. infestans* colonization and enhanced

salt tolerance, while transgenic potato plants overexpressing *StERF3* lead to enhanced colonization and reduced salt tolerance, confirming 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 re-localization between the cytoplasm and nucleus. This shifting in localization may affect the fate of StERF3 in regulating plant response. The present researches enrich our knowledge for ERFs in regulating plant response to biotic and abiotic stresses and provide a new clue, particularly, for 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 Rapid cDNA Amplification End (RACE) method. *StERF3* encodes a deduced protein of 223 amino acids which shows highly similar (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 to 60 amino acids (Fig. 1). Additionally, the StERF3 protein also contains a conserved ERF-associated amphiphilic repression (EAR) motif in the C-terminal region characterizing StERF3 as a member of the class II ERF transcription factors which have been shown to act as repressors of transcription (Ohta *et al.* 2001), suggesting that the StERF3 might negatively regulate the defense in potato.

Fig. 1

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

The promoter region of *StERF3* was cloned to investigate the expression pattern of *StERF3* (Supplementary Fig. S1). Promoter *cis*-elements analysis showed that the 1067 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*, SA, ethephon, ABA and NaCl separately. The results showed that the GUS was expressed around the inoculated site region after infection of *P. infestans* (Fig. 2B), and could be induced by NaCl (Fig. 2C), ABA (Fig. 2D), as well as ethephon (Fig. 2E) and SA (Fig. 2F). It suggests that StERF3, as a transcriptional factor, might functions through different signaling pathways in potato.

Fig. 2

StERF3 interacts with the GCC box in yeast cells

Plant ERF proteins contain a highly conserved DNA-binding domain which could specifically bind to GCC-box presenting in the promoter of many functional genes and manipulate their expressions (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 into the upstream of the *HIS3* reporter gene in pHIS2.1 vector （pHIS-5×GCC）for the Yeast one-hybrid assay. If StERF3 could bind to GCC-box on 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. 4, the yeasts carrying pHis2.1-5×GCC and pGADT7-Rec2-StERF3 grew well on minimal medium lacking histidine and containing 3-AT. The positive control that containing 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 GCC-box and it is same as other ERFs.

Fig. 3

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 (RNA interference) lines were obtained for each cultivar. Two of 'Zhuanxinwu' RNAi lines (ZXW-Ri-1 and ZXW-Ri-2) and three of 'E-potato-3' RNAi lines (E3-Ri-2, 3 and 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 lesions size were remarkably decreased in the StERF3-RNAi lines compared to their corresponding control formed by pathogen infection at 4 dpi (days post inoculation by *P. infestans*). To confirm the possible negative role for StERF3 in basal immunity to *P. infestans*, two of each 'Zhuanxinwu' and 'E-potato-3' overexpress lines (OE), showing elevated transcripts accumulation (5-to-8-fold) (Supplementary Fig. S2A, B), were selected for further pathogen

inoculation. Each OE lines of both cultivars displayed an increase in lesion size relative to control (Fig. 4A and B).

To further assess differences in late blight resistance, we determined mean lesion areas at 5 dpi. As shown in Fig. 4C and D, significant 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. 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 in consistent with the lesion expansion of the transgenic lines. The resistance level of both RNAi and OE lines are consistent with the role for *StERF3* in negatively regulating potato defense to *P. infestans*.

Fig. 4

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

StERF3 can be induced by NaCl and ABA treatments, (Fig. 2 D and E), suggesting that *StERF3* may be involved in potato stresses tolerance. So nodal cuttings of the 'Zhuanxinwu' and 'E-potato-3' transgenic lines and untransformed controls were cultured on 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 two cultivars were seriously suppressed on the medium containing 150 mM NaCl (Fig. 5A and B). But, it is obvious that 'Zhuanxinwu' RNAi lines showed an elevated level of tolerance to NaCl compared with the OE lines and control indicted by roots regeneration on RNAi lines. Even few roots regenerated in both transformed and control of 'E-potato-3', higher plant height and larger fresh weight were observed in RNAi lines grown on the medium containing

150 mM NaCl (Fig. 5C and D). These results indicate that silencing of *StERF3* enhance potato salt tolerance *in vitro*.

Fig. 5

***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 salicylic acid (SA)-mediated defense marker gene *PR1* (pathogen related protein 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 to corresponding controls (Fig. 6A). Similar expression patterns were observed for the SA signaling regulatory genes *NPR1* and *WRKY1* which were upregulated in the RNAi lines and repressed in the OE lines (Fig. 6B and C). Besides the SA-mediated defense marker genes, genes encoding MAP 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 the significance was not detected in RNAi lines for *MAPK* (Fig. 6D). Whereas, expression of *Osmotin* and *PAL* showed no differences in both OE and RNAi lines (data not show). These findings imply that the *StERF3* gene has impacts on the SA-depended gene expression.

Fig. 6

Interaction proteins of StERF3

Our results showed that StERF3 may be involved in different pathways to execute its regulatory functions in plant bio- and abiotic stress responses. To substantially evidence possible interaction network, StERF3 was used as bait to screen the interaction proteins from the potato yeast two-hybrid cDNA library. Totally 6 proteins, aconitatehydratase (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 to putatively 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 stresses response (CAD and CYN) (Trabucco et al. 2013), plant development (CIP3) (Ben-Naim et al. 2006) and protein synthesis (60S ribosomal protein L19).

To further confirm the interactions, three proteins (KIN1, CYN and CIP3) were subjected to the 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) well developed on SD/–Trp/–Leu/–His/–Ade plates and exhibited positive in X-α-Gal assay (Fig. 7A), indicating that StERF3 can physically interact with StKIN1, StCYN and StCIP3.

To further confirm the potential interaction between StERF3 and StKIN1, StCYN or StCIP3 *in planta*, bimolecular fluorescence complementation (BiFC) assay was utilized. BiFC assay demonstrated that tobacco BY2 cells co-transfected with StERF3:nYFP/StCYN:cYFP, 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.

Fig. 7

Subcellular localisations 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 of *35S: StERF3: GFP* and *35S: GFP* were transiently introduced into *N. benthamiana* epidermal cells by Agroinfiltration. Fluorescence analysis indicated that 35S: StERF3: GFP expressed only in the nuclei, while 35S: GFP was detected in both the nuclei and cytoplasm (Fig. 8). These results indicate that StERF3 is exclusively localized to the nucleus. Transient expression showed that 35S: StKIN1: GFP is localized to 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 at the cytoplasm when StERF3: nYFP was co-expressed with StKIN: cYFP, StCYN: cYFP or StCIP: cYFP (Fig. 7B). Typically, when StERF3: nYFP was expressed with cytoplasm-localized StKIN1, implicating that protein interaction might lead to re-localisation of StERF3. These observations imply that the subcellular re-localization of StERF3 caused by the interaction with target proteins may be important for a subtle manipulation of StERF3. However, the biological significance of this is worthy of investigation in the future.

Fig. 8

Discussion

ERFs have been reported responsive to biotic and abiotic stimuli and consequently modulate defense and stress related genes' 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). For example, AtERF4, AtERF9 and

OsERF922, are known to negatively regulate transcript levels of defense-related genes and subsequent plant stress tolerance (Maruyama et al. 2013; McGrath et al. 2005; Liu et al. 2012). In this study, StERF3 is characterized a member of 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 most important blight disease, and to salt stress. *StERF3* could responses to diverse stimuli including *P. infestans*, SA, ABA, ethephon and salt stress through analysis of the responses of 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 *StERF3* gene in potato shows increased resistance against *P. infestans* (Fig. 4) and enhanced salt tolerance (Fig. 5) suggesting that StERF3 function as a repressor-type regulator that links multiple signaling networks within 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 consist with that SlERF3 acts 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., GCC-box or DRE) in the promoters of target genes and then initiates a transcriptional cascade and leads to activation of downstream genes (Cheng et al. 2013; Hao et al. 1998). ERF transcription factors directly regulate pathogenesis-related (PR) gene expression by binding DNA with the GCC-box, such as *PR1* to *PR5* (Ohme-Takagi and Shinshi 1995). Our results show that StERF3 confers the ability to bind to GCC-box sequences and as a transcriptional activator in yeasts (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 that overexpression of *SlERF3* leads to repression of GCC-mediated transcription such as *PR1*, *PR2* and *PR5* (Pan et al. 2010). How GCC-box binding 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 ethylene, SA, and JA defense-signaling pathways (Liu et al. 2012; Zarei et al. 2011; Pre et al. 2008; McGrath et al. 2005). The present research evidenced that StERF3 RNAi potato lines activated the expression of the salicylic acid (SA)-mediated defense marker gene PR1 and WRKY1 while StERF3 overexpression transgenic lines suppressed their expression after *P. infestans* inoculation. Another key regulator gene NPR1 of SA signaling transduction pathway showed similar expression pattern in the StERF3 RNAi and OE lines (Fig.6). These results imply that SA signaling transduction pathway 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 present study. The situation is same as overexpression of an ERF member of the EREBP/AP2 family gene DREB2A did not result in significant induction of downstream genes in *Arabidopsis* (Umezawa et al. 2006). Maybe some modification could be required for StERF3 to induce the expression of some downstream target genes.

Modifications including interacting 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 be reported that plant repressor-type ERF might recruit a co-repressor and interact with histone deacetylases to block transcriptional activation of the target genes (Kagale and

Rozwadowski 2011; Song et al. 2005). 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 the *Arabidopsis* ERF6 could interact physically with mitogen-activated protein kinase 6 (MPK6) and phosphorylated by MPK6, which affected the dynamic alternation of the ERF6 protein and resulted in changes in 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 transcription regulation such as protein-protein interaction is needed for StERF3 protein to perform its regulation functions in potato.

Re-localization is a common mode-of-action for nuclear-localized TFs regulates target gene expression in plant. For example, during *P. infestans* infecting potato, *P. infestans* secreted effectors can prevent culture filtrate-triggered re-localization of potato transcription factors 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 the protein interaction affects StERF3 re-localization from the nucleus to cytoplasm. Wang et al. (2013) reported that phosphorylation modification 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 protein, is a SNF1-related protein kinase (Ghillebert et al. 2011), suggesting phosphorylation may be essential for StERF3 regulating potato defense. Since StERF3 is a negative regulator, it was hypothesized that protein interactions in 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*-overexpressing lines were more susceptible to *P. infestans* (Fig. 5). The question is why 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 plant. Plant-pathogenic effectors modulate host immunity 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 host gene *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 further determine whether any effectors or secreted proteins from *P. infestans* are involved in manipulating of negative regulatory StERF3 to attenuate host defense responses. Besides, StERF3 could also play a role in preventing over activation 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 genome DNA of a Chinese potato cultivar (cv.) 'E-potato-3' using hiTAIL-PCR (Liu and Cheng 2007). The longest PCR fragment (1067 bp) was used to analyses it inducing expression pattern. The detailed *pStERF3* sequence and predicted *cis*-elements using PlantCARE database (Lescot et al. 2002) are showed in supplementary Fig. S1. For construction of *pStERF3* controlled *GUS* vector, 1067 bp promoter fragment was digested from pUC18-*pStERF3* using *Bam*HI and *Hind* III and inserted into corresponding sites of pBI121 to replace the CaMV 35S promoter. For construction of the overexpressing (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 binary vector pBI121 through *Bam*HI and *Sac* I sites to produce OE vector pBI-*35S: StERF3*. To construct the RNA interference (RNAi) vector, a 236 bp fragment located in non- conserved region of *StERF3* (335-571 bp) was amplified by *StERF3*-RNAi-F and *StERF3*-RNAi-R primers (Supplementary Table S1). BP recombination enabled cloning into binary vector pHGRV (modified pHELLSGATE2 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 the YEB medium containing appropriate antibiotics.

Plant growth and transformation

Binary plasmid pBI-pStERF3-*GUS* was used to transform *Nicotiana benthamiana* leaf discs as described by Clemente (2006). Transformed clones were selected on Murashige & Skoog (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℃ and a 16 h photoperiod on MS medium.

Two Chinese potato cultivars 'E-potato-3' and 'Zhuanxinwu' were used for transformation of OE vector pBI-*35S:StERF3* and RNAi vector pHGRV-*StERF3*. Transgenic potato plants were obtained by *Agrobacterium*-mediated microtuber discs 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℃ 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 to 25℃ in controlled greenhouse under 16/8-h light-dark photoperiod.

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

For clarifying the induction pattern of *StERF3* promoter by biotic and abiotic factors, leaves of five-week-old transgenic and control *N. berthamiana in vitro* plantlets were detached for the treatments. For induction of signaling compounds, the leaves were sprayed separately with 10 μM salicylic acid (SA), 50 μM abscisic acid (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 10 μl droplet of freshly prepared *P. infestans* sporangia suspension (1×10^5 sporangia ml^{-1}) onto the abaxial side of the leaf and kept in petri dishes on wet paper disc. As control, water was pipetted onto the leaves. Five days post inoculation (5 dpi), the inoculated leaves were undertaken 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 ORF of these genes without stop code 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 drived constitutive overexpression plant expression vector pB7FWG2 using LR clonase (Invitrogen). Then vectors were transformed into *Agrobacterium* GV3101. *A. tumefaciens* containing each construct was pressure infiltrated into leaves of 4-week-old *N. benthamiana* as described methods (Wang et al. 2014). OD600 of *A. tumefaciens* was adjusted to 0.1. Cells expressing fluorescent protein fusions were observed using Carl Zeiss AXIO Observer A1 inverted fluorescence microscope 2 days post-infiltration.

Yeast one-hybrid (Y1H) assay

The yeast one-hybrid assay was performed using MATCHMAKER One-Hybrid Library Construction and Screening Kit (Clontech, USA) to examine the binding ability of StERF3 to 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), pHis2.1-5×GCC and pGADT7-Rec2-*StERF3* were introduced into yeast strain Y187 and the transformants were selected on synthetic dropout (SD) medium lacking Leu and Trp. Transformed colonies were then streaked on SD-His-Leu-Trp medium with 50 mm 3-AT (3-amino-1, 2, 4-triazole) and cultured at 28℃ 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, USA) according to the User Manual. For constructing a DNA-BD fusion, *StERF3* coding region was amplified by PCR using primers StERF3-BD-F and StERF3-BD-R (Supplementary Table S1) and then subcloned into *Bam*HI and *Pst*I sites of pGBKT7 to generate 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. After that approximately 3×10^6 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℃ 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 were put in supplementary Fig. S3). Primers used for the vector construction are presented in 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-β-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. Targets gene and fusion YFP terminus were controlled by 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 were showed in the 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 particle bombarded 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, Hercules, CA, USA). For staining the nuclei, 10 mg/mL 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. *P. infestans* isolates were routinely grown on rye agar medium supplemented with 2% sucrose at 18℃ in the dark. *P. infestans* sporangia were collected as described by Champouret et al. (2009). *P. infestans* inoculum

concentrations were adjusted before leaf inoculation to 1×10^5 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℃, 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 days post inoculation (dpi), lesion areas were measured as described (Vleeshouwers et al. 1999). Inoculation experiment was repeated three times. At least twenty leaves from four plants in each repeat. Statistical analysis was carried out using ANOVA.

For dynamically testing growth of the pathogen biomass around inoculated sites, DNA of leaf discs cut from the inoculation sites from the first day to the four day after *P. infestans* inoculation as described by Llorente et al. (2010) was extracted for qPCR to monitor the *P. infestans* growth. The pathogen biomass was quantified from 5 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, Japan）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 5 inoculation sites.

Salt tolerance assay of *in vitro* plantlets

In vitro transgenic and control plantlets of 'E-potato-3' and 'Zhuanxinwu' were used for salt tolerance assay. Single-node cuttings were cultured on MS medium or MS medium containing 150 mM NaCl, respectively. Shoot length, root length and fresh

weight of the plants were measured after 4 weeks. The salt tolerance assays was triplicated and each replication contained at least 36 plantlets (4 box×9 plantlets). Significant difference (*=p, 0.05) in plantlets fresh weight and height compared to 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, Shanghai, China). Synthesis of cDNA was conducted using an oligo (dT) primer and a M-MLV reverse transcriptase kit (Takara, Dalian, China) according to the manufacturer's instructions. qRT-PCR was performed on an ABI7300 PCR machine (Applied Biosystems, Foster City, CA) using the SYBR® Green Realtime PCR Master 282 Mix (Toyobo, Japan) according to the manufacturer's protocol. Six genes: *StPR1*, *StNPR1*, *StWRKY1*, 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 *Ef-1α* gene. Relative expression levels were calculated by a comparative Ct method as described by Cikos *et al*. (2007).

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Disclosures

The authors have no conflicts of interest to declare.

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Figure Legends

Fig. 1 Structure and conservation of StERF3 protein sequence in alignment with four class II ERF transcription factors.

AP2/ERF domain and EAR motif were 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 dark. Dashes show gaps in the amino acid sequences introduced to optimize alignment. NtERF3 (protein ID BAJ72664) is derived from *N. berthamiana*. OsERF3 (BAB03248) is derived from *O. sativa*. *SlERF3* is derived from *S. lycopersicum* (AAO34705) and StERF3 (ABK96798) is derived from *S. tuberosum*. *AtERF4* (AAM98171) is derived from *A. thaliana*.

Fig. 2 Promoter of *StERF3* responses to various biotic and abiotic stimuli.

Histochemical analysis of *pStERF3:GUS* gene activities in *N. berthamiana* leaves. Leaves of five-week-old transgenic and control *N. berthamianain vitro* plantlets were detached for various treatments. (A) Positive control (*35S:GUS*). (B) the leaf 5 d post inoculation with *P. infestans*. (C) The leaf treated with 200 mM NaCl solution for 12 h. (D, E and F) The leaves treated for 5 h with 50 μM abscisic acid (ABA), 10 μM ethephon and 10 μM salicylic acid (SA), respectively. The leaves were then 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 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. 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 medium SD/-Trp/-Leu/-His containing 50 mM 3-amino-1, 2, 4-triazole (3-AT). the yeast were grown for 3 d at 28℃.

Fig. 4 Overexpression and suppression of *StERF3* alter potato resistance to *Phytophthora infestans*.

(A) and (B) Images of representative detached leaves test of 'E-potato-3' and 'Zhuanxinwu' transgenic plants for resistance to *P. infestans* at 4 dpi. Leaves were detached from 6-week-old potato plants and inoculated with sporangia suspensions 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 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 SD of three independent tests. Ri- and OE- represent RNA interference and overexpression lines, respectively. WT, untransgenic plant. EV, empty vector (*35S:GUS*) transformation. (E) Dynamic growth of the pathogen biomass around inoculated sites on 'E-potato-3' (E3) RNAi and OE potato line leaves. 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 SEM. Three biological replicates were performed, each using 5 inoculation sites.

Fig. 5 Performance of *StERF3* transgenic potato lines grow on MS medium without or supplemented with 150 mM NaCl.

In vitro plantlets were cultured on MS medium or 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 untransgenic plant. (C) Plantlets height of 'E-potato-3' transgenic lines cultured on MS medium + 150 mM NaCl. (D) Plantlets fresh weight of 'E-potato-3' transgenic lines grown on MS + 150 mM NaCl medium. Salt tolerance assays were repeated three times with similar results and the results obtained from one representative experiment are shown. Error bars represent SEM, and significant difference (*=p, 0.05) in plantlets fresh weight and height compared to control was 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* gene 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) represents *PR1*, *NPR1*, *WRKY1* and *MAPK* relative expression level separately. Quantitative analysis was performed using qRT-PCR. The 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 SD.

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.

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Fig.1

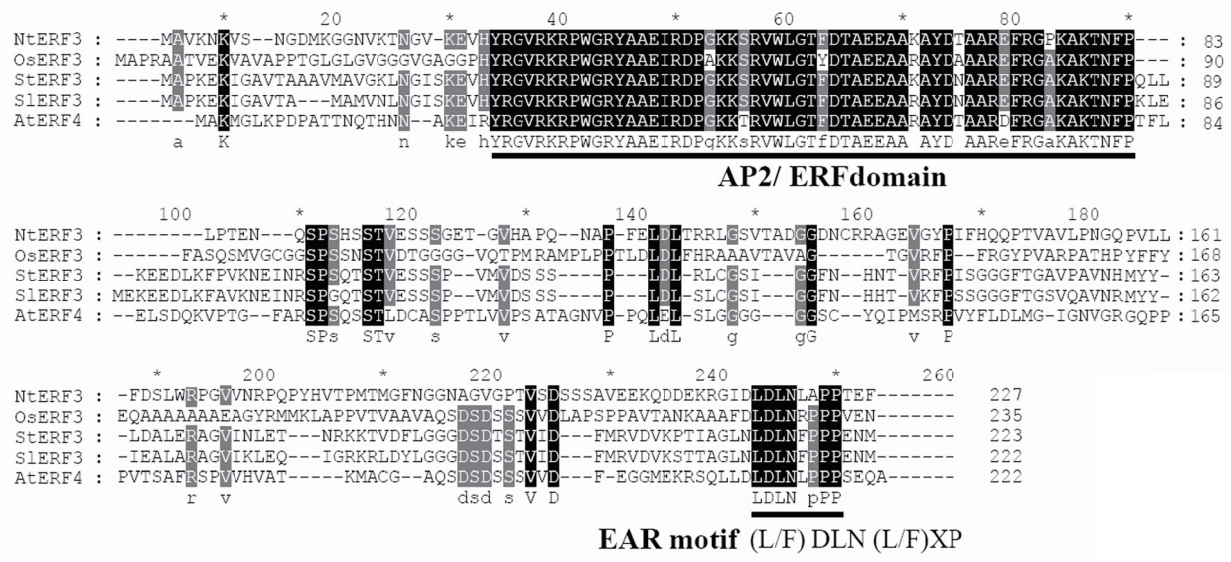

Fig. 1 Structure and conservation of StERF3 protein sequence in alignment with four class II ERF transcription factors.

AP2/ERF domain and EAR motif were 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 dark. Dashes show gaps in the amino acid sequences introduced to optimize alignment. NtERF3 (protein ID BAJ72664) is derived from *N. berthamiana*. OsERF3 (BAB03248) is derived from *O. sativa*. *SlERF3* is derived from *S. lycopersicum* (AAO34705) and StERF3 (ABK96798) is derived from *S. tuberosum*. *AtERF4* (AAM98171) is derived from *A. thaliana*.

Fig.2

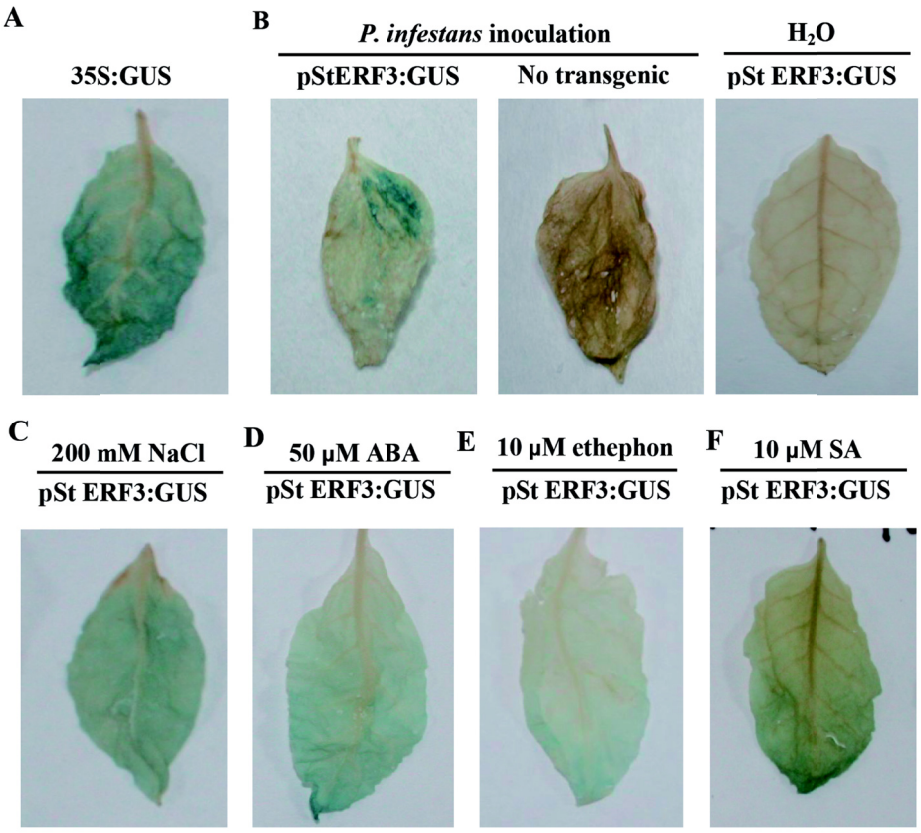

Fig. 2 Promoter of *StERF3* responses to various biotic and abiotic stimuli.

Histochemical analysis of *pStERF3:GUS* gene activities in *N. berthamiana* leaves. Leaves of five-week-old transgenic and control *N. berthamianain vitro* plantlets were detached for various treatments. (A) Positive control (*35S:GUS*). (B) the leaf 5 d post inoculation with *P. infestans*. (C) The leaf treated with 200 mM NaCl solution for 12 h. (D, E and F) The leaves treated for 5 h with 50 μM abscisic acid (ABA), 10 μM ethephon and 10 μM salicylic acid (SA), respectively. The leaves were then 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

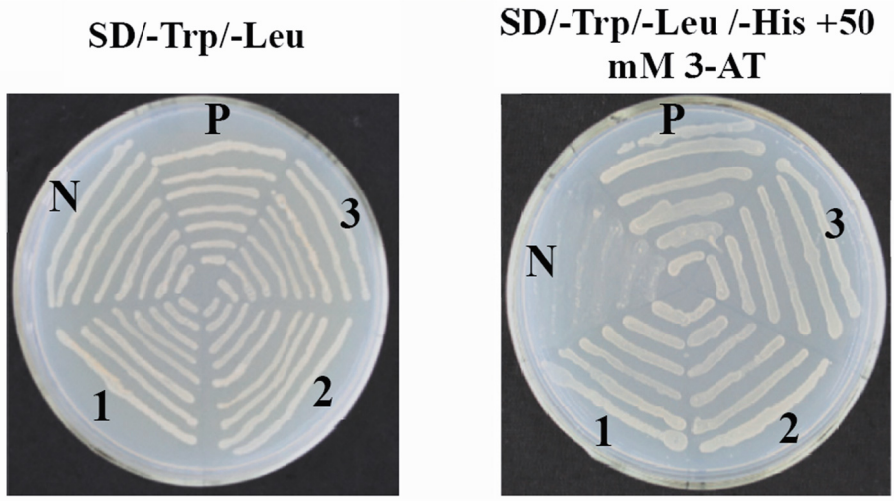

Fig. 3 StERF3 binds to GCC box in yeast.

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. 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 medium SD/-Trp/-Leu/-His containing 50 mM 3-amino-1, 2, 4-triazole (3-AT). the yeast were grown for 3 d at 28℃.

Fig.4

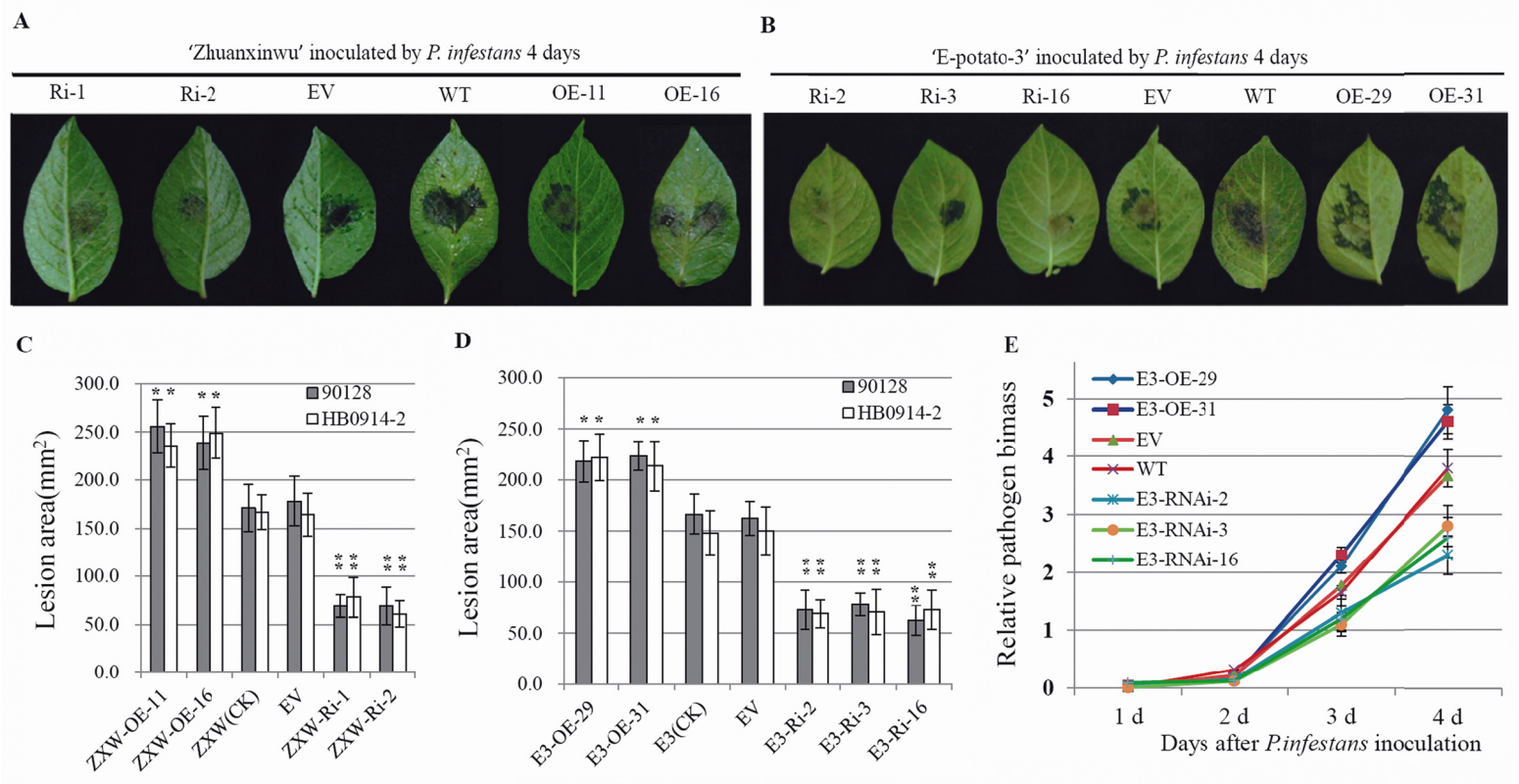

Fig. 4 Overexpression and suppression of *StERF3* alter potato resistance to *Phytophthora infestans*.

(A) and (B) Images of representative detached leaves test of 'E-potato-3' and 'Zhuanxinwu' transgenic plants for resistance to *P. infestans* at 4 dpi. Leaves were detached from 6-week-old potato plants and inoculated with sporangia suspensions 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 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 SD of three independent tests. Ri- and OE- represent RNA interference and overexpression lines, respectively. WT, untransgenic plant. EV, empty vector (*35S:GUS*) transformation. (E) Dynamic growth of the pathogen biomass around inoculated sites on 'E-potato-3' (E3) RNAi and OE potato line leaves. 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 SEM. Three biological replicates were performed, each using 5 inoculation sites.

Fig.5

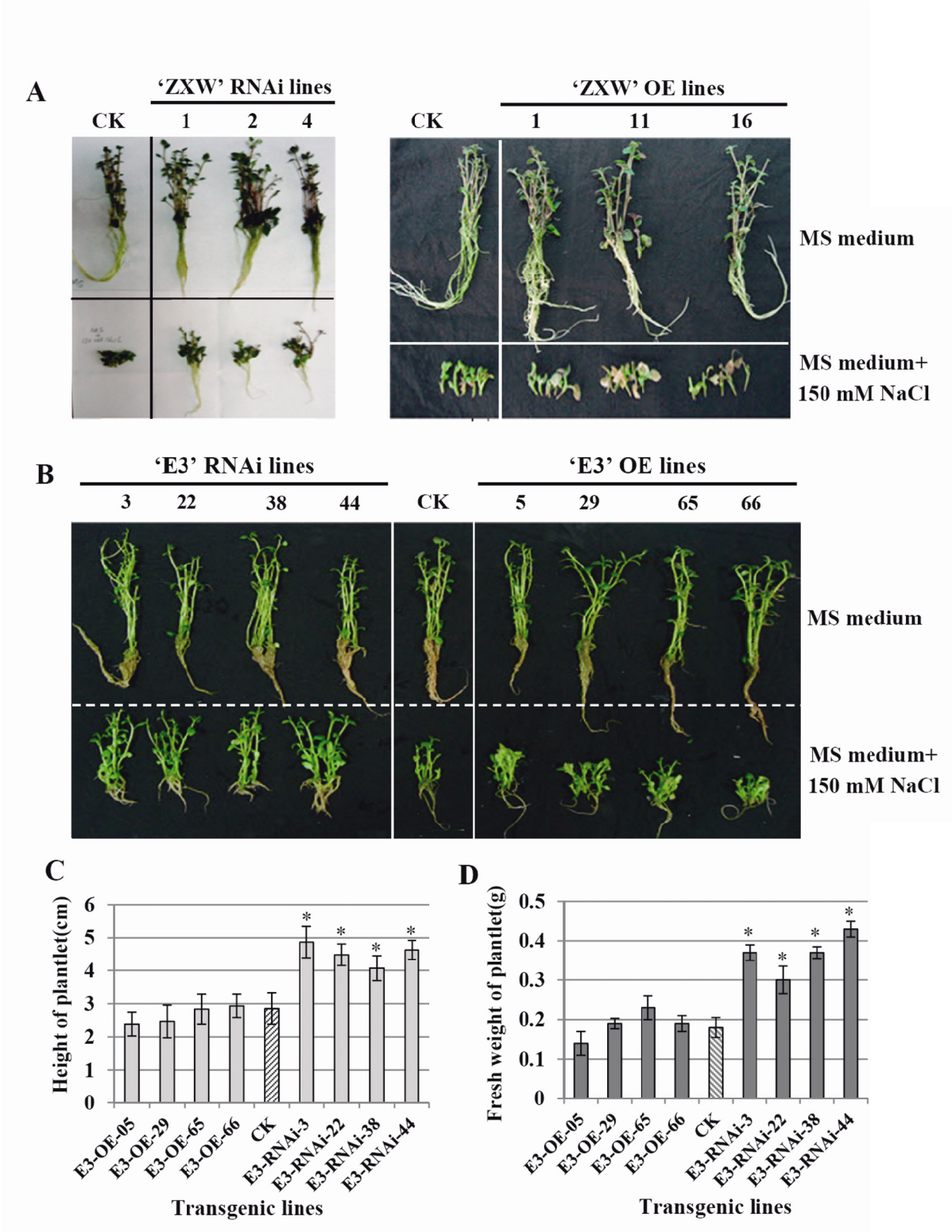

Fig. 5 Performance of *StERF3* transgenic potato lines grow on MS medium without or supplemented with 150 mM NaCl.

In vitro plantlets were cultured on MS medium or 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 untransgenic plant. (C) Plantlets height of ‘E-potato-3’ transgenic lines cultured on MS medium + 150 mM NaCl. (D) Plantlets fresh weight of ‘E-potato-3’ transgenic lines grown on MS + 150 mM NaCl medium. Salt tolerance assays were repeated three times with similar results and the results obtained from one representative experiment are shown. Error bars represent SEM, and significant difference (*=p, 0.05) in plantlets fresh weight and height compared to control was determined by one-way ANOVA. Data are based on at least 36 plantlets per transgenic line.

Fig. 6

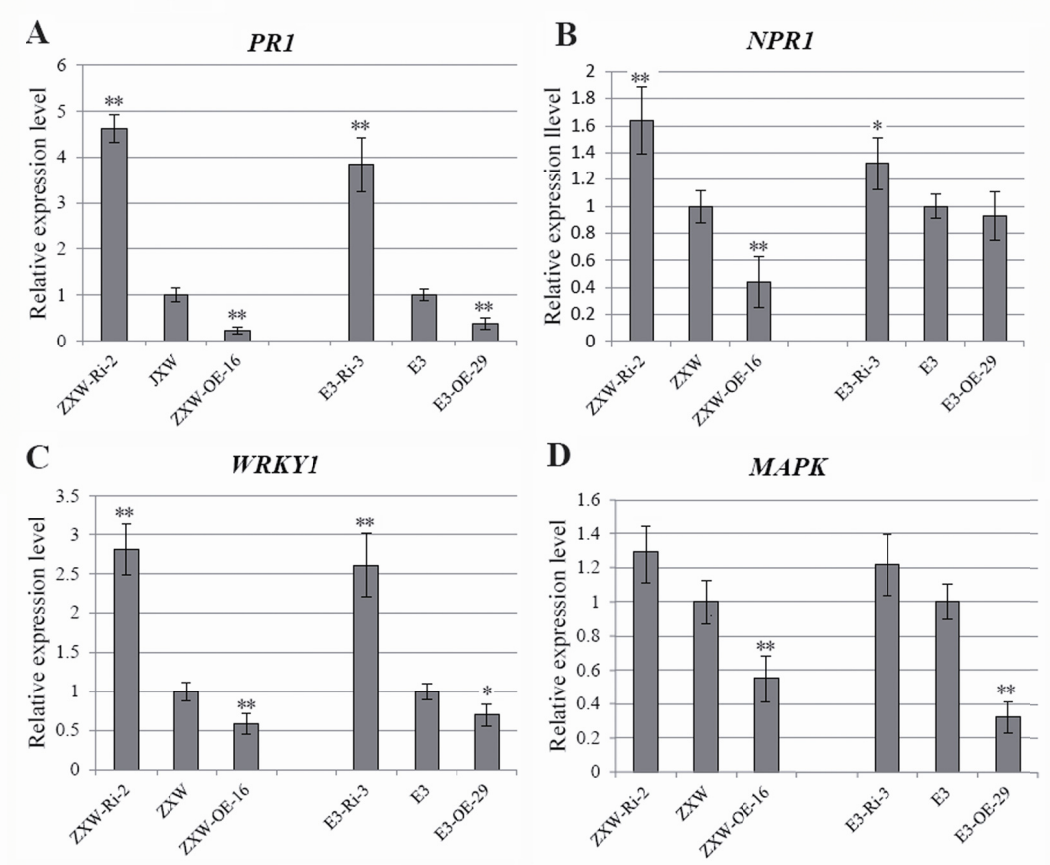

Fig. 6 Expression of *PR1*, *NPR1*, *WRKY1* and *MAPK* gene in *StERF3* RNAi and OE potato lines after *P. infestans* inoculation.

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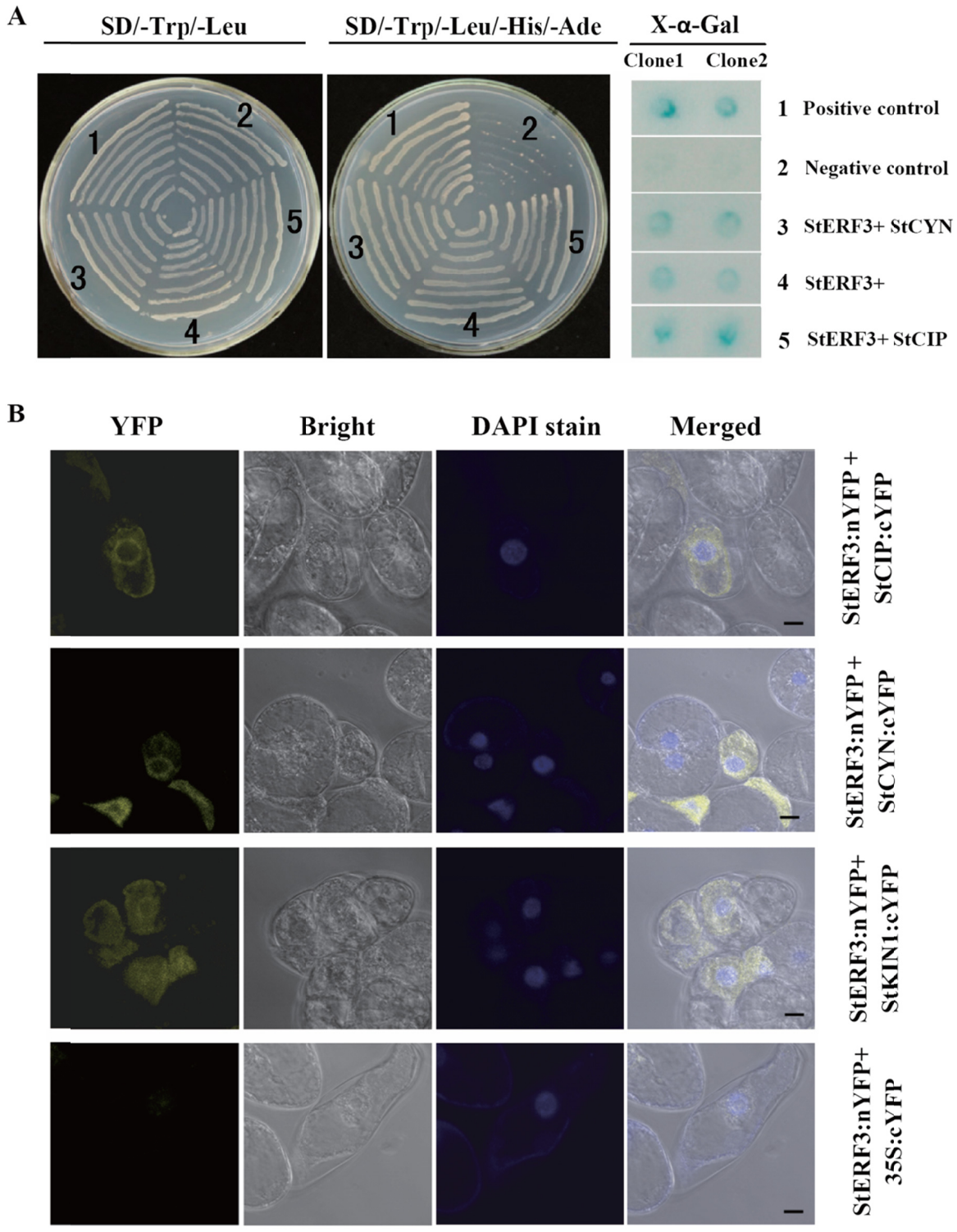

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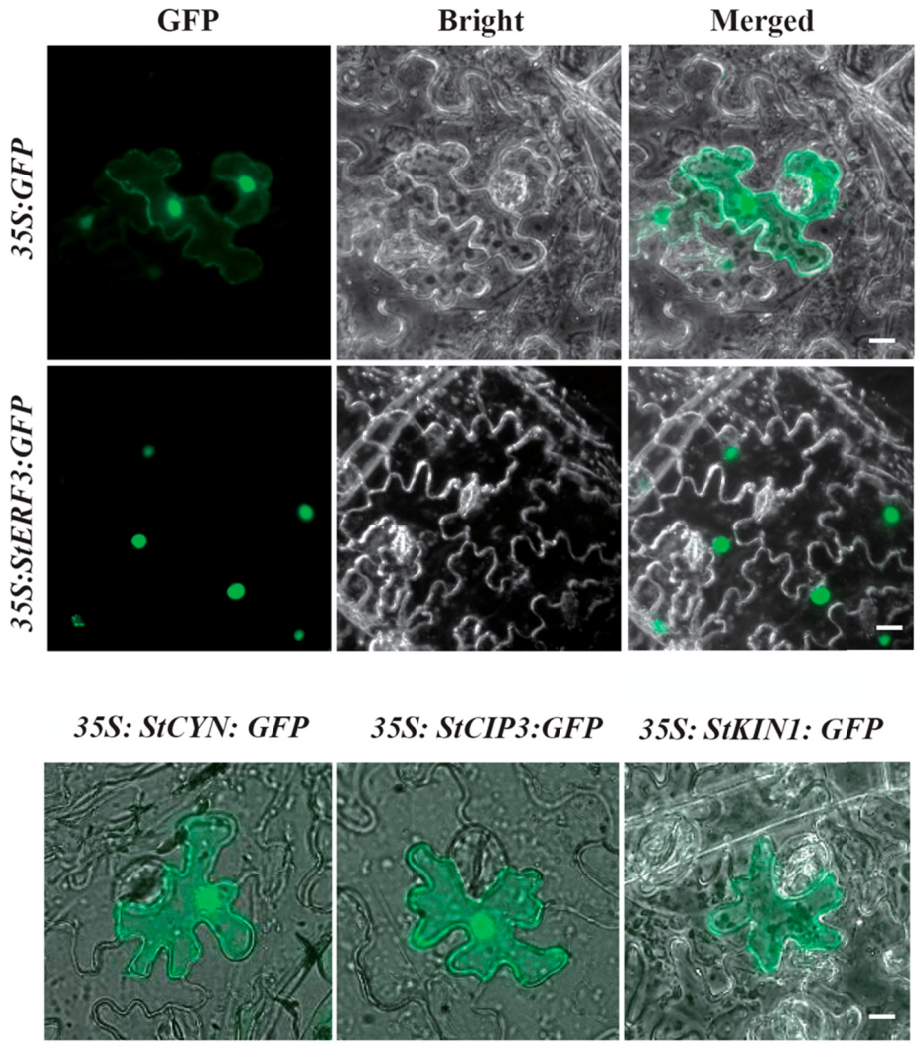

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