***RS_CRZ1*, a C2H2-Type Transcription Factor Is Required for Pathogenesis of *Rhizoctonia solani* AG1-IA in Tomato**

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Rhizoctonia solani is a necrotrophic fungal pathogen that causes disease in diverse plant species. In recent years, the genomic and transcriptomic studies have identified several candidate pathogenicity determinants of *R. solani*; however, most of them remain to be validated. In this study, we report a viral vector-based host-induced gene silencing (HIGS) as well as a dsRNA (double-stranded RNA)-based approach to effectively downregulate genes of *R. solani* AG1-IA (BRS1 strain) during pathogenesis in tomato. We tested a few of the in-planta upregulated *R. solani* genes and observed that silencing of one of them, i.e., *RS_CRZ1* (a C2H2 type zinc finger transcription factor) significantly compromises the pathogenesis of *R. solani* in tomato. The *RS_CRZ1*-silenced plants not only exhibited significant reduction in disease symptoms, but the depth of pathogen colonization was also compromised. Furthermore, we identified the *R. solani* genes that were coregulated with *RS_CRZ1* during the pathogenicity process. The HIGS-mediated silencing of a few of them [CL1756Contig1; subtilisin-like protease and CL1817Contig2; 2OG-Fe(II) oxygenase] compromised the pathogenesis of *R. solani* in tomato. The ectopic expression of *RS_CRZ1* complemented the *crz1* mutant of yeast and restored tolerance against various metal ion stress. Overall, our study reveals the importance of

RS_CRZ1 in managing the hostile environment encountered during host colonization. Also, it emphasizes the relevance of the HIGS and dsRNA-based gene silencing approach toward functional characterization of pathogenicity determinants of *R. solani*.

Keywords: infection cushion, metal ion stress, pathogenicity determinant, sheath blight disease, VIGS

Nucleotide sequence data for *RS_CRZ1* is available in the GenBank database under accession number MN686215.

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Rhizoctonia solani is a major phytopathogen that causes huge economic loss worldwide (Molla et al. 2020; Singh et al. 2019). It causes diseases such as sheath blight, damping off, brown patch, stem rot, and root rot in various agriculturally important crops (Yang and Li 2012). Although external application of chemical fungicides can control this disease, there are no sources of complete disease resistance known. The availability of genome and transcriptome sequence of *R. solani* AG1-IA have facilitated the discovery of its pathogenicity determinants (Ghosh et al. 2018, 2019; Zheng et al. 2013). Besides genomics, a proteomics approach has also been explored to identify the pathogenicity-associated proteins of *R. solani* (Anderson et al. 2016a and b). Previously, using interaction transcriptome analysis, we had identified certain proteases, transcription factors, ABC transporters, sugar transporters, and carbohydrate-active enzymes of *R. solani* to be upregulated during pathogenesis in rice (Ghosh et al. 2018).

The roles of transcription factors such as the C2H2 type zinc finger transcription factors in regulating the growth as well as pathogenesis of fungal pathogens have been widely reported (Cao et al. 2016; Todd et al. 2014). Among them, a calcineurin-responsive zinc finger 1 (CRZ1) transcription factor is regulated by calcium-dependent signaling (Espeso 2016). Under normal conditions, CRZ1 remains inactive and is present in the cytosol in a phosphorylated state. However, upon accumulation of intracellular Ca^{2+} ions, it gets dephosphorylated by calcineurin (Ca^{2+} calmodulin-dependent serine/threonine phosphatase) at certain serine and tyrosine residues. Subsequently, the dephosphorylated (active) CRZ1 gets translocated into the nucleus and regulates the expression of various target genes (Chow et al. 2017; Thewes 2014). Various studies have highlighted the multifaceted role of CRZ1 in regulating cellular activities, such as cell-wall biogenesis, ion homeostasis, metabolic reprogramming, vesicle trafficking, stress response, and more, in fungal pathogens (Thewes 2014). Disruption of a CRZ1 ortholog, *BcCRZ1*, in *Botrytis cinerea*, imparted abnormal hyphal morphology, retarded sclerotial formation, and reduced penetration in the host (bean) plants (Schumacher et al. 2008). Similarly, the mutants of CRZ1 orthologs of *Magnaporthe oryzae* (*MoCRZ1*) and *Fusarium graminearum* (*FgCRZ1a*)

exhibited reduced conidiation as well as pathogenesis in their respective hosts (rice and wheat) (Chen et al. 2019; Choi et al. 2009).

Due to lack of a standardized genetic transformation methodology, characterization of pathogenicity-associated genes of *R. solani* remains a challenge. The multinucleated (two to five nuclei in a cell) and filamentous nature of *R. solani* further add complexity in genetic manipulation studies. Hence, there is an urgent need to develop robust assays to functionally characterize the candidate pathogenicity determinants of *R. solani*. Host-induced gene silencing (HIGS) has been widely used to validate the function of pathogen genes during the infection process in fungi, oomycetes, nematodes and insects (Govindarajulu et al. 2015; Huang et al. 2006; Koch et al. 2016; Mao et al. 2007; Song and Thomma 2018; Zhu et al. 2017). In this approach, RNA interference (RNAi)-mediated down-regulation of a targeted pathogen gene is carried out by a host-derived pathogen gene-specific small interfering RNAs (siRNAs) that get translocated into the pathogen (Chen et al. 2016b; Nowara et al. 2010; Panwar et al. 2018). Recently, RNAi-mediated silencing of some of the essential genes (such as cohesin complex subunit Psm1, importin beta-1 subunit, ubiquitin E3 ligase, and RNA polymerase) of *R. solani* have been shown to reduce its pathogenesis in transgenic tall fescue grass (Zhou et al. 2016). Similarly, transgenic rice expressing RNAi against the pathogenicity-associated mitogen-activated protein kinase 1 homologs of *R. solani* (*RPMK1-1* and *RPMK1-2*) exhibited reduced disease symptoms upon *R. solani* infection (Tiwari et al. 2017). However, as the generation of transgenic RNAi lines is a tedious and time-consuming process, it is not amicable for large-scale functional screening of pathogenicity determinants. The viral vector-mediated HIGS approach has been found to be a robust, quick, and efficient method for silencing pathogen genes during host colonization (Hua et al. 2018). In this approach, a viral-based vector (such as tobacco rattle virus [TRV]) is used to generate siRNA against a pathogen gene, using host machinery to knock-down and study its functions during the infection process (Panwar et al. 2013; Zhang et al. 2016; Zhu et al. 2014, 2017).

In the present study, we have established tomato as a model host to characterize the pathogenicity determinants of *R. solani*. Using a TRV-based HIGS approach; we have successfully knocked down some of the *R. solani* genes during pathogenesis in tomato. Among various genes (*RS_CRZ1*, *RS_GH16*, and *RS_TF3*) analyzed during the study, the silencing of *RS_CRZ1* significantly compromised the pathogenesis of *R. solani*. We further identified several *RS_CRZ1* coregulated genes and observed that silencing of a few of them could also compromise its pathogenesis. Overall, data presented in this study highlights the importance of *RS_CRZ1* in regulating the expression of pathogenicity determinants of *R. solani* and protecting the pathogen from various metal ion stresses encountered during plant colonization.

RESULTS

Tomato as a model system to characterize pathogenicity determinants of *R. solani*.

R. solani AG1-IA not only infects rice but also causes disease in several other important crop plants, including members of family Solanaceae (Yang and Li 2012). We observed that, similar to the pathogenesis in rice, the *R. solani* AG1-IA strain BRS1 produces infection cushions at the site of infection in tomato (Supplementary Fig. S1). Also, enhanced accumulation of reactive oxygen species (ROS) and cell death at the site of disease lesions were observed in rice as well as tomato. Generally, a transient gene silencing approach (viral vector-based)

does not work very efficiently in rice, while it has been extensively used for functional studies in tomato (Kant et al. 2019; Ramegowda et al. 2014; Scofield and Nelson 2009). Considering this, we selected tomato as a suitable host to transiently silence and study the function of candidate pathogenicity-associated genes of *R. solani*.

Previously, we have predicted certain pathogenicity determinants of *R. solani* that are upregulated during establishment or the necrotrophic phase of sheath blight disease in rice (Ghosh et al. 2018). We randomly selected a few of them, including a zinc finger C2H2 type (*RS_CRZ1* [calcineurin-regulated zinc finger 1]) transcription factor, a glycoside hydrolase 16 domain-containing gene (*RS_GH16*), and a GAL4-like Zn₂Cys₆ domain-containing transcription factor (*RS_TF3*) to study their involvement during pathogenesis of *R. solani*. The quantitative reverse transcription PCR (qRT-PCR) analysis reflected that, similar to pathogenesis in rice, the *RS_CRZ1* and *RS_TF3* genes get induced during the establishment phase (12, 24, and 48 h postinoculation [hpi]), while *RS_GH16* gets upregulated during the necrotrophic phase (72 hpi) of *R. solani* pathogenesis in tomato (Supplementary Fig. S2).

Silencing of candidate pathogenicity determinants in *R. solani*.

Using TRV vectors, we attempted to silence the *PDS* (phytoene desaturase) gene of tomato and observed a characteristic photo-bleaching phenotype in the emerging leaves at 15 to 20 days postinoculation (dpi) (Supplementary Fig. S3). Further, we analyzed whether the TRV-based HIGS approach can be used to effectively silence the *R. solani* genes during its pathogenesis in tomato. For this, a unique gene fragment of each of the target genes (*RS_CRZ1*, *RS_GH16*, and *RS_TF3*) were selected to avoid off-target silencing (Supplementary Fig. S4). The selected fragments were cloned into pTRV2 and the recombinant vector (pTRV:*RS_CRZ1*, pTRV:*RS_GH16*, pTRV:*RS_TF3*, pTRV:*RS_GT34* and pTRV:0) along with pTRV1 and were agroinfiltrated in tomato seedlings, to silence the respective genes (Supplementary Fig. S4). Subsequently, these plants were infected with *R. solani* and disease progression was monitored. The *RS_CRZ1*, *RS_GH16*, and *RS_TF3* silenced lines showed reduction in disease symptoms, as compared with the vector control as well as the wild-type tomato (Fig. 1A). However, the disease reduction was most drastic in *RS_CRZ1*-silenced plants, wherein a majority of the infected leaves (approximately 90%) showed minimal infection and only a few leaves had merged lesions (approximately 10%). On the other hand, the *RS_GH16*- and *RS_TF3*-silenced plants had a majority of the infected leaves (70 to 80%) with merged lesions (Fig. 1B). Similarly, the lesion area of infected leaves was significantly lower in *RS_CRZ1*-silenced plants compared with the vector control plants (Supplementary Fig. S5). The semi-quantitative PCR analysis reflected downregulation of target genes in the respective silenced plants, as compared with the vector control (pTRV:0) plants (Fig. 1C to E). Overall, this suggested *RS_CRZ1* to be an important pathogenicity determinant of *R. solani*.

To rule out the possibility that HIGS may induce basal host defense, we analyzed the expression of some of the defense marker genes (*NPR1*, *PR1*, *AOS2*, and *EIN3*) in *RS_CRZ1*-silenced plants using qRT-PCR. The analysis reflected similar level of expression of these genes in both the vector control as well as *RS_CRZ1* silenced plants (Supplementary Fig. S6). Moreover, the HIGS of *R. solani* genes do not necessarily compromise its pathogenesis. We observed that silencing of *RS_GT34* (a glycosyl transferase family 34 protein), a previously reported in-plant upregulated gene of *R. solani* (Ghosh et al. 2018), did not alter its pathogenesis in tomato (Supplementary

Fig. S7). The disease symptoms, disease index, lesion area, as well as the pathogen load in the *RS_GT34*-silenced plants was similar to that of vector control plants (Supplementary Fig. S7a-d). On the other hand, silencing of *RS_CRZ1* significantly compromised the pathogenesis of *R. solani* (Supplementary Fig. S7a-d). The qRT-PCR analysis reflected efficient silencing of both the genes, using a HIGS approach (Supplementary Fig. S7e and f). Overall, this reinforced that a TRV-based HIGS approach is suitable to study the functions of pathogenicity-associated genes of *R. solani*.

Silencing of *RS_CRZ1* compromises colonization of *R. solani* in tomato.

Host penetration is a fundamental step that governs colonization and the disease establishment process of fungal pathogens (Agrios 2005). We analyzed the colonization pattern of *R. solani* in tomato, under a confocal microscope upon WGA-FITC staining (a fluorescent chitin binding dye that selectively stains fungal mycelia) of the infected leaves. The analysis revealed deep penetration of *R. solani* mycelia in the infected wild-type tomato (up to 43 μ m), while only limited penetration (up to 26 μ m) was observed in the case of *RS_CRZ1*-silenced plants (Fig. 2A). The three-dimensional (3D) topological imaging of infected leaves revealed a thick mat of mycelial growth in the wild-type (Fig. 2B) and vector-control (Fig. 2C) plants, while only scant growth of fungal mycelia was observed in the case of *RS_CRZ1*-silenced plants (Fig. 2D). Moreover, compared with the wild type (Fig. 3A) as well as the vector control (Fig. 3B), the infection cushions area was significantly less in the *RS_CRZ1*-silenced tomato plants (Fig. 3C). Taken together, the data suggested that silencing of *RS_CRZ1* compromises the ability of *R. solani* to effectively colonize and penetrate inside the host.

Conservation of *RS_CRZ1* orthologs in different fungal species.

The *RS_CRZ1* protein contains a zinc finger C2H2-type transcription factor domain (Supplementary Fig. S8a). We identified a total of 35 different zinc finger C2H2 type

transcription factors in *R. solani* AG1-IA (BRS1 strain), by analysis of its transcriptome sequence (Ghosh et al. 2018). Notably, many of them were upregulated during the establishment phase while others were induced at necrotrophic phase of *R. solani* pathogenesis in rice (Supplementary Fig. S8b).

The *RS_CRZ1* bears sequence similarity (at amino acid level) with previously characterized CRZ1 orthologs in other fungi, such as *Saccharomyces cerevisiae* (48%), *Aspergillus nidulans* (49%), and *Cryptococcus neoformans* (65%). However, high sequence similarity (80 to 90%) was observed among *RS_CRZ1* orthologs in *R. solani* strains belonging to different anastomosis groups. Further, phylogenetic analysis of *RS_CRZ1* orthologs in different fungi, revealed formation of a *R. solani*-specific clade (Supplementary Fig. S9). As phosphorylation status of CRZ1 is known to regulate its functions (Thewes 2014), we analyzed the occurrence of phosphorylation sites (SP and TP residues) in the *RS_CRZ1* protein. Notably, several of the potential phosphorylation sites (15 SP and four TP residues) were conserved among *RS_CRZ1* orthologs in different *R. solani* strains belonging to different anastomosis groups (Supplementary Fig. S10).

RS_CRZ1 coregulated pathogenicity determinants of *R. solani*.

Recently, several CRZ1-regulated genes in *C. neoformans* had been reported (Chow et al. 2017). We identified the orthologs of some of these genes in the transcriptome sequence of the BRS1 strain of *R. solani* (Ghosh et al. 2018) and observed 22 of them to be coexpressed with *RS_CRZ1* during the establishment phase of its pathogenesis in rice (Fig. 4A). These genes encoded important functions, such as metabolism, cell-wall strengthening, cell-wall degradation, protein degradation, signaling, and more. We randomly selected five of them (namely, c17182_g1_i1, CL2868Contig1, CL1756Contig1, CL3021Contig2, CL1817Contig2), and qRT-PCR analysis revealed each of them to be coregulated with *RS_CRZ1* during pathogenesis in rice (Fig. 4C). Notably, we observed the expression of these genes to be downregulated during pathogenesis

Fig. 1. Silencing of *RS_CRZ1* compromised the pathogenesis of *Rhizoctonia solani* in tomato. **A**, Disease symptoms in *R. solani*-infected leaves of *RS_CRZ1*, *RS_GH16*, and *RS_TF3* HIGS plants at 3 days postinoculation. The vector (pTRV:0 infiltrated) as well as wild-type (WT) tomato plants were used as control. **B**, Disease severity index reflecting percentage of leaves with minimal infection, merged lesions, and total necrosis. **C**, Semi-quantitative PCR showing transcript levels of respective genes in the silenced *RS_CRZ1*, **D**, *RS_GH16*, **E**, *RS_TF3*, and vector control plants. The 18S ribosomal DNA gene of *R. solani* was used as endogenous control. Graph shows mean values of three independent biological replicates.

of *R. solani* in the *RS_CRZ1*-silenced tomato plants when compared with that of vector control plants (Fig. 4D).

Further, using a yeast one-hybrid (Y1H) assay, we analyzed whether *RS_CRZ1* binds to the promoter of the coregulated genes of *R. solani*. We could amplify the promoter region of three of five *RS_CRZ1* coregulated genes (c17182_g1_i1, CL1756Contig1, and CL1817Contig2) and cloned them into a bait vector (pAbAi) to obtain recombinant pAbAi:promoter constructs. Moreover, the *RS_CRZ1* was cloned into a prey vector (pGADT7) to obtain a pGADT7:*RS_CRZ1* construct. The Y1H Gold bait reporter yeast strain expressing both the recombinant bait and prey vectors grew on aureobasidin (AbA) containing double drop out (SD-Ura-Leu [synthetic defined medium lacking uracil and leucine]) plates (Fig. 4B). On the contrary, the strain coexpressing recombinant bait (pAbAi:promoter) and empty prey (pGADT7:0) vectors failed to grow. Based upon this, we speculate them to be potential targets of *RS_CRZ1* transcription factor.

Functional characterization of selected *RS_CRZ1* coregulated genes in *R. solani*.

To understand the role of *RS_CRZ1* coregulated genes during pathogenesis of *R. solani*, we silenced two of them (CL1756Contig1 and CL1817Contig2), using a TRV-mediated HIGS approach (Fig. 5D and E). Compared with vector control plants, both silenced lines exhibited reduced disease symptoms upon *R. solani* infection (Fig. 5A and C). Also, the pathogen

load (abundance of *R. solani* biomass) was significantly less in CL1756Contig1- and CL1817Contig2-silenced plants, compared with the vector control plants (Fig. 5B). Together, these observations suggested that silencing of *RS_CRZ1* coexpressed genes (CL1756Contig1 and CL1817Contig2) effectively retards the pathogenesis of *R. solani* in tomato.

Double-stranded RNA (dsRNA)-mediated silencing of *RS_CRZ1* compromises the pathogenesis of *R. solani* in tomato.

The dsRNA-based approach to silence one or more fungal genes is emerging to be an efficient method to combat diseases in crop plants (Dubrovina and Kiselev 2019; Wang and Jin 2017). We synthesized a dsRNA that can efficiently silence *RS_CRZ1* in *R. solani* and tested whether the treatment compromises the pathogenesis of *R. solani* in tomato. Notably, significant reduction in disease symptoms was observed in tomato leaves that were infected with dsRNA-pretreated mycelia, as compared with the buffer-treated mycelia (Fig. 6A). This was further supported by the disease index (Fig. 6B) and lesion area (Fig. 6C) as well as the pathogen load (Fig. 6D) being significantly less in the dsRNA-treated leaves, compared with the buffer treated leaves. However, the dsRNA-mediated silencing of *RS_GT34* did not compromise the pathogenesis of *R. solani* in tomato. The qRT-PCR analysis revealed that dsRNA treatment effectively silences *R. solani* genes (Fig. 6E and F).

Fig. 2. The depth of host penetration is compromised in *RS_CRZ1*-silenced tomato plants. **A**, Depth of host penetration by *Rhizoctonia solani* mycelia in infected tomato leaves stained with WGA-FITC, using confocal microscopy. Each biological setup comprises mean values $\pm$ standard error of at least five infected leaf samples. Topological overview showing *R. solani* penetration in **B**, infected wild type (WT), **C**, vector control (pTRV:0 infiltrated), and **D**, *RS_CRZ1*-silenced tomato leaves. The z axis represents the depth of host penetration and is indicated by the colored index bars.

dsRNA-mediated silencing of *RS_CRZ1* confers sensitivity to metal ion stress during growth in laboratory media.

Several studies have reported that the CRZ1 transcription factor is activated upon metal ion (Na^+ , Ca^{2+} , Mn^{2+}) stress in various fungi (Cyert 2003; Karababa et al. 2006). We envisaged testing the role of *RS_CRZ1* in combating metal ion stress in *R. solani*. The dsRNA as well as buffer-treated mycelia were grown on laboratory media, with and without metal ion (Ca^{2+} , Mn^{2+} , and Na^+) stress. The qRT-PCR analysis revealed efficient silencing of *RS_CRZ1* in the dsRNA-treated *R. solani* mycelia (Fig. 7A). Further, we analyzed the accumulation of ROS in the *R. solani* mycelia upon staining with dihydrorhodamine123 (a fluorescence dye) under confocal microscope. We observed that, under control conditions (without metal ion stress), both buffer- as well as dsRNA-treated mycelia exhibited only limited ROS accumulation (Fig. 7B). On the other hand, enhanced ROS accumulation was observed in the dsRNA-treated mycelia upon Ca^{2+} (Fig. 7C), Mn^{2+} (Fig. 7D), and Na^+ (Fig. 7E) ion stress, compared with the buffer-treated (control) mycelia. The quantification of the fluorescence intensity also reflected enhanced ROS accumulation upon metal ion stress in the dsRNA-treated mycelia. Overall, this suggested *RS_CRZ1* enables *R. solani* mycelia to withstand metal ion stress.

RS_CRZ1 functionally complements *crz1* mutant in yeast.

Various reports suggest that a *crz1* mutant of yeast (*Saccharomyces cerevisiae*) is susceptible to Ca^{2+} (Dudley et al. 2005), Li^+ (Stathopoulos and Cyert 1997), and Na^+ (Huang et al. 2013) ion stress. We tested whether *RS_CRZ1* can complement the *crz1* mutant of yeast and restore its ability to combat Li^+ , Ca^{2+} , and Na^+ ion stress. As reported earlier, the *crz1* mutant was unable to grow under metal ion stress. However, ectopic expression of *RS_CRZ1* restored the ability of *crz1* mutant to grow, under metal stress, similarly to wild-type or vector control yeast cells (Fig. 8). Overall, our findings establish *RS_CRZ1* to be a bona fide ortholog of CRZ1 transcription

factor of yeast and highlights its role in combating metal ion stress.

DISCUSSION

Due to lack of a source of complete disease resistance, controlling *Rhizoctonia solani* remains a challenge for sustainable agriculture. Also, efforts to understand the ability of *R. solani* to cause disease on a wide range of plant species have practical limitations in terms of the inability to genetically transform the pathogen. The data presented in this study suggests that a TRV-based HIGS as well as a dsRNA-mediated gene silencing approach can be effectively used to study the role of *R. solani* genes during pathogenesis in tomato. We report that *R. solani* adopts similar mode of colonization in both rice and tomato. Also, the expression pattern of candidate pathogenicity-associated genes of *R. solani* was similar in both the hosts. Based upon this, we propose that tomato can be used as a model system to study functions of *R. solani* genes during host colonization.

Using a TRV-based HIGS approach, we could individually silence three of the *R. solani* genes (*RS_CRZ1*, *RS_GH16*, and *RS_TF3*) during its pathogenesis in tomato. The impact of gene silencing was reflected on disease-causing ability of *R. solani*, and, among the tested genes, silencing of *RS_CRZ1* was found to significantly reduce the disease in tomato compared with that observed in vector control plants. However, compared with wild-type plants, the vector control plants had lesser disease symptoms. We anticipate that this may be due to limited induction of host defense responses due to infiltration of agrobacterial strain GV3101 during delivery of TRV vector (Grosse-Holz et al. 2018; Sheikh et al. 2014). However, we observed that the extent of upregulation of certain defense marker genes (*NPR1*, *PR1*, *AOS2*, and *EIN3*) was comparable in the vector control as well as *RS_CRZ1* silenced plants. This reinforced that observed difference in the pathogenesis of

Fig. 3. Formation of infection cushions is reduced in *RS_CRZ1*-silenced tomato leaves. WGA-FITC staining reflects infection cushions developed during *Rhizoctonia solani* pathogenesis in **A**, wild type, **B**, vector control (pTRV:0), and **C**, *RS_CRZ1*-silenced tomato plants, observed under fluorescent microscope. The small panel on the right side of each image shows an enlarged confocal microscopic view of infected tissues. **D**, Total area covered by infection cushions in infected leaves under 2× magnification. Graph shows mean values $\pm$ standard error of three biological replicates. Different letters indicate significant difference at $P \leq 0.05$.

R. solani is predominantly due to the silencing of *RS_CRZ1*. The formation of limited infection cushions as well as reduced host penetration in *RS_CRZ1*-silenced plants further reinforced its importance during pathogenesis. It is worth mentioning that infection cushion formation and host penetration are considered to be important for pathogenesis of *R. solani* (Lakshman et al. 2012). Although *R. solani* is a necrotrophic pathogen, it can adopt a brief biotrophic phase to penetrate and establish inside the host, before entering into the necrotrophic phase (Ghosh et al. 2017). We speculate that silencing of *RS_CRZ1* may halt *R. solani* in the establishment phase of its pathogenesis.

RS_CRZ1 is a calcineurin-regulated zinc finger transcription factor (C2H2 type). Studies have shown that fungal pathogens like *Candida albicans* and *Cryptococcus neoformans* utilize CRZ1 as an important virulence factor to activate various metabolic as well as biological processes inside the cell (Chow et al. 2017; Karababa et al. 2006). We anticipate that *R. solani* may utilize *RS_CRZ1* to modulate the expression of its pathogenicity-associated genes. Indeed, various *R. solani* genes that are potentially involved in cell-wall modification, proteolysis, and various signaling events associated with pathogenesis were coregulated with *RS_CRZ1*. Interestingly, the expression of some of the coregulated genes was down-regulated in *RS_CRZ1*-silenced tomato plants. Using a Y1H assay, we demonstrated that *RS_CRZ1* can transactivate the selected coregulated genes. Further, TRV-based HIGS mediated silencing of two of the *RS_CRZ1* coregulated genes, encoding

subtilisin-like protease (CL1756Contig1) and 2OG-Fe(II) oxygenase (CL1817Contig2), restricted the pathogenesis of *R. solani* in tomato. In some other pathosystems, subtilisin-like proteases are secreted by pathogens to degrade the host extracellular matrix and promote host penetration (Kennan et al. 2010; Pannkuk et al. 2015). We anticipate that the *RS_CRZ1* mediated induction of a subtilisin-like protease along with other cell wall-degrading enzymes may facilitate host penetration during the establishment phase of *R. solani* pathogenesis.

It has been reported that the *Saccharomyces cerevisiae* *crz1* mutant has growth defects and is sensitive to metal ion stress (such as Mn^{2+} , Ca^{2+} , Li^+ and Na^+) (Karababa et al. 2006; Thewes 2014). To analyze the role of *RS_CRZ1* during growth of *R. solani* in laboratory conditions, we standardized a dsRNA-mediated gene silencing approach. As shown in Figure 7, silencing of *RS_CRZ1* led to enhanced ROS accumulation in *R. solani* mycelia upon various metal ion stress. On the other hand, wild-type *R. solani* was able to manage the stress. Previous study has reported increased accumulation of metal ions in host cells during the infection process (Poschenrieder et al. 2013). The metal ions serve as cofactors in various metabolic processes that is altered during host colonization (Mithöfer et al. 2004). Also, there are reports that suggest that influx of metal ions induce ROS-mediated defense responses in host cells (Poschenrieder et al. 2006, 2013). Considering the above, we speculate that *RS_CRZ1* may assist *R. solani* in better management of metal ion stress-induced ROS accumulation in its mycelia cells during host colonization. Indeed, we observed

Fig. 4. Expression dynamics of *RS_CRZ1* coregulated genes of *Rhizoctonia solani* during pathogenesis and transactivation of the selected genes by *RS_CRZ1*. **A**, Heat map showing expression dynamics of potential *RS_CRZ1* coregulated genes in different rice genotypes. The genes are categorized according to their function. The color-coded scale bar represents the level of gene expression. **B**, Yeast one-hybrid-based transactivation assay wherein growth of different prey and bait constructs reflects promoter binding ability of *RS_CRZ1*. **C**, The quantitative reverse transcription PCR (qRT-PCR)-based quantification of the expression of selected *RS_CRZ1* coregulated genes during pathogenesis of *R. solani* in rice, at 1 and 3 days postinoculation. The primary axis represents qRT-PCR-based relative expression and the secondary axis represents fragment per kilobase per million reads value, as quantified by RNA-seq analysis. **D**, Expression dynamics of selected *RS_CRZ1* coregulated genes of *R. solani* in *RS_CRZ1*-silenced and vector control tomato plants. Graph shows mean values $\pm$ standard error of three biological replicates. Different letters indicate significant difference at $P \leq 0.05$.

that complementation of *crz1* mutant strain of yeast with *RS_CRZ1* restores tolerance to various metal ion stress. This reinforces the role of *RS_CRZ1* in combating metal ion toxicity in *R. solani*. A model summarizing the *RS_CRZ1* functions during pathogenesis of *R. solani* is summarized in Figure 9.

In conclusion, the present study demonstrates that *RS_CRZ1* is required for successful pathogenesis of *R. solani*. It transcriptionally regulates various pathogenicity-associated genes and assists the pathogen to combat various stresses that are encountered during host colonization.

MATERIALS AND METHODS

Biological materials and pathological assays.

R. solani AG1-IA (BRS1 strain) sclerotia were cultured on potato dextrose agar (PDA) (39 g per liter of PDA [Himedia]) plates at 28°C (Ghosh et al. 2014). Tomato (*Solanum lycopersicum* ‘Pusa Ruby’) was grown in a plant growth chamber at 26°C temperature, 60% relative humidity, and 12-h-day and 12-h-night cycle. Tomato plants (30 days old) were used for *R. solani* infection in a plant infection chamber (at temperature 26°C). Sclerotium was placed on the ventral surface of a healthy tomato leaf and was wrapped with a strip of aluminum foil. The plants were periodically sprayed with sterile distilled water to maintain high humid conditions (80%). The disease progression was measured in terms of disease index on a scale of 1 to 3. The scores are as follows: 1 = absence or presence of small necrotic lesions (minimal lesions), 2 = when these necrotic

spots enlarge (merged lesions), and 3 = when several necrotic spots coalesce (total necrosis). The samples for expression analysis were collected at different timepoints of 0, 12, 24, 48, and 72 hpi. A total of five plants (three leaves per plant) were infected and each experiment was repeated three times.

To infect rice (PB1), freshly prepared equal-sized *R. solani* sclerotia were inoculated in the sheath as per method described earlier (Ghosh et al. 2014). The infected tissues (including 1 cm up and down from the site of infection) were harvested for expression analysis.

Pathogenic developmental study of *R. solani* infection in tomato.

To study pathological development of *R. solani*, tomato leaves were stained with 0.1% Calcofluor white M2R (Sigma Aldrich) in 0.05 M phosphate-buffered saline (pH 8.5) for 30 min. Samples were washed twice with MilliQ (MQ) water and were observed under a DAPI (4',6-diamidino-2-phenylindole) filter in a laser scanning confocal microscope (AOBS TCS-SP5; Leica), using 20× objective. The depth of optical sections ranged from 50 to 70 μm and images were analyzed using a Leica Application Suite (LAS) AF version 2.6.0 build 7266 software. To visualize ROS accumulation, DAB (3,3'-diaminobenzidine) staining was performed (Ghosh et al. 2017). The infected leaves were stained with a 1 mg/ml DAB solution (prepared in 50 mM Tris acetate buffer, pH 5.0) and were incubated at 25°C for 24 h in the dark. Following staining, the leaves were de-stained, overnight, in a 3:1 ratio of ethanol/acetone

Fig. 5. Silencing of selected *RS_CRZ1* coregulated genes compromised the pathogenesis of *Rhizoctonia solani* in tomato. **A**, The disease index in the silenced lines of *RS_CRZ1* coregulated genes (CL1756Contig1 and CL1817Contig2) and vector control plants. The percentage of leaves with minimal infection was more in the host-induced gene silencing plants, as compared with vector control plants. **B**, A bar graph reflecting fungal load, estimated through quantification of 18S ribosomal DNA (rDNA) of *R. solani* in the infected samples, using tomato actin gene for normalization. **C**, Lesion area, calculated as percentage of necrotic area in the infected leaves. **D**, The relative expression of *RS_CRZ1* targets CL1817Contig2 and **E**, CL1756Contig1 in silenced and vector control plants reflects effective gene silencing. The 18s rDNA was used as endogenous control. Graph shows mean values $\pm$ standard error of three biological replicates. Different letters indicate significant difference at $P \leq 0.05$.

solution. H_2O_2 was visualized as brown colored patches in de-stained leaves, due to DAB polymerization, and leaves were photographed with a Nikon D3200 DSLR camera. The experiment was independently repeated three times and, in each experiment, three leaves from independent infected plants were analyzed.

Trypan blue staining was used for the cell-death assay (Ghosh et al. 2017). The leaves were stained in a 0.01% solution of trypan blue (Himedia) and were incubated overnight, with shaking at low speed. Following washing, the leaves were de-stained in chloral hydrate solution for 24 h, with slow shaking, and were observed in bright field under a Nikon stereo zoom microscope (Nikon AZ100) coupled with a Nikon camera (Nikon Digital Sight DS-Ri1).

Construction of HIGS vectors to silence *R. solani* genes.

For silencing *R. solani* target gene, the corresponding gene sequence was analyzed, using siFi21 software (available online), to select unique gene fragments having no off-target silencing effect of the generated siRNAs in both *R. solani* and the tomato genome (RefSeq National Center for Biotechnology Information [NCBI]) (Supplementary Fig. S3). We selected a 460-bp region of *RS_CRZ1*, a 411-bp region of *RS_GH16*, a 364-bp region of *RS_TF3*, and 385-bp region of *RS_GT34* and PCR-amplified the gene fragments from the genomic DNA of *R. solani*, using gene-specific primer pairs having suitable restriction sites incorporated (Supplementary Table S1). Subsequently, these fragments were individually cloned into pTRV2 vector using restriction enzymes. As a visual marker for gene silencing, 374 bp of cDNA sequence of *PDS* (accession NM_001247166.2) gene that encodes phytoene desaturase (a carotenoid biosynthesis enzyme in plants) was cloned into pTRV2. All plasmid DNA samples were isolated using the GeneJET plasmid miniprep kit (Thermo Scientific). Upon confirmation by Sanger sequencing, the positive constructs (recombinant pTRV2) as well as pTRV1 and pTRV2 were mobilized into *Agrobacterium tumefaciens* GV3101 through chemical transformation.

Agroinfiltration in tomato plants.

To conduct agroinfiltration in tomato plants, primary cultures of *A. tumefaciens* (supplemented with appropriate antibiotics) harboring pTRV1, pTRV2, and recombinant pTRV2 vectors were raised overnight, at 28°C, under shaking conditions. The secondary cultures of the respective clones were raised using 0.1% primary culture as inoculum. The bacterial cultures were centrifuged at 3,000 rpm (TOMY centrifuge MX-301) and the cells were resuspended in induction buffer (10 mM $MgCl_2$, 10 mM morpholine ethane sulfonic acid and 200 μM acetosyringone). Upon adjusting the optical density at 600 nm to 1, the cells were incubated for 3 h at 28°C with gentle shaking. The pTRV1 cells were mixed with recombinant pTRV2 (having respective gene fragments cloned) cells in 1:1 ratio and were infiltrated into the abaxial surface of leaves of 10-day-old tomato seedlings, using a 1-ml needleless syringe. The pTRV1 along with pTRV2 cells were also infiltrated into tomato leaves as vector control. Further the seedlings were kept at 25°C and 80% humidity, under 16-h day and 8-h night conditions in a growth chamber (Adaptis, Conviron). The target gene silencing and phenotype in the plants were evaluated at 15 to 20 dpi. The plants infiltrated with recombinant pTRV:*PDS* constructs were observed for the photo-bleaching phenotype in emerging leaves. Further, newly emerging leaves from the TRV-infiltrated plants were harvested at 21 dpi and DNA was isolated to analyze viral multiplication by PCR amplification of the movement protein-encoding gene of TRV. The experiment was performed in ten independent biological replicates with at least 15 different leaves infected each time.

Semiquantitative-based expression studies of *R. solani* genes in HIGS tomato plants.

The TRV positive tomato lines were inoculated with *R. solani* sclerotia. At 3 dpi, total RNA was isolated from the infected leaves, using RNeasy plant mini kit (Qiagen). cDNA was synthesized using 2 μg of RNA using High-Capacity cDNA reverse transcription kit (Applied Biosystems). Semi-quantitative PCR was performed, to quantify the transcript level of each of the genes in the respective HIGS plants, using DynazymeII *Taq* polymerase (Thermo Scientific). Thermal

Fig. 6. *RS_CRZ1* dsRNA-treated *Rhizoctonia solani* is compromised for pathogenesis in tomato. **A**, Disease symptoms of dsRNA treated *R. solani* in tomato plants, at 3 days postinoculation. **B**, Disease severity index reflecting percentage of total leaves with minimal infection, merged lesion and total necrosis. **C**, Lesion area calculated as percentage of necrotic area in the infected leaves. **D**, quantification of fungal load in buffer and dsRNA treated *R. solani* infected leaves. Quantitative reverse transcription PCR based expression analysis showing effective gene silencing in **E**, *RS_CRZ1* and **F**, *RS_GT34* silenced plants. Graph shows mean values $\pm$ standard error of three biological replicates. Different letters indicate significant difference at $P \leq 0.05$.

cycling conditions were initial denaturation at 95°C for 3 min, followed by 30 cycles of 95°C denaturation for 30 s, annealing at 56°C for 30 s, and extension at 60°C for 30 s. 18S ribosomal DNA (rDNA) of *R. solani* was used as endogenous control. The semiquantitative PCR was performed in three independent plant samples (one leaf per plant). The experiment was repeated in three independent biological replicates (Supplementary Fig. S11).

qRT-PCR based expression studies.

To validate the expression pattern of genes during pathogenesis of *R. solani*, expression analysis based on qRT-PCR was carried out at different timepoints (0, 12, 24, 48, and 72 hpi) during pathogenesis on susceptible rice plant cultivar PB1 and tomato (Pusa Ruby). qPCR primers were designed in such a manner that they selectively amplify target genes but not host (rice or tomato) genes (Supplementary Table S1). qRT-PCR was performed, using PowerUp SYBR Green master mix (Applied Biosystems), according to manufacturer instructions. The relative expression was calculated using the cycle threshold (Ct) $2^{-\Delta Ct}$ method, wherein ΔCt is the difference between Ct values of target and reference (18S rDNA) genes of *R. solani* (Kiirika et al. 2012).

We also estimated fold change of expression of these genes at 12, 24, 48, and 72 hpi compared with 0 hpi by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). To quantify fungal load in the infected samples, fold change was calculated using the $2^{-\Delta Ct}$ method, wherein ΔCt is the difference between Ct values of fungal 18S rDNA (target) and host actin gene (as reference), as described by Kant et al. (2019). The data from three

technical replicates were averaged and the mean for three independent biological replicates were used to calculate standard error. One-way analysis of variance was performed using Sigma Plot 11.0 software (SPSS, Inc.) to estimate the statistical significance between different groups, using the Student-Newman-Keuls test. The significance was determined at $P \leq 0.05$, wherein different letters indicate significant difference.

Quantification of infection depth.

Infected tomato leaves were collected from pTRV:RS_CRZ1, pTRV:0 inoculated and wild-type plants at 3 dpi. The infected portions of leaves were cut into small strips, which were dipped into 1 M KOH (potassium hydroxide; Amresco Inc.) and were autoclaved (Ayliffe et al. 2013). The samples were neutralized with 50 mM Tris (pH 7.0), followed by incubation with WGA-FITC stain (20 µg/ml; Sigma Aldrich) for 30 min, and were washed with sterile MQ water. Subsequently, the samples were analyzed under the green fluorescent protein (GFP) filter of the confocal laser scanning microscope (Leica AOBs TCS-SP5) using 20× objective. The images were analyzed using LAS AF version 2.6.0 build 7266 software and topological 3D image was created to reflect the depth of pathogen penetration in the host tissue. The leaf samples were visualized under a EVOS FL microscope (Thermo Fisher Scientific) under 2× magnification to obtain a wider image of the infected area. The area under the infection cushions was measured using ImageJ software. The experiment was performed in five independent biological replicates with at least ten leaves analyzed in each replicate.

Fig. 7. The silencing of *RS_CRZ1* enhances sensitivity to metal ion stress in *Rhizoctonia solani*. **A**, The quantitative reverse transcription PCR based expression of *RS_CRZ1* gene in double-stranded RNA (dsRNA) and buffer-treated *R. solani* mycelia. **B**, Confocal micrograph of dihydrorhodamine 123 (a reactive oxygen species [ROS] indicator)-stained *RS_CRZ1*-silenced (dsRNA treated) as well as control (buffer-treated) *R. solani* mycelia. Compared with the control, increased green fluorescence reflecting enhanced ROS accumulation was observed in *RS_CRZ1*-silenced (dsRNA treated) as well as control (buffer-treated) *R. solani* mycelia. Compared with the control, increased green fluorescence reflecting enhanced ROS accumulation was observed in *RS_CRZ1*-silenced *R. solani* upon various metal ion stresses, such as **C**, Ca^{2+} **D**, Mn^{2+} , and **E**, Na^{+} stress. Fluorescence intensity (calculated across colored region of interest [ROI]) quantifying ROS accumulation in *R. solani* mycelia is drawn as line graph wherein orange represents control and blue represents dsRNA-treated samples. Graph shows mean values $\pm$ standard error of three biological replicates. Different letters indicate significant difference at $P \leq 0.05$.

Identification of C2H2 zinc finger transcription factors in *R. solani*.

Using a keyword-based search, we have identified several zinc finger transcription factors from the FungiDB database (Basenko et al. 2018). The protein sequences of these transcription factors were subjected to Conserved Domains Database (CDD) search (NCBI), and those containing a zinc finger C2H2 domain were selected. Further, they were subjected to

motif discovery using MEME software (Bailey et al. 2015). The consensus motif generated was searched in the *R. solani* transcriptome (Ghosh et al. 2018), using FIMO software (MEME website). The transcripts showing a positive hit (E value $< e^{-5}$) were subjected to CDD search and those with Zn-C2H2 domain were selected. The fragment per kilobase per million reads values of the selected transcripts were plotted in the form of a heat map.

Fig. 8. Ectopic expression of *RS_CRZ1* complements the *crz1* mutant strain of yeast. **A**, Growth of *crz1* + pYES2:*RS_CRZ1* and *crz1* + pYES2:0 expressing yeast (*Saccharomyces cerevisiae*) strains on synthetic defined medium lacking uracil containing 2% galactose media or on media supplemented with **B**, LiCl **C**, NaCl and **D**, CaCl₂. The *RS_CRZ1*-complemented yeast exhibited enhanced growth, as compared with the *crz1* mutant, under metal ion stress.

Fig. 9. A model summarizing the role of *RS_CRZ1* during pathogenesis of *Rhizoctonia solani*. During the establishment phase of pathogenesis, *R. solani* upregulates a *RS_CRZ1* transcription factor. Upon potential dephosphorylation by calcineurin-like protein, the *RS_CRZ1* gets translocated into the nucleus and regulates the expression of several *R. solani* genes, including a serine protease and a 2OG-Fe(II) oxygenase. The silencing of *RS_CRZ1* or any one of its coregulated genes [serine protease and 2OG-Fe(II) oxygenase] compromised the ability of *R. solani* to cause disease. Further, ectopic expression of *RS_CRZ1* could restore metal ion stress tolerance ability in a *crz1* mutant of yeast. We anticipate that induction of *RS_CRZ1* provides the ability to the pathogen to better manage hostile environment that is encountered during host colonization.

Phylogenetic analysis of RS_CRZ1.

The orthologs of RS_CRZ1 proteins in different fungal species were downloaded from the NCBI database. The sequences were aligned using ClustalW algorithm. The aligned sequences were subjected to phylogenetic analysis using MEGAX, following a neighbor-joining algorithm with 500 bootstrap values.

Identification of RS_CRZ1 coregulated genes.

The transcriptome analysis of wild-type and *crz1* mutant had identified several potential targets of CRZ1 in *C. neoformans* (Chow et al. 2017). We extracted the nucleotide sequence of 86 potential targets of CRZ1 in *C. neoformans*. Using the Bioedit tool (Hall 1999), the selected targets of *C. neoformans* CRZ1 were searched (E value $< e^{-5}$) in the previously reported transcriptome sequence of *R. solani* AG1-IA (BRS1 strain) (Ghosh et al. 2018). In this process, we identified 44 *R. solani* genes that are orthologous to the CRZ1 targets in *C. neoformans*. The expression dynamics of these orthologs were analyzed, and a total of 22 genes were upregulated during the establishment phase of *R. solani* infection in rice, similar to the expression of RS_CRZ1. These 22 genes were considered potential RS_CRZ1 coregulated genes. Among them, we randomly selected five genes (namely c17182_g1_i1, CL2868Contig1, CL1756Contig1, CL3021Contig2, CL1817Contig2) for further characterization.

Y1H assay.

Y1H assay was performed using Matchmaker Gold Y1H library screening system (Clontech). The nucleotide sequences of the promoter region of selected RS_CRZ1 coregulated genes in *R. solani* were retrieved, using the RSIADB database (Chen et al. 2016a). The promoter sequence (approximately 2,000 bp) from each of the genes (Supplementary Data 1) was cloned in pAbAi bait vector (Clontech) upstream of the AbA antibiotic resistance gene. The plasmid was linearized with *Bst*BI restriction enzyme and was transformed into Y1H Gold yeast strain, per manufacturer protocol. The positive transformants (Y1H-bait) were selected on AbA (1,000 ng of AbA per milliliter) plate. Subsequently, a full-length copy of RS_CRZ1 was cloned in pGADT7-AD (Clontech) prey vector as the GAL4 transcription activation domain fusion protein. The recombinant prey plasmid was transformed into the Y1H Gold yeast strain that was pretransformed with the recombinant bait plasmid. Positive transformants (Y1H – bait + prey) were selected by growing serially diluted (10^{-1} , 10^{-2} , 10^{-3} , and 10^{-4}) yeast cells at 30°C on AbA (0 to 2,000 mg/ml) containing double drop out (SD-Ura-Leu) plates.

dsRNA-mediated silencing of R. solani genes.

The 460-bp region of RS_CRZ1 and 385 bp of RS_GT34 that were used for HIGS were selected for dsRNA synthesis. The forward and reverse primers were designed such that they contain a 5' flanking region of the T7 RNA polymerase promoter. Partial fragments of the genes were amplified from the genomic DNA of *R. solani*. Purified gene fragments (1 µg) were used for in-vitro transcription to produce dsRNA, using the MEGascript T7 transcription kit (Thermo Scientific), according to manufacturer protocol. dsRNA (50 µg) was used to treat *R. solani* mycelial mats grown on nitrocellulose polyvinylidene difluoride membrane (Axiva) for 16 h at 28°C. The mycelial mats were harvested, and the expression was quantified by qRT-PCR, using gene-specific primers (Supplementary Table S1). The experiment was performed in at least three independent biological replicates.

dsRNA treatment of R. solani and pathogenicity assays.

The *R. solani* sclerotia were treated with 30 µg of dsRNA for 24 h. These sclerotia along with buffer-treated sclerotia were

inoculated in 30-day-old tomato plants in the infection chamber at 28°C. After 3 dpi, the infected plants were photographed, and samples were harvested for fungal load quantification. At least five plants (three leaves per plant) were analyzed for each treatment and the experiment was independently repeated three times (biological replicates).

Metal ion stress in R. solani.

The RS_CRZ1 dsRNA-treated mycelial mats of *R. solani* were treated with either NaCl (1.3 M), CaCl₂ (700 mM), or MnCl₂ (4 mM) for 24 h at 28°C. The buffer-treated mycelial mats were used as control. The mycelial mats were washed twice in MQ water and were stained in dihydrorhodamine123 (Merck) solution (10 mg/ml) to visualize ROS accumulation. The samples were washed twice with sterile MQ water and were observed under a GFP filter of the confocal laser scanning microscope (Leica AOBs TCS-SP5) under a 100× oil immersion objective. The images were analyzed using LAS AF version 2.6.0 build 7266 software. Microscopy was performed in three treated mycelial samples using five independent biological replicates.

Yeast complementation assay.

Homozygous diploid yeast knockout *crz1* strain (Y35353) was procured from the Euroscarf Yeast Collection. The cDNA was prepared using Superscript III (Invitrogen) reverse transcription from *R. solani*-infected PB1 rice genotype (3 dpi). The RS_CRZ1 (approximately 2.5 kb) was PCR-amplified from the cDNA of 3-dpi infected rice sheaths, using Phusion high fidelity DNA polymerase (Thermo Fisher Scientific). The PCR product was initially cloned into pJET1.2 (Thermo Fisher Scientific) cloning vector. Upon digestion with *Kpn*I and *Xba*I restriction enzymes, the product was cloned into similarly digested pYES2 vector (containing GAL1 as galactose-inducible promoter and ampicillin as bacterial selectable marker). Plasmids were transformed into competent *Escherichia coli* (DH5α) cells and the recombinant plasmid (pYES2:RS_CRZ1) was selected on Luria Bertani agar plates with 50 µg of ampicillin per milliliter. Further the pYES2:RS_CRZ1 was transformed into *crz1* yeast mutant using a yeast transformation kit (Yeastmaker Yeast Transformation System 2; Takara) according to manufacturer protocol. The transformed colonies were selected on SD-Ura plates. An overnight culture of yeast grown in SD-Ura medium (MP Biochemicals) was serially diluted (1:10) and was spotted on to SD-Ura medium plates containing 2% galactose as a sole carbon source. For metal toxicity assays, the SD-Ura plates were supplemented with NaCl (0.65 M [Sigma Aldrich]), LiCl (0.2 M [Himedia]) and CaCl₂ (0.35M [Sigma Aldrich]).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

MEME software: <http://meme-suite.org/tools/meme>
siFi21 software: <http://labtools.ipk-gatersleben.de/>

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