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LeSPL-CNR negatively regulates Cd acquisition through repressing nitrate reductase-mediated nitric oxide production in tomato

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Abstract

Main conclusion **An SPL-type transcription factor, LeSPL-CNR, is negatively involved in NO production by modulating *SlNR* expression and nitrate reductase activity, which contributes to Cd tolerance.**

Cadmium (Cd) is a highly toxic pollutant. Identifying factors affecting Cd accumulation in plants is a prerequisite for minimizing dietary uptake of Cd from crops grown with contaminated soil. Here, we report the involvement of a *SQUAMOSA* PROMOTER-BINDING PROTEIN-LIKE (SPL) transcription factor LeSPL-CNR in Cd tolerance in tomato (*Solanum lycopersicum*). In comparison with the wild-type *Ailsa Craig* (AC) plants, the *Colourless non-ripening* (*Cnr*) epimutant displayed increased Cd accumulation and enhanced sensitivity to Cd, which was in well accordance with the repression of *LeSPL-CNR* expression. Cd stress-induced NO production was inhibited by nitrate reductase (NR) inhibitor, but not NO synthase-like enzyme inhibitor. Expression of *LeSPL-CNR* was negatively correlated with *SlNR* expression and the NR activity. We also demonstrated that LeSPL-CNR inhibited the *SlNR* promoter activity in vivo and bound to *SlNR* promoter sequence that does not contain a known SBP-binding motif. In addition, expression of an *IRON-REGULATED TRANSPORTER1*, *SlIRT1*, was more abundant in *Cnr* roots than AC roots under Cd stress. LeSPL-CNR may thus provide a molecular mechanism linking Cd stress response to regulation of NR-dependent NO production, which then contributes to Cd uptake via *SlIRT1* expression in tomato.

Keywords Cadmium toxicity · Iron uptake · *Solanum lycopersicum* · Transcription factor

Abbreviations

AC	Ailsa Craig
Cnr	*Colourless non-ripening*
DAF-FM DA	4-Amino-5methylamino-2′,7′-difluorescein diacetate
IRT1	IRON-REGULATED TRANSPORTER1
NO	Nitric oxide
NOS	Nitric oxide synthase
NR	Nitrate reductase
SlNR	*Solanum lycopersicum* nitrate reductase
SPL	*SQUAMOSA* PROMOTER-BINDING PROTEIN-LIKE
TF	Transcription factor

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Introduction

Cadmium (Cd) is a highly toxic heavy metal for most organisms including plants, animals, and humans (Lux et al. 2011). However, the Cd contamination is widely distributed in soils and water owing to the rapid industrial development and release of agrochemicals into the environment. Being highly soluble in water, Cd can be easily taken up by

plants and enters the human food chain, therefore, threatening human health (Ueno et al. 2010). Thus, identifying strategies to lower Cd accumulation in crops grown in Cd-contaminated soils to ensure the human health is an urgent task for many today's scientists. A substantial understanding of the responses of plants to Cd exposure is a prerequisite for successful ensuring to perform the above work.

Many plant physiological processes involved in alleviating Cd toxicity have been identified. Upon Cd exposure, plant roots secrete chelating compounds into rhizosphere to reduce Cd^{2+} bioavailability (Zhu et al. 2011; Guo et al. 2017), and inhibit the expression of transporters for Cd^{2+} to minimize Cd entry into root cells (Mao et al. 2014; Fan et al. 2014; He et al. 2017), pump Cd out at the plasma membrane (Kim et al. 2007), and immobilize Cd at apoplast (Krzeslowska 2011). Once having entered the cytoplasm, Cd^{2+} can be bound to various thiol compounds such as glutathione, phytochelatin, and metallothionein (DalCorso et al. 2008), and can be also sequestered into vacuoles (Lux et al. 2011). When the Cd is over-accumulated, plants also up-regulate the antioxidative system to combat with Cd-induced oxidative stress (Verbruggen et al. 2009). Recent studies have shown that many of the above responses are initiated by transcription regulation which depends on the action of transcription factors (TFs). The class A4 heat shock TF, HsfA4a in wheat, regulates Cd tolerance by up-regulating metallothionein gene expression (Shim et al. 2009); the BjCdR15, a bZIP TF in *Brassica juncea*, enhances Cd tolerance and accumulation in plants possibly by up-regulating expression of several metal transporter genes (Farinati et al. 2010); a zinc-finger TF, ZAT6, positively regulates Cd tolerance by regulating *GSH1* expression in *Arabidopsis* (Chen et al. 2016). Nevertheless, how TFs control Cd responses needs further investigations, because Cd is able to affect expression of many TFs in various plant species (Weber et al. 2006; Romero-Puertas et al. 2007; Tamas et al. 2008).

The *SQUAMOSA* PROMOTER-BINDING PROTEIN-LIKE (SPL) TF family is involved in diverse aspects of development and metabolic processes in plants (Chen et al. 2010b; Guo et al. 2011; Preston and Hileman 2013). Recently, the role of SPL proteins in plant biotic and abiotic stress is also emerging. For example, AtSPL14 is reported to be involved in the sensitivity to fumonisin B1, a fungal toxin (Stone et al. 2005). AtSPL7 up-regulates *miRNA398* expression, which, in turn, down-regulates the expression of Cu/Zn SOD and the chloroplast stroma SOD (Kliebenstein et al. 1998; Sunkar et al. 2006; Yamasaki et al. 2007; Abdel-Ghany and Pilon 2008). Genomewide sequencing analysis revealed that AtSPL7 regulates *FRO4* and *FRO5* expression, which is necessary for the reduction of Cu(II) to Cu(I) (Bernal et al. 2012). AtSPL7 has also been demonstrated to target copper transporter, COPT6, thus, controlling Cu uptake under Cu deficiency

(Jung et al. 2012). Recently, Lei et al. (2015) reported that AtSPL3 is involved in P deficiency responses in *Arabidopsis*. These results prompted us to investigate whether SPL proteins are also involved in plants' response to Cd stress.

In this study, we used tomato plants to investigate the role of LeSPL-CNR, an SPL protein previously characterized to control the ripening of fruit (Thompson et al. 1999; Manning et al. 2006), in regulating the Cd acquisition and tolerance. We found, for the first time, that LeSPL-CNR functions as a transcriptional repressor to down-regulate *nitrate reductase* (*NR*) expression, and control the NO production by NR; thus, inhibiting the LeIRT1-mediated Cd acquisition by roots.

Materials and methods

Plant materials and growth conditions

Tomato (*Solanum lycopersicum*) cultivar Ailsa Craig (AC) and *Colourless non-ripening* (*Cnr*) mutant (AC background) are the same as described in Chen et al. (2015a). Seeds were sterilized by 10% NaClO (v/v) for 15 min and thoroughly washed with deionized water to remove the residual NaClO. Seeds were then incubated in deionized water with a shaker at 200-rpm overnight and transferred to petri dish containing 1/5 strength-modified Hoagland nutrient solution and 1% agar (pH 5.5 with 10-mM Mes) for germination. The nutrient solution contains macronutrients in mM: KNO_3, 1.0; $Ca(NO_3)_2$, 1.0; $MgSO_4$, 0.4; $NH_4H_2PO_4$, 0.01, and micronutrients in μM: NaFeEDTA, 20; H_3BO_3, 3.0; $MnCl_2$, 0.5; $CuSO_4$, 0.2; $ZnSO_4$, 0.4; $(NH_4)_6Mo_7O_{24}$, 1.0. After germination, seedlings were kept at petri dish for 2 days until the primary root length was about 3–4-cm long. Uniform seedlings were then transferred to a plastic mesh floating on 1/5 strength-modified Hoagland nutrient solution for 2–day acclimation. Cd treatment was performed by adding different concentrations of $CdCl_2$ into nutrient solution. For pharmacological experiments, either 50-μM *S*-nitrosoglutathione (GSNO) prepared according to Chen et al. (2010a), 100-μM sodium nitroprusside (SNP; Sangon, Shanghai, China), 20-μM tungstate (Sangon), 100-μM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO; Dojin Laboratories), or 100-μM N^ω-nitro-L-arginine methyl ester hydrochloride (L-NAME; Sigma) was added to the treatment solution. The treatment solution was renewed every other day. All the experiments were carried out in an environmentally controlled growth room with a 16-h/26 °C day and an 8-h/23 °C night regime, a light intensity of 250–300-μmol photon m^{-2} s^{-1}, and a relative humidity of 70%.

Root growth assay and metal determination

Root growth was evaluated by measuring the primary root length with a ruler before and after treatment, which was repeated at least twice each with ten seedlings. For metal determination, five plants were combined into one sample, and plants were separated into roots and shoots after treatment. Root samples were washed twice with 1-mM EDTA-Na$_2$ solution to remove root surface contaminated Cd^{2+}, while shoot samples were rinsed in deionized water and blotted dry, and dried at 60 °C for 3 days. Dried tissues were weighed and digested for the measurement of elemental concentrations by inductively coupled plasma atomic emission spectrometry (AIRIS/AP, TJA, Thermo Fisher Scientific).

RNA extraction and quantitative real-time RT-PCR

Total RNA extraction and quantitative real-time RT-PCR were carried out as previously described (Chen et al. 2015b), with primer sequences provided in Table S1. Tomato *GAPDH*, *ACTIN*, and *18S rRNA* were used as a control to normalize the relative expression of target genes. Gene expression levels were calculated using the delta cycle threshold method.

Measurement of NO

Nitric oxide was visualized using 4-amino-5methylamino-2′,7′-difluorescein diacetate (DAF-FM DA). After treatment, roots were rinsed gently with deionized water and root tips were loaded with 10-μM DAF-FM DA in 20-mM Hepes/NaOH buffer (pH 7.4) for 30 min. After washed three times in fresh buffer, fluorescence was observed under a microscope (Nikon Eclipse E600, Nikon, excitation 488 nm, and emission 495–575 nm). Exposure settings (1 s) were constantly maintained during fluorescence microscopy. Signal intensities of green fluorescence in the images were quantified by Photoshop software (Adobe Systems).

Measurement of NR activity

NR activity was measured according to Jin et al. (2011). Briefly, whole roots were excised and placed in each test tube. 5 mL of assay solution comprising 2% 1-propanol, 100-mM KH$_2$PO$_4$ (pH 7.5), and 30-mM KNO$_3$ were added to each tube. Samples were vacuum-infiltrated for 5 min and incubated in a shaking water bath at 25 °C for 30 min in the dark. After incubation, 1 mL aliquot from each sample was transferred to a new tube, followed by the addition of 1 mL of sulphanilamide (1% w/v in 1.5 M HCl) and 1 mL *N*-(1-naphthyl)-ethylenediaminedihydrochloride

(0.02% w/v in 0.2 M HCl). The samples were incubated at room temperature for 30 min. The absorbance at 540 nm was measured with a spectrophotometer.

Yeast one-hybrid assay

The yeast one-hybrid assay was performed following our previous report (Fan et al. 2015). To investigate the interaction between LeSPL-CNR and the *SlNR* promoter, we amplified the promoter segments of *SlNR* (Table S1) by PCR from tomato genomic DNA. The amplified promoter region was cloned upstream of the Aureobasidin A (AbA) resistance reporter gene (AUR1-C) in the pAbAi vector. The open-reading frame of LeSPL-CNR was cloned in frame after the transcriptional-activation domain of the yeast transcription factor GAL4 in pGADT7 (pAD-LeSPL-CNR). Two of these plasmids, pAbAi-ProSlNR and pAD-LeSPL-CNR, or the positive controls p53-AbAi and pAD-p53, were introduced into yeast strain Y1HGold and cultured on SD medium without Ura and containing 0 or 150 ng mL^{-1} AbA at 30 °C for 3 days according to the manufacturer's instructions.

Transactivation potential assay in yeast

The full-length LeSPL-CNR, truncated LeSPL-CNR fragments were amplified using specific primers with *Nde*I and *Sal*I restriction digestion sites that flanked both terminals of each full-length/truncated sequence (Table S1). Amplified sequences were inserted into the corresponding sites of pGBKT7 (Clontech). Each plasmid (and vector control) was transformed separately into yeast strain AH109 (carrying the GAL4-responsive GAL1 promoter and the HIS3 reporter gene) and cultured on either SD-Trp or SD-Trp-His medium at 30 °C for 3 days according to the supplier's protocols.

Transcriptional repression assay in *Nicotiana benthamiana* leaves

The full-length LeSPL-CNR was amplified and cloned into the binary vector (pCambia2300) under the control of CaMV 35S promoter. The 2- or 1406-kb promoter of *SlNR* was amplified using specific primers (Table S1). For dual-luciferase assay, a 2-kb promoter sequence was ligated into the reporter vector pGreenII0800-LUC (Hellens et al. 2005). The reporter plasmid and effector plasmid were, respectively, transformed into *Agrobacterium tumefaciens* GV3101. 100 μL of *Agrobacterium* carrying the reporter plasmid and 900 μL of *Agrobacterium* carrying the 35S:LeSPL-CNR were co-infiltrated into the tobacco leaves. The firefly and renilla luciferase activities were measured using a Dual-Luciferase assay kit (Promega). The analysis was executed using the Luminoskan Ascent Microplate Luminometer (Thermo Fisher Scientific). For GFP reporter

assay, the promoter sequences were cloned into pCAM-BIA1300 vector. A pair of plasmids was transformed into *A. tumefaciens* strain GV3101. Agrobacterial cells resuspended in the infiltration buffer (10-mM $MgCl_2$, 0.2-mM acetosyringone, and 10-mM Mes, pH 5.6) were infiltrated into leaves of *N. benthamiana*. The final optical density of bacteria solution at 600 nm value was 0.5 for reporter, or 0.5 for effector. GFP fluorescence was observed by a confocal laser scanning microscope (LSM710; Karl Zeiss, Jena, Germany).

Statistical analysis

Data are given as mean ± standard deviation (SD). Each result in transcriptional expression and metal contents had at least three biological replicates. Each result in the root growth assay and NO fluorescence observation had at least ten replicates. Each experiment was independently carried out at least twice. All statistical analyses were conducted by Tukey's test among treatments or one-way ANOVA test between genotypes ($P < 0.05$) with DPS 11.0 edition for windows (Tang and Zhang 2012).

Results

Repressed expression of *LeSPL-CNR* by Cd stress

We first carried out a dose experiment to investigate the effects of Cd stress on the growth of tomato (*S. lycopersicum* cv. AC) seedlings. The primary root elongation was slightly but not significantly inhibited by 5 μM Cd over the entire 4-day-treatment duration (Fig. 1a). The root growth was moderately inhibited by 10-μM Cd over the entire treatment time, but almost completely arrested by 20-μM Cd. The relative root growth was 80% after 1 day of 10-μM Cd treatment, and progressively decreased to 50% at day 4 (Fig. 1b). Next, to investigate whether SPL genes are involved in Cd stress responses in tomato, we analyzed the expression changes of 16 SPL gene family members in response to 10-μM Cd stress at the transcriptional level. To ensure the reliability of expression levels of real-time PCR analysis, we first checked the stability of three reference genes, namely, *GAPDH*, *ACTIN*, and *18S rRNA*. In AC
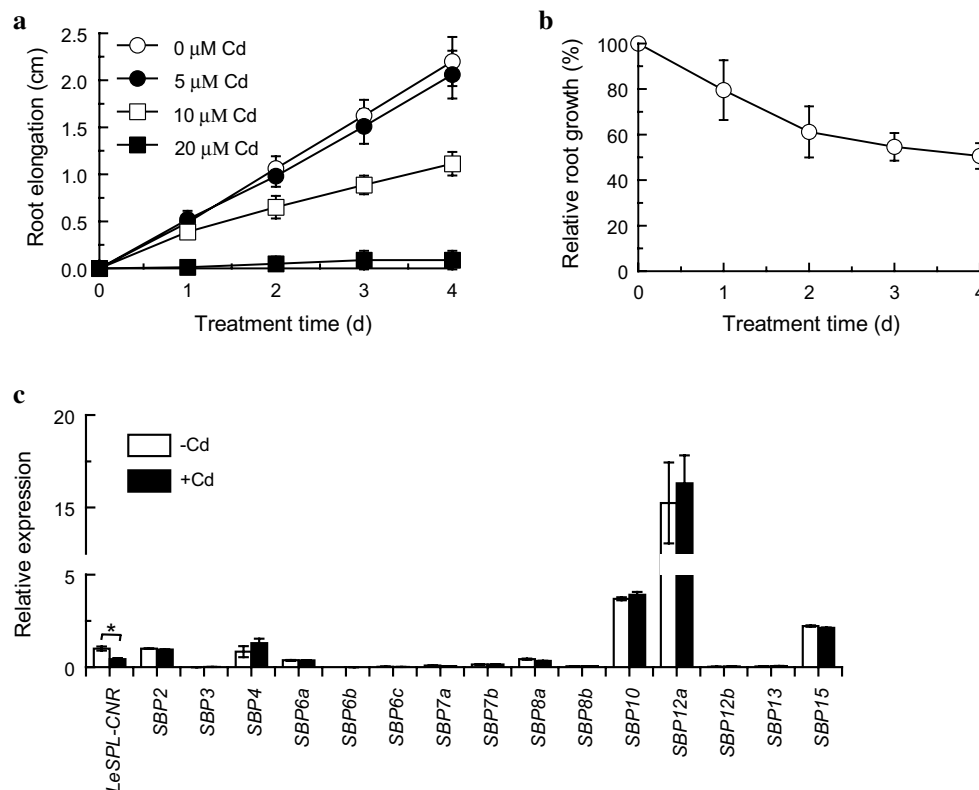

Fig. 1 Effects of Cd stress on primary root elongation and the expression of *SPL* genes in tomato. **a** Four-day-old seedlings of AC plants were exposed to nutrient solution containing 0-, 5-, 10-, or 20-μM $CdCl_2$ for different days. Root length was measured before and after treatment. Data are mean ± SD ($n = 10$). **b** Relative root growth in response to 10-μM $CdCl_2$. Data are mean ± SD ($n = 10$). **c** Expression of *SPL* genes in response to Cd stress. 4-day-old seedlings were exposed to nutrient solution containing 10 μM $CdCl_2$ for 1 day. *18S rRNA* was used as internal control of gene expression. Data were normalized as relative expression to that of *LeSPL-CNR* without Cd treatment, which was designated as 1. Data are mean ± SD ($n = 3$ biological replicates). Asterisk represents significant difference between treatments (one-way ANOVA, $P < 0.05$)

plants, all three reference genes kept constant in response to Cd (Fig. S1a); however, in *Cnr* plants, Ct values of *GAPDH* and *ACTIN* increased 3 and 5.7%, respectively, by Cd stress (Fig. S1b). The expression level of *18S rRNA* was hardly affected by Cd stress in *Cnr* plants. Therefore, *18S rRNA* was more stable, although all three genes were suitable for qRT-PCR analysis. Interestingly, among 16 SPL genes, only the transcript abundance of *LeSPL-CNR* was significantly repressed by Cd stress (Fig. 1c).

Cnr mutant displayed increased Cd accumulation in roots and enhanced sensitivity to Cd Stress

The specific repression of *LeSPL-CNR* transcript abundance by Cd stress led us to wonder whether LeSPL-CNR is positively or negatively involved in Cd stress response. To investigate the function of *LeSPL-CNR* in Cd stress, a naturally occurring epigenetic mutant, *Cnr*, was used along with its wild-type AC to compare Cd sensitivity. *Cnr* contains 18 hypermethylated cytosines in a 286-bp region of the *LeSPL-CNR* promoter, which results in the arrest of *LeSPL-CNR* expression during tomato fruit ripening (Manning et al. 2006). We investigated the expression of *LeSPL-CNR* mRNA in roots of AC and *Cnr* either in the absence or in the presence of Cd stress. The transcript abundance of *LeSPL-CNR* was significantly down-regulated in *Cnr* in comparison with AC in the absence of Cd stress (Fig. 2), suggesting that the *Cnr* mutant is suitable for functional characterization of LeSPL-CNR with respect to Cd tolerance. In the presence of Cd stress, the transcript abundance of *LeSPL-CNR* was significantly reduced both in AC and *Cnr*, but the level was much lower in *Cnr* than that in AC (Fig. 2).

Next, we examined the root growth of AC and *Cnr* under Cd stress. As shown in Fig. 3a, there was no significant
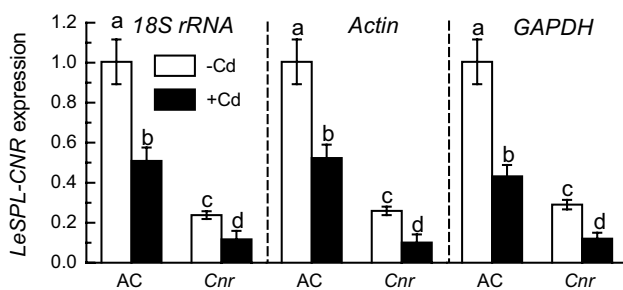
difference in root growth during the entire 4-day treatment duration. However, the root growth became more severely inhibited in *Cnr* than AC after 1 day of Cd treatment and such difference became much larger with prolonged treatment duration (Fig. 3b), suggesting that repression of *LeSPL-CNR* results in increased sensitivity to Cd stress in tomato roots.

To determine whether increased Cd sensitivity in *Cnr* is associated with Cd accumulation, we then investigated Cd content in roots and shoots. Without Cd stress, there was a trace amount of Cd in both roots and shoots, but the difference was not obvious between AC and *Cnr*. In response to Cd stress, both AC and *Cnr* accumulated a substantial amount of Cd in roots and shoots (Fig. 3c, d). However, the root Cd content in *Cnr* was 47% higher than that in AC, whereas there was no significant difference in shoot Cd content between *Cnr* and AC, suggesting that the repression of *LeSPL-CNR* resulted in the increased accumulation of Cd in roots. Thus, it appears that LeSPL-CNR acts as a positive regulator involved in Cd stress tolerance, though *LeSPL-CNR* expression is repressed by Cd stress.

NO accumulation is associated with increased Cd sensitivity

Emerging evidence indicates that NO plays an important role in Cd accumulation (Besson-Bard et al. 2009; Kan et al. 2016; Yuan and Huang 2016). To investigate whether NO is involved in increasing Cd accumulation and Cd sensitivity in *Cnr* root, we examined the NO production using the NO-specific fluorescence probe DAF-FM DA (Fig. 4a). In the absence of Cd stress, the fluorescence intensity was greater (42%) in *Cnr* than AC. In comparison with their respective -Cd control roots, Cd stress induced the fluorescence intensity by 2.27- and 3.54-fold in AC and *Cnr* roots, respectively (Fig. 4b). It is noteworthy that endogenous NO concentration was much greater in *Cnr* mutant than that in AC after Cd stress (Fig. 4b).

Both NR and nitric oxide synthase (NOS)-like enzyme have been suggested as the key enzymes for the production of NO in plants (Neill et al. 2003). However, a recent report demonstrated that land plants are lack of NOS sequences (Jeandroz et al. 2016). To investigate the origins of Cd stress-induced production of NO in tomato roots, we carried out pharmaceutical experiments. The Cd stress-induced accumulation of NO in both AC and *Cnr* was significantly inhibited by the NR inhibitor, tungstate, to the levels similar to that in AC without Cd treatment, whereas the NOS-like enzyme inhibitor, ʟ-NAME, failed to inhibit Cd-induced NO production (Fig. 5a, b). For comparisons, addition of the NO scavenger cPTIO also effectively abolished Cd-induced NO production in both AC and *Cnr* roots (Fig. 5a, b). We also observed the inhibitory effects of other NR inhibitors, NH_4^+
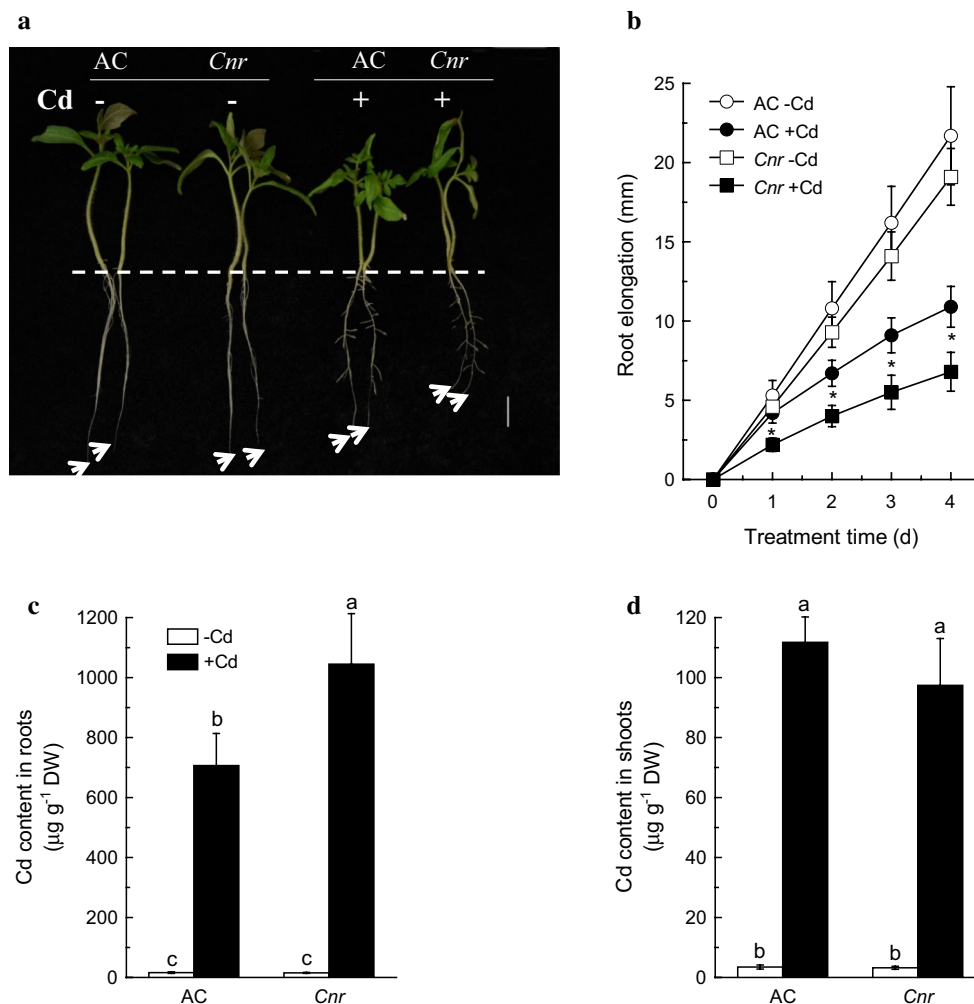

Fig. 2 Differential *LeSPL-CNR* expression levels between AC and *Cnr* in response to Cd stress. Four-day-old seedlings of AC and *Cnr* were treated with 0- or 10-μM CdCl₂ for 1 day. Roots were collected after treatment for RNA extraction and qRT-PCR analysis. *18S rRNA*, *Actin*, or *GAPDH* was used as internal control of gene expression. Data were normalized as relative expression to that of AC plants without Cd treatment, which was designated as 1. Data are mean ± SD ($n = 3$ biological replicates). Different lowercases above the error bars represent significant differences (Tukey's test, $P < 0.05$)

Fig. 3 Phenotypic difference between AC and *Cnr*. **a** Representative photo of AC and *Cnr* after 4-day treatment with or without 10-μM CdCl$_2$. Dashed line indicates root–shoot junction and arrow heads represent position of root tip. **b** Root elongation assays. Four-day-old seedlings were subjected to nutrient solution containing 0- or 10-μM CdCl$_2$ for 4 days. Root elongation was measured every day with a ruler. Data are mean±SD (n=10 seedlings). Asterisk represents sig-

nificant difference between genotypes after Cd treatment (Tukey's test, $P < 0.05$). **c** Cd content in roots. **d** Cd content in shoots. After 4-day treatment, both roots and shoots were harvested, dried, and digested for Cd analysis by ICP-OES. Data are mean±SD (n=4 biological replicates). Different letters above the error bars indicate significant difference (Tukey's test, $P < 0.05$)

and Gly, on Cd-induced NO production in both AC and *Cnr* plants (Fig. S2). These results suggest that NR rather than NOS-like enzyme is involved in NO production in response to Cd stress in tomato roots. Intriguingly, in the absence of Cd stress, tungstate could significantly reduce NO production in *Cnr* mutants but not in AC plants (Fig. 5c), implying that LeSPL-CNR may regulate NO production through NR pathway irrespective of Cd stress.

Since *Cnr* mutant accumulated more NO in roots than AC, we wanted to know whether further increase in NO in AC plants or decrease in NO in *Cnr* mutants will correspondingly result in increased or decreased Cd sensitivity.

Thus, we performed NO donor or NR inhibitor application experiments. Application of tungstate (20 μM) had a little inhibitory effect on *Cnr* root elongation in the absence of Cd. However, tungstate partially alleviated Cd-induced inhibition of root elongation (Fig. 6a). Likewise, treatment of AC roots with NO donors, GSNO or SNP, had no effects on root growth in the absence of Cd stress, but the root elongation was much greater inhibited in the presence of Cd (Fig. 6b; Fig. S3). These results indicate that NO production is responsible for Cd stress-induced root growth inhibition, and the increased Cd sensitivity in *Cnr* is related to increased production of NO under Cd stress.
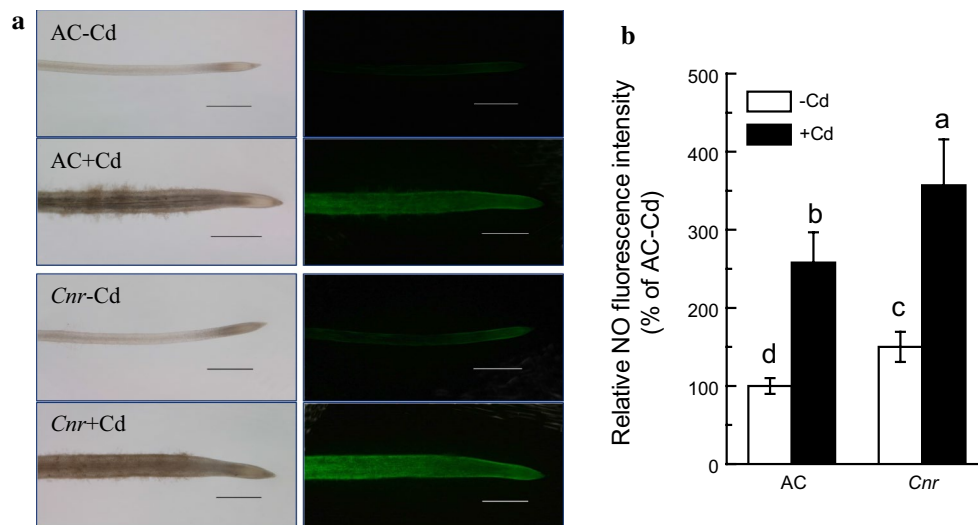
Fig. 4 Effects of Cd stress on NO accumulation in tomato roots of AC and *Cnr*. **a** Detection of endogenous NO levels. 4-day-old seedlings of AC and *Cnr* were treated with 0- or 10-μM CdCl₂ for 4 days. The NO-specific fluorescence probe DAF-FM DA was used to detect NO fluorescence intensity. At least ten seedlings were observed for each treatment and the representative photos were shown. Bar 0.5 mm. **b** Quantification of the fluorescence intensity based on Adobe Photoshop software. Data are mean ± SD ($n = 10$ seedlings). Different letters indicate significant difference (Tukey's test, $P < 0.05$)

LeSPL-CNR negatively regulates NR activity

Considering that NR inhibitors are effective in repressing NO production in the absence and presence of Cd stress, we examined whether NR activity will be regulated by Cd stress in tomato roots. In the absence of Cd, NR activity was higher in *Cnr* than in AC (Fig. 7a), which is in accordance with differential NO contents (Fig. 5). Although Cd stress induced significantly the NR activity in both, AC and *Cnr*, the activity was significantly higher in *Cnr* than AC (Fig. 7a). It is noteworthy that there was no significant difference in the relative increment of NR activity between Cnr and AC because of higher basal NR activity in *Cnr* plants.

The activity of the NR can be modulated at transcriptional, translational, and post-translational levels. To test whether *LeSPL-CNR* regulates NR activity at transcriptional level, we first identified genes coding for NR in tomato. In *Arabidopsis*, two genes, *NIA1* (At1g77760) and *NIA2* (At1g37130), encode NR. Orthologous gene search (https://phytozome.jgi.doe.gov/pz/portal.html) indicated that Solyc11g013810 is the strongest candidate encoding NR (named as SlNR hereafter). We next investigated the effect of Cd stress on *SlNR* gene expression. After treatment with Cd for 4 days, the expression of the reference gene was constant (Fig. S4). The expression level of *SlNR* was more abundant in *Cnr* than AC in the absence of Cd stress. Although Cd stress increased the transcript abundance of *SlNR* in both AC and *Cnr*, the level was more abundant in *Cnr* than that in AC after Cd stress (Fig. 7b). The expression pattern of *SlNR* was in well accordance with NR activity, implying that LeSPL-CNR could regulate NR activity at transcriptional levels.

LeSPL-CNR directly binds to *SlNR* promoter in yeast

To determine if LeSPL-CNR could interact with the promoter of *SlNR*, we performed yeast one-hybrid assays. It has been reported that SPL proteins bind to the GTAC core motif in their target genes (Birkenbihl et al. 2005; Yamasaki et al. 2009). There are 4 GTAC motifs in the 2-kb sequence of *SlNR* promoter (Fig. 8a; Fig. S5). We dissected the promoter into seven fragments, A1 (− 2000 to − 1679), A2 (− 1719 to − 1470), A3 (− 1416 to − 1070), A4 (− 1075 to − 753), A5 (− 770 to − 422), A6 (− 543 to − 223), and A7 (− 237 to + 13) (Fig. 8a). Interestingly, we found that LeSPL-CNR interacts strongly with A2 that has no GTAC motif, and much weakly interacts with A7 that has a GTAC motif (Fig. 8b).

LeSPL-CNR represses *SlNR*

The negative correlation of transcript abundance between *LeSPL-CNR* and *SlNR* as well as the interaction between LeSPL-CNR and the promoter of *SlNR* suggest that LeSPL-CNR functions as a repressor of *SlNR* expression. Phylogenetic analysis showed that tomato LeSPL-CNR is homologous to AtSPL3 in *Arabidopsis* (Fig. S6). They contain the conserved SBP domain encompassing two putative zinc-finger DNA-binding sites, and have less similar N-terminal region (Fig. 9a). To examine

Fig. 5 Nitrate reductase-mediated NO production under Cd stress. **a** Detection of endogenous NO-level change in AC roots. **b** Detection of endogenous NO-level change in *Cnr* roots. 4-day-old seedlings of AC and *Cnr* were treated with 0- or 10-μM CdCl$_2$ for 4 days. The NO-specific fluorescence probe DAF-2 DA was used to detect NO fluorescence intensity. At least ten seedlings were observed for each treatment and the representative photos were shown. Bar 0.5 mm. **c** Quantification of the fluorescence intensity based on Adobe Photoshop software. Data are means of ten roots $\pm$ SD. Uppercase and lowercase above the bars indicate significant differences among treatments in AC and Cnr, respectively (Tukey's test, $P < 0.05$). Asterisks indicate significant difference between genotypes with in a treatment (one-way ANOVA test, $P < 0.05$)
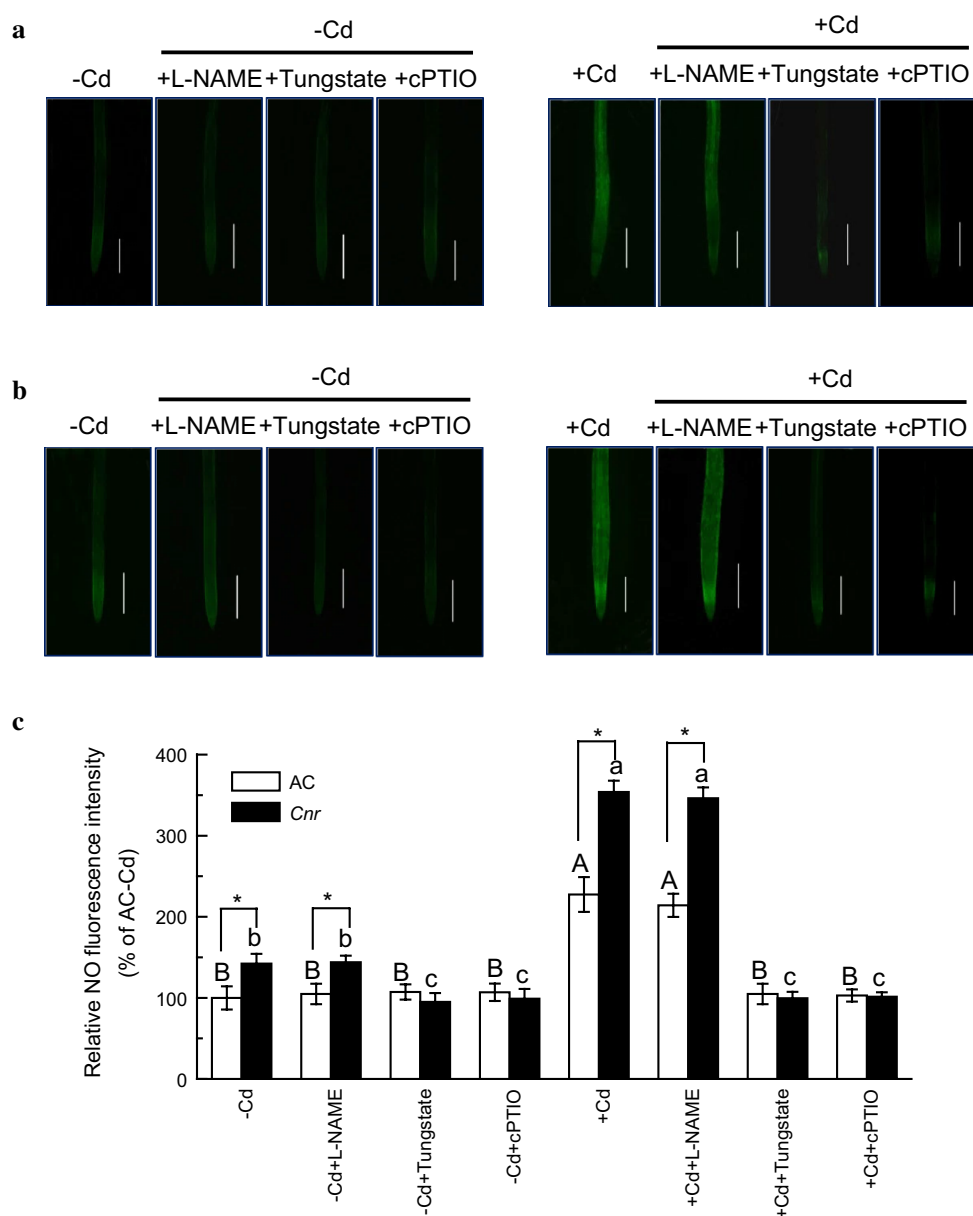
how LeSPL-CNR regulates *SlNR* transcription, we first analyzed transactivation potential of LeSPL-CNR in a yeast expression system. Yeast strain AH109 transformed with the fusion plasmid pGBKT7/LeSPL-CNR [refer to I full (1–136 aa)] grew much worse than yeast cells transformed with the empty control pGBKT7 vector [refer to VI pGBKT7 (control)] in SD medium even in the presence of His (Fig. 9b). In the growth medium-lacking His, all yeast strains could not grow (Fig. 9b). These results suggest that LeSPL-CNR is lack of transactivation potential in yeast; instead, it may act as a transcriptional repressor. We then separated the LeSPL-CNR protein into NTR (1–48 aa) and SBP-box + CTR regions (49–136 aa). Yeast cells containing NTR region grew as well as control in the medium with His, whereas the growth of yeast cells containing

SBP-box + CTR region was greatly reduced similar to those containing LeSPL-CNR full protein (Fig. 9b).

To investigate how LeSPL-CNR regulates *SlNR* transcription, we cloned *SlNR* promoter into the LUC reporter vector and LeSPL-CNR into pCambia2300 under the control of CaMV35S promoter. The pCambia2300 vector was used as the vector control (Fig. 9c). When co-transformed with vector control, *SlNR* promoter could drive the expression of LUC. However, the promoter activity was drastically repressed when LUC reporter vector co-transformed with *LeSPL-CNR*, suggesting that LeSPL-CNR inhibits *SlNR* promoter activity (Fig. 9d).

To further confirm that LeSPL-CNR negatively regulates *SlNR* transcription by binding to A2 region of its promoter, we carried out comparative transient assays
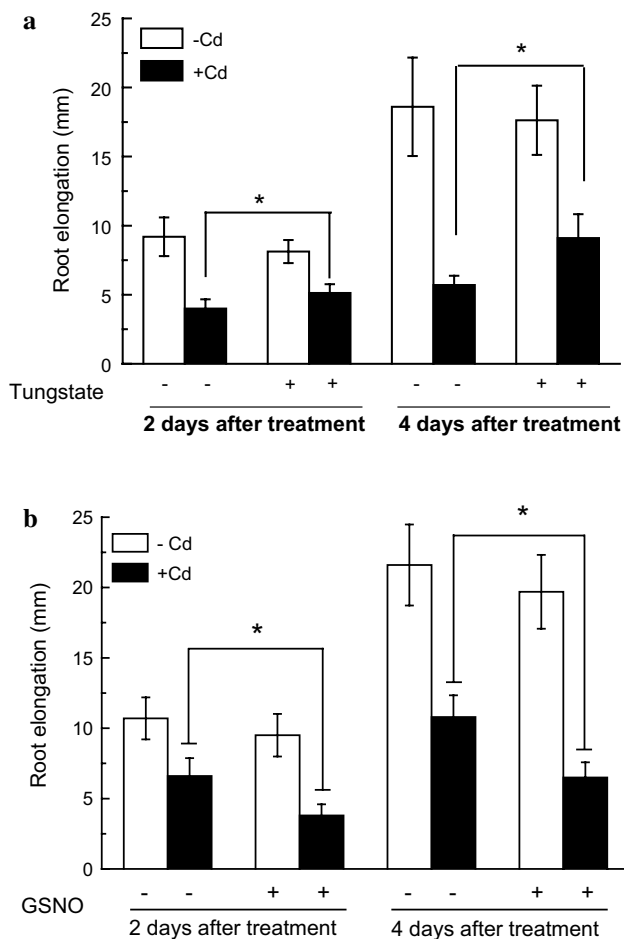
Fig. 6 Effects of tungstate or NO donor on Cd-induced root elongation inhibition. **a** Four-day-old seedlings of *Cnr* mutants were treated with nutrient solution containing 0- or 10-μM $CdCl_2$ in the absence or presence of 20-μM tungstate for 2 or 4 days. **b** Four-day-old seedlings of AC plants were treated with nutrient solution containing 0- or 10-μM $CdCl_2$ in the absence or presence of 50-μM GSNO for 2 or 4 days. Root elongation was measured with a ruler. Data are mean ± SD ($n = 10$ seedlings). Asterisk represents significant difference between treatments (one-way ANOVA, $P < 0.05$)

in leaves of *N. benthamiana*. The reporter green fluorescence protein (GFP) expression was driven by either − 2000 bp ($SlNR_{Pro-2000bp}$:GFP) or − 1406 bp ($SlNR_{Pro-1406bp}$:GFP) sequence length of *SlNR* promoter. In the absence of LeSPL-CNR, there were no discernable differences in the GFP signals between $SlNR_{Pro-2000bp}$:GFP and $SlNR_{Pro-1406bp}$:GFP. Interestingly, the reporter fluorescence signal of $SlNR_{Pro-2000bp}$:GFP was markedly repressed by LeSPL-CNR protein, whereas that of $SlNR_{Pro-1406bp}$:GFP was not (Fig. 10). This result indicates that LeSPL-CNR binds to the A2 region of *SlNR* promoter, which suppresses its transcription.

Increased Cd uptake is mediated by an iron-uptake system

The finding that LeSPL-CNR regulates Cd tolerance by negatively affecting NO production led us to wonder whether Cd accumulation is related to iron (Fe) uptake, because NO has been well documented to positively regulate Fe deficiency responses in plants (Chen et al. 2010a). The Fe content in *Cnr* roots was more abundant than AC roots particularly under Cd stress (Fig. 11a). While Cd stress slightly reduced Fe accumulation in AC roots, it induced Fe accumulation in *Cnr* roots (Fig. 11a). We further investigated the expression of an *IRON-REGULATED TRANSPORTER1* (*SlIRT1*) that contributes to Fe uptake. Consistent with Fe content, the expression of *SlIRT1* was constitutively higher in *Cnr* roots than AC roots (Fig. 11b). These results suggest that the increased Cd accumulation in *Cnr* roots is due, at least in part, to the up-regulation of *SlIRT1*.

Discussion

In this study, we demonstrated that an SBP-box TF, LeSPL-CNR, is involved in Cd tolerance in tomato. Previously, LeSPL-CNR was found to play a central role in tomato fleshy fruit ripening (Manning et al. 2006; Chen et al. 2015b). Recent evidence suggests that SPL box proteins are engaged in plant mineral nutrition such as Cu homeostasis and P deficiency responses in *Arabidopsis* (Preston and Hileman 2013). Phylogenetic analysis from representative seed plants suggests that SPL proteins can be grouped into nine clades (Preston and Hileman 2013). LeSPL-CNR belongs to the clade VI of SPL proteins, in which only AtSPL3 has recently been reported to function in P deficiency responses (Lei et al. 2015). The expression level of *LeSPL-CNR* was significantly repressed by Cd stress (Figs. 1, 2). Furthermore, *Cnr* displayed increased sensitivity to Cd stress in comparison with AC plants (Fig. 3). In addition, *Cnr* roots accumulated significantly more Cd than AC roots (Fig. 3c). These results suggest that LeSPL-CNR is positively involved in Cd tolerance, though *LeSPL-CNR* transcription is repressed by Cd stress.

Nitric oxide is a gaseous cell-signaling molecule that plays important roles not only in growth and development, but also in the regulation of multiple responses to biotic and abiotic stresses (Lamattina et al. 2003; Neill et al. 2003). While the involvement of NO in response to either metal toxicity or mineral element deficiency has been extensively reported, the underlying basis of NO production remains ambiguous. In the present study, we revealed that NO production is negatively regulated by LeSPL-CNR in response to Cd stress in tomato roots. Cd stress induced NO production in roots of both AC and *Cnr* plants, but *Cnr* plants
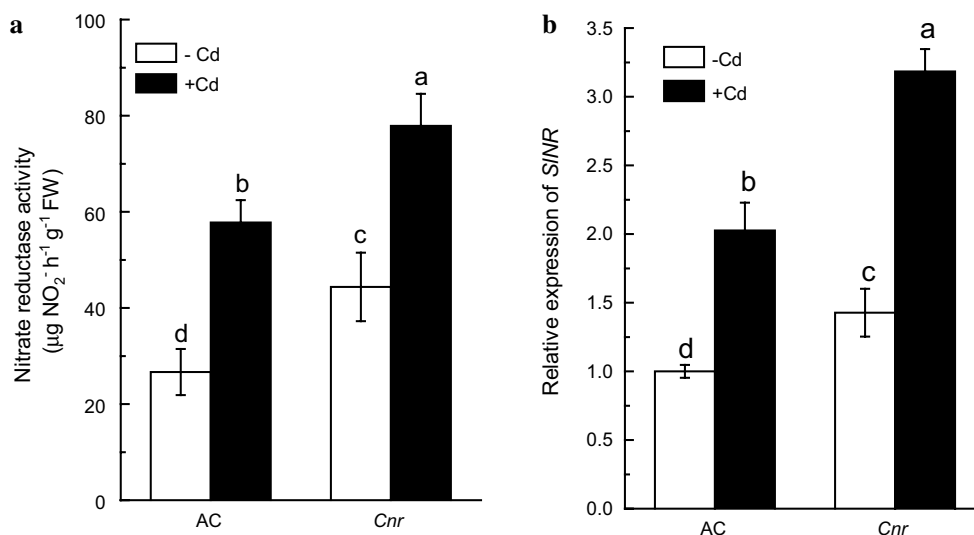
Fig. 7 Effects of Cd stress on NR activity and *SlNR* transcript abundance. **a** NR activity. 4-day-old seedlings of AC and *Cnr* were treated with 0- or 10-μM CdCl$_2$ for 4 days. Data are mean ± SD ($n = 4$ biological replicates). **b** qRT-PCR analysis of *SlNR* transcript abundance. 4-day-old seedlings of AC and *Cnr* were treated with 0- or 10-μM CdCl$_2$ for 4 days. *18S rRNA* was used as internal control of gene expression. Data are expressed as relative levels to that of AC without Cd stress. Data shown are mean ± SD ($n = 3$ biological replicates). Different letters indicate significant difference (Tukey's test, $P < 0.05$)
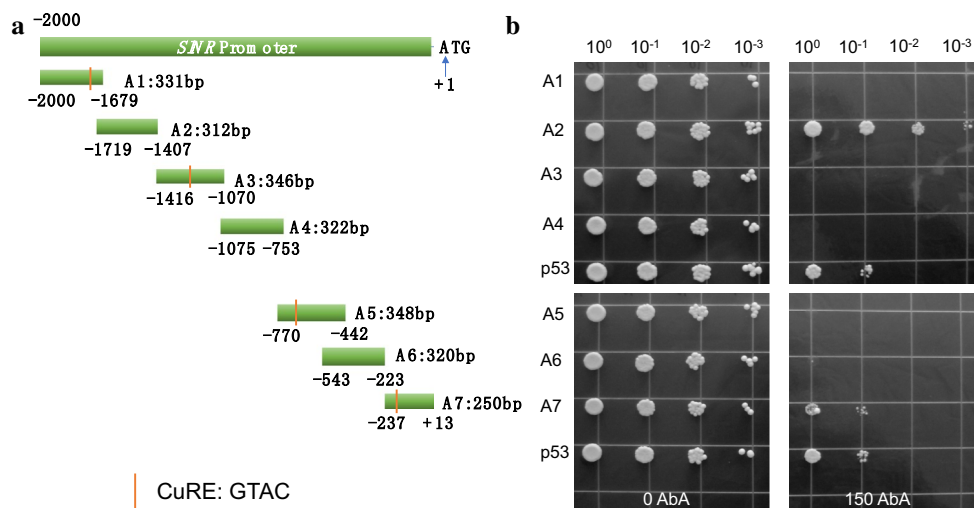

Fig. 8 Yeast one-hybrid analysis of transcriptional regulation of SlNR by LeSPL-CNR. **a** Schematic diagram of the bait fragments (A1–A7) used to construct the reporter vectors in the yeast one-hybrid assay. The vertical red bar indicates the site of canonical GTAC SBP proteins *cis*-acting motif. **b** Yeast one-hybrid assay. A pair of plasmids, pAbAi-ProSlNR (containing the Aureobasidin A (AbA) resistance reporter gene driven by the SlNR promoter regions A1–A7) and pGAD-LeSPL-CNR (a fusion of the yeast transcription factor GAL4 activation domain and LeSPL-CNR), were introduced into yeast strain Y1HGold and cultured on SD medium without Ura contain 0- or 150-ng mL^{-1} AbA at 30 °C for 3 days. p53 was used as a positive control (a yeast strain containing pGADT7-p53 and p53-AbAi plasmids)

accumulated more NO than AC either in the presence or absence of Cd stress (Fig. 4), which is in accordance with their differential *LeSPL-CNR* expression levels in response to Cd stress (Fig. 2). Moreover, we revealed that LeSPL-CNR directly binds to the promoter of *SlNR* (Fig. 8), thereby repressing its expression and NR activity (Figs. 9, 10). It

is worth to note that LeSPL-CNR acts as a transcriptional repressor of *SlNR* transcription in regulating tomato Cd tolerance. Supporting the notion are findings showing the negative expression relationship between *LeSPL-CNR* and *SlNR* under Cd stress (Figs. 2, 7). Compelling evidence also comes from *Cnr* mutant plants in which the repressed *LeSPL-CNR*
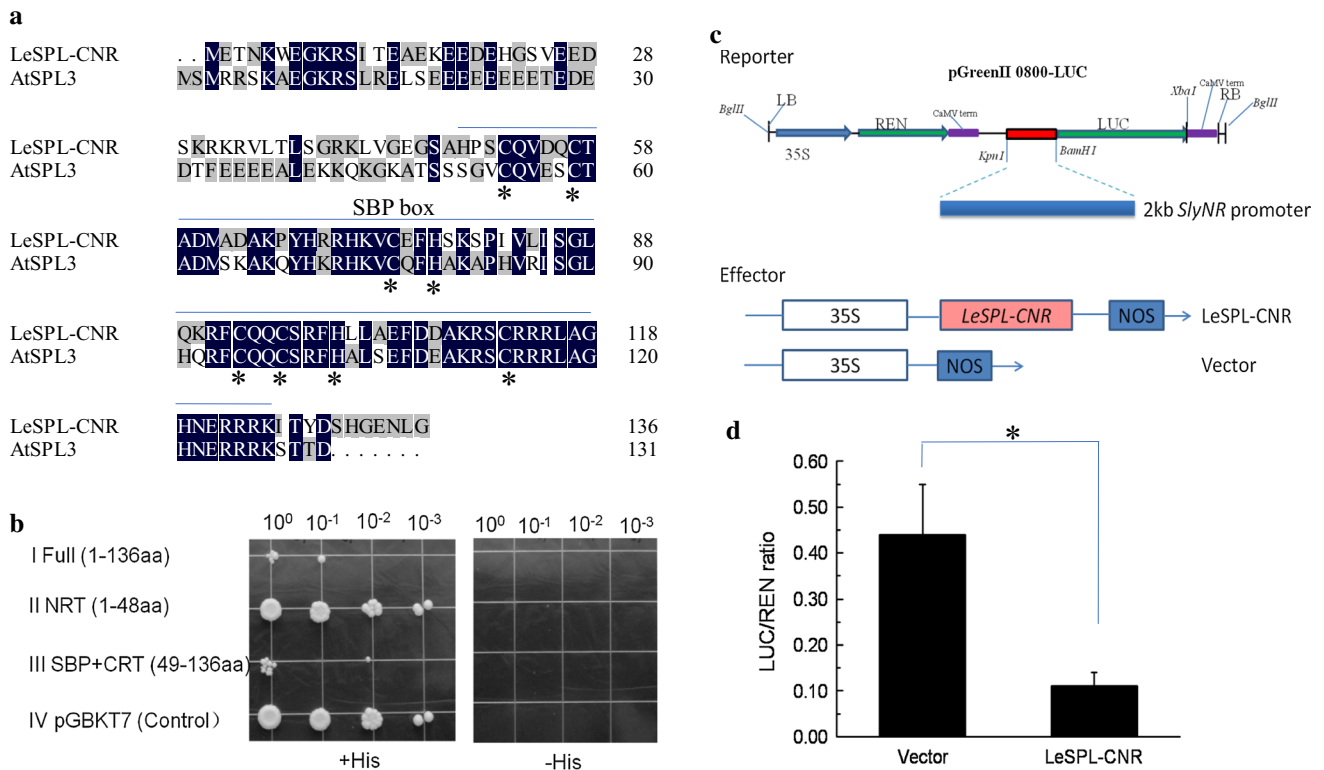
Fig. 9 Transcriptional repression analysis. **a** Alignment of amino acid sequences between LeSPL-CNR and Arabidopsis AtSPL3. The conserved SBP-box domain is shown and putative zinc-binding sites are marked with asterisks. **b** Transactivation potential assay in yeast. Yeast strain AH109 carrying fusion gene of GAL4 DNA-binding domain and different fragments of LeSPL-CNR or DB_{GAL4} alone as control were cultured on SD medium with or without His at 30 °C

for 3 days. **c** Schematic diagram of reporter plasmid and effector plasmid transformed into *N. benthamiana* leaves. **d** Relative LUC activity. *LUC* firefly luciferase activity, *REN* renilla luciferase activity (as transfection efficiency control). Data shown are ratios of LUC to REN. Data are mean ± SD ($n = 3$ biological replicates). Asterisk in **d** represents significant difference between treatments (one-way ANOVA test, $P < 0.05$)

transcript abundance is associated with increased *SlNR* expression levels (Figs. 2, 7). Furthermore, LeSPL-CNR inhibited *SlNR* promoter activity (Figs. 9, 10). SPL proteins have been previously reported to act as a transcriptional activator. For example, Kim et al. (2012) reported that AtSPL3 protein directly binds to GTAC motifs of the *FLOWERING LOCUS T* promoter, thereby participating in flowering. AtSPL3 has also been reported to target directly the GTAC motifs in the *PLDZ2*, *Pht1;5*, and *miR399f* promoters (Lei et al. 2015). This is, however, the first report that LeSPL-CNR acts as a transcriptional repressor of *SlNR* transcription under Cd stress in tomato.

Unexpectedly, the binding site of LeSPL-CNR in the promoter of *SlNR* is not limited to regions that contain typical GTAC core motif. Our yeast one-hybrid assay demonstrated that LeSPL-CNR binds not only to A2 (-1719 to -1470) but also to A7 (-237 to $+13$) of *SlNR* promoter (Fig. 8). LeSPL-CNR contains highly conserved DNA-binding SBP domain and two Zn^{2+}-binding sites (Fig. 9a), which are characteristics of SPL proteins (Yamasaki et al. 2004). Transcription repressors can be grouped into either passive or

active repressors (Mitsuda and Ohme-Takagi 2009). While passive repressors have neither an activation domain nor a repression domain, the active repressors have repression domains that confer repressive activity to the TF. Thus, there are two possibilities to explain the present result that LeSPL-CNR repressed *SlNR* expression. One is that the binding of LeSPL-CNR to the *SlNR* promoter directly represses its transcription, and the other is that the binding of LeSPL-CNR indirectly represses *SlNR* expression by competing with other transcription activators that bind to the same *cis*-elements. Since LeSPL-CNR binds preferentially to A2 region without GTAC core motif (Fig. 8), it is much likely that LeSPL-CNR competes with unknown transcriptional activator, which in turn represses *SlNR* expression. Although the underlying mechanism requires future investigation, a similar scenario has been reported in AtSPL9 which suppresses *DIHYDROFLAVONOL REDUCTASE* expression by interfering with the integrity of a MYB-bHLH-WD40 transcriptional-activation complex (Guo et al. 2011).

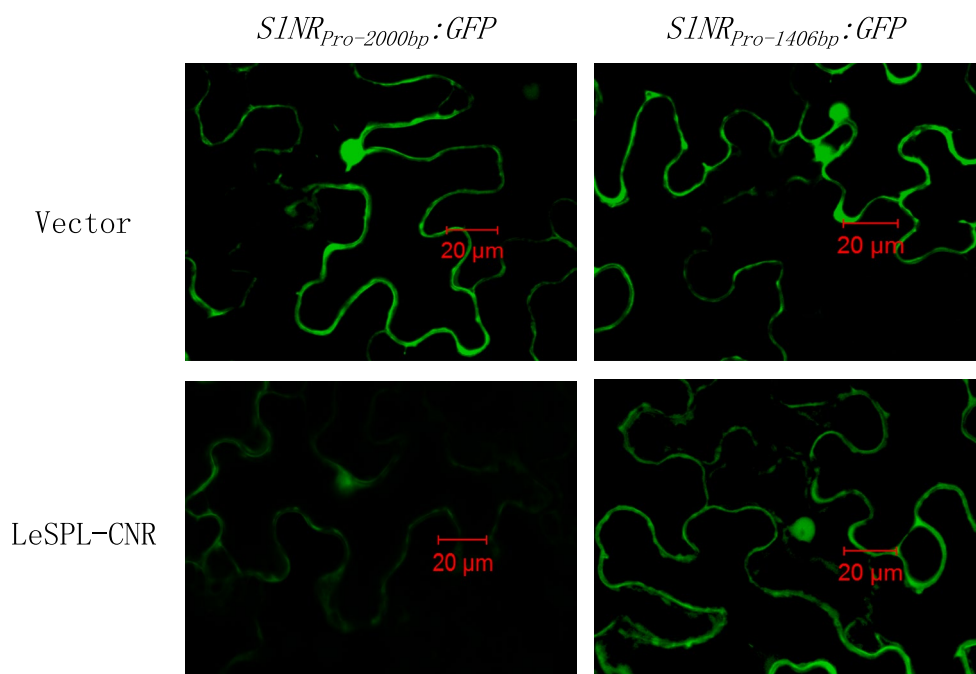We found that NO accumulation contributes to Cd toxicity in tomato. This notion is evidenced by the finding that

$SlNR_{Pro-2000bp}{:}GFP$ $SlNR_{Pro-1406bp}{:}GFP$

Vector

LeSPL-CNR

Fig. 10 Effects of interaction between *SlNR* promoter and LeSPL-CNR on reporter (GFP) activity in *N. benthamiana* leaves. A pair of plasmids, SlNR promoter reporter plasmids, and the effector plasmids (vector or 35S:LeSPL-CNR) were transformed into *N. benthamiana* leaves. SlNR$_{Pro-2000bp}$:GFP and SlNR$_{Pro-1406bp}$:GFP represent reporter plasmid containing *GFP* driven by −2000- or −1406-bp length of *SlNR* promoter, respectively. Bar 20 μm
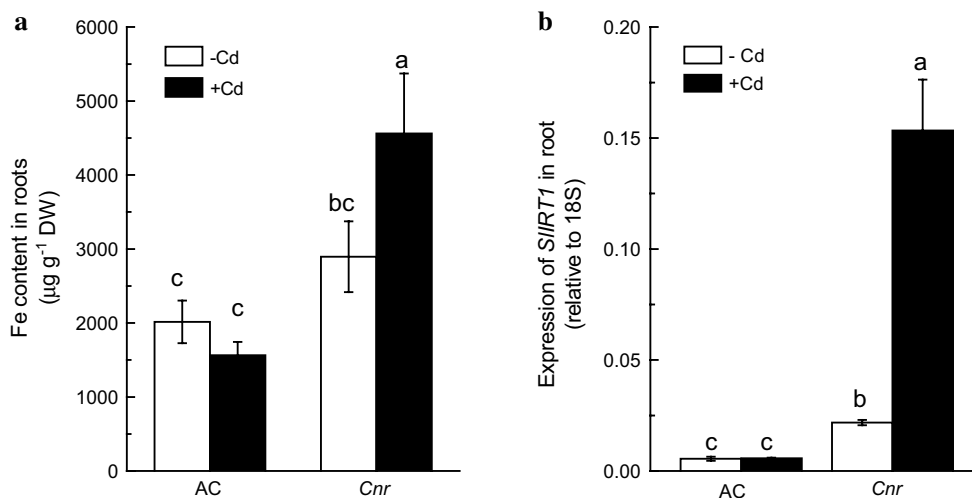

Fig. 11 Effects of Cd stress on Fe accumulation and *SlIRT1* expression. **a** Fe content. Data are mean ± SD (*n* = 5 biological replicates). **b** qRT-PCR analysis of *SlIRT1* expression. Four-day-old seedlings of AC and *Cnr* were treated with 0- or 10-μM CdCl$_2$ for 4 days. *18S rRNA* was used as internal control of gene expression. Data are mean ± SD (*n* = 3 biological replicates). Different letters indicate significant difference (Tukey's test, $P < 0.05$)

exogenous application of GSNO aggravated, but tungstate alleviated Cd-induced root growth inhibition (Fig. 6). The more compelling evidence comes from the comparative analysis of Cd sensitivity between AC and *Cnr* plants where they displayed differential endogenous NO content (Figs. 4, 5). While a growing body of evidence indicates that Cd

stress induced NO production in a number of plant species, it is a matter of debate whether NO is positively or negatively involved in Cd tolerance. For example, NO has been reported to be involved in either reducing Cd accumulation or activating the enzymatic antioxidant system, therefore enhancing Cd tolerance in rice (Xiong et al. 2010). On the

contrary, elevated NO production was reported to be associated with Cd toxicity in *Arabidopsis* (Besson-Bard et al. 2009; Yuan and Huang 2016), barley (Valentovičová et al. 2010), and wheat (Groppa et al. 2008). Such discrepancy in terms of the role of NO in plant abiotic stress responses is not limited to Cd. For example, both positive and negative regulation of NO in plants response to Al toxicity has also been reported (Zhou et al. 2012; Sun et al. 2014). While the discrepancy may be attributable to different NO concentrations and plant species, the exogenous application of NO may not be suitable for manipulating internal NO concentrations. Here, the finding that LeSPL-CNR regulates NO production provides us many opportunities to reassess the role of NO in plant biotic and abiotic stress responses.

Despite accumulating evidence suggesting the importance of NO in response to Cd stress in plants, the origin of Cd-induced NO remains poorly understood, because there are generally two enzymatic pathways, NOS-like enzyme and NR, accounted for NO production (Lamattina et al. 2003; Neill et al. 2003; Graziano and Lamattina 2007). For instance, Xu et al. (2010) reported that both NOS-like enzyme and NR pathways are responsible for Cd-induced NO production in *Medicago truncatula* seedlings. However, in Arabidopsis, Cd-induced NO production can be reversed by the inhibitors of NOS-like enzyme, which does not involve NR (Besson-Bard et al. 2009). In this study, we found that NR rather than NOS is the major enzyme involved in Cd-induced NO production in tomato roots, as evidenced by the inhibitory effects of NR inhibitors in Cd-induced NO production (Fig. 5; Fig. S2). In accordance with our result, Kan et al. (2016) found that NR-mediated NO production is the major route in *Panax notoginseng* roots under Cd stress. Recent report that land plants are lack of NOS sequences also suggests that higher plants have developed the efficient production of NO from NR as the main strategy of survival (Jeandroz et al. 2016).

Cadmium can enter plant roots as cationic Cd^{2+} through Fe, Zn, and Ca transporters/channels (Clemens 2006). The involvement of NO in regulating Fe uptake through transcriptional regulation of Fe uptake system in Strategy I plant species has been well documented (Chen et al. 2010a; Jin et al. 2011). Here, the finding that LeSPL-CNR negatively regulates NO production under Cd stress suggests that increased Cd accumulation and sensitivity in *Cnr* mutant is attributed to Fe uptake system. This was supported by the finding that the expression of *SlIRT1* was significantly higher in *Cnr* roots than AC roots (Fig. 11b), which was in accordance with higher Fe accumulation in *Cnr* roots (Fig. 11a). However, in AC roots, Cd stress had no significant effects on both *SlIRT1* expression and Fe accumulation (Fig. 11), although it induced NO accumulation (Figs. 4, 5). This can be explained by the complicated interactive effects between Cd stress and Fe homeostasis. Actually, the previous studies provided conflicting results concerning the effects of Cd stress on *IRT1* expression and Fe content in roots. In line with our supposition, Besson-Bard et al. (2009) reported that Cd-induced root growth inhibition is attributed to NO-mediated up-regulation of *IRT1* expression in *Arabidopsis*.

In summary, we demonstrated that an SPL TF, LeSPL-CNR, is involved in NO metabolism through regulation of *SlNR* expression. NO seems to be critical for triggering Fe uptake system, namely *SlIRT1*, which facilitates uptake of Cd (Fig. S7). Characterization of LeSPL-CNR in negatively regulating NO production also provides a new route to unravel the role of NO in response to Cd stress.

Compliance with ethical standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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