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The elicitor-responsive gene for a GRAS family protein, *CIGR2*, suppresses cell death in rice inoculated with rice blast fungus via activation of a heat shock transcription factor, *OsHsf23*

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We show that a rice GRAS family protein, CIGR2, is a *bonafide* transcriptional activator, and through this function, targets the B-type heat shock protein-encoding gene *OsHsf23* (Os09g0456800). *CIGR2* (Os07g0583600) is an *N*-acetylchitooligosaccharide elicitor-responsive gene whose activity, through the direct transcriptional control of *OsHsf23*, is required for mediating hypersensitive cell death activation during pathogen infection. RNAi lines of *CIGR2* and *OsHsf23* similarly exhibited the higher level of granulation in the epidermal cells of leaf sheath inoculated with an avirulent isolate of rice blast fungus. Interestingly, we did not observe altered levels of resistance, suggesting that CIGR2 suppresses excessive cell death in the incompatible interaction with blast fungus via activation of *OsHsf23*.

Key words: GRAS family; heat shock transcription factor; cytoplasmic granulation; rice

Higher plants employ a suite of complex mechanisms to activate resistance signaling processes in response to pathogen infection. In most cases, these responses can be functionally categorized based on timing, as well as on the mechanism(s) associated with the initial recognition and signaling events associated with their activity. For example, during the initial stages of pathogen perception, the plant innate immunity system is engaged via the recognition of conserved cellular components derived from the pathogen. In brief, pathogen-associated molecular patterns (PAMPs) elicit a rapid basal defense response via the recognition by host-derived pathogen recognition receptors (PRRs). As a result of this interaction, basal resistance—typically referred to as PAMPs-triggered immunity (PTI)—functions in the rapid induction of host responses aimed at abrogating pathogen growth and the elicitation of disease.[1)] In the second layer of the host immune signaling, termed effector-triggered immunity, the activity of pathogen-derived effectors, whose function is to block PTI, is recognized by the host, and this process leads to the induction of robust immune signaling to prevent pathogen immune subversion and the proliferation.

Among the best-characterized elicitors of PTI are *N*-acetylchitooligosaccharides (GNn), hydrolyzed fragments of chitin, a major component of the fungal cell wall. As highly potent elicitors of PTI, GNns are perceived by the plasma membrane-localized PRRs CEBiP[2)] and OsCERK1[3)] in rice, or by AtCERK1[4)] and AtLYK5[5)] in *Arabidopsis*. Once bound to its receptor, GNns initiate a highly regulated and specific suite of defense responses, including the induction of dynamic changes in host gene expression.[2,6)]

Members of the plant-specific *GRAS* gene family encode transcriptional regulators with functions in a wide array of signaling mechanisms, including response to growth and development, hormone signaling, and plant defense. We previously reported that the expression of two genes encoding members of the GRAS family of proteins, *CIGR1* and *CIGR2* (Os07g0583600), were up-regulated in response to GNn in rice cells, and their respective gene products were localized within the nucleus.[7,8)] In total, these data support our hypothesis that these PTI-inducible genes may encode proteins that function as transcriptional regulators. Since the first discovery of *SCARECROW* in *Arabidopsis*,[9)] genes encoding plant-specific

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Abbreviations: SD, standard deviation; hpi, hs post-inoculation.
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GRAS family proteins have been identified as key regulators in numerous developmental process, as well as in response to biotic and abiotic stimuli.[10,11] As noted above, the GRAS family of proteins is hypothesized to be transcriptional regulators, and numerous efforts have been made to identify GRAS target genes. For example, *SHORT-ROOT*, a transcription factor associated with developmental processes required for root formation in *Arabidopsis*, was shown to directly activate eight genes, including *SCARECROW*, that are responsible for the radial patterning and development of root tissue.[12] Similarly, *RGA*, encoding a DELLA-type GRAS family protein in *Arabidopsis* that negatively regulates gibberellin (GA) signaling, was shown to induce a set of genes involved in GA-signaling.[13]

In addition to growth and developmental processes, GRAS proteins have also been shown to function in a wide array of biotic signaling processes. For example, two *Medicago truncatula* genes, *NSP1* and *NSP2*, encoding GRAS family proteins that are indispensable to nodule formation, were shown to activate an early nodulin gene, *ENOD11*, by binding of the heteropolymer of NSP1 and NSP2 to the promoter region of *ENOD11*.[14] Similarly, during pathogenic interactions, a tomato *SIGRAS6* was shown to mediate the resistance (*R*) gene-dependent resistance to *Pseudomonas syringae* pv. *tomato*.[15] In the current study, we show that CIGR2 activates the expression of *OsHsf23* (Os09g0456800), a gene encoding a B-type heat shock transcription factor (HST). Transgenic expression of *CIGR2* and *OsHsf23* was found to suppress cell death induced in the incompatible interactions of rice with rice blast fungus, *Magnaporthe oryzae*, suggesting a regulatory role for these proteins during plant–pathogen interactions.

Materials and methods

Fungal materials and preparation of conidia. *M. oryzae* field isolates Ai79-142 (race 037.7; MAFF #101 520;http://www.gene.affrc.go.jp/databases-micro_search_en.php) and P91-15B (race 001.0) were grown on oatmeal agar plates at 26 °C. For plant inoculation assays, a conidial suspension was prepared as reported previously.[16] Washed conidia were obtained from the conidial suspension by centrifugation and re-suspended in sterile distilled water as described previously.[17]

Pathogen inoculation assays. *Oryza sativa* cv. Nipponbare BL2, susceptible and resistant to Ai79-142 and P91-15B, respectively, were grown in hydroponic culture for three weeks as previously described.[18] The fourth leaves were sprayed with a conidial suspension (1 x 10^5 conidia/mL) and incubated at 25 °C in a moist chamber for 24 h and then transferred to a growth chamber at 25 °C. In some experiments, noted in the text, the excised fourth leaves from rice plants were spot-inoculated with 5 µL of conidia suspension and incubated in the same way as described above. For the leaf sheath assay, excised leaf sheaths were inoculated with the conidial suspension (1 x 10^5 conidia/mL) and incubated at 25 °C for the times indicated. Evaluation of hyphal infection in epidermal cells of the leaf sheaths was carried out as described previously.[18] The resultant vector was introduced into rice via *Rhizobium radiobacter* strain EHA105 as previously described.[19]

Histological techniques. To measure the longitudinal length of lesions, inoculated leaves were treated with a solution of lactophenol/trypan blue followed by destaining with chloral hydrate, as previously described.[20] For quantification of cell death *in planta*, the number of appressoria beneath which the epidermal cells exhibit granulation was counted.

For pathogen assays using suspension-cultured cells, cell death was evaluated by Evan's blue staining.[21] In brief, suspension cells (ca. 100 mg) were sub-cultured for 36 h in fresh medium and stained with 0.05% Evans blue in 500 mM HEPES-KOH (pH 7.2) for 20 min, followed by four washes with 500 mM HEPES-KOH (pH 7.2). The Evan's blue dye was eluted by *N*,*N*-dimethylformamide and the spectral absorbance at 595 nm was measured.

Construction of binary vectors and rice transformation. All PCR products for the construction of plasmids were generated using gene-specific primers (Table S1) and the resultant products were verified by DNA sequencing. The cDNA fragment encoding the ORF of *CIGR2* (1712 bp) was amplified by PCR using gene-specific primers (Table S1) and cloned into pGEM-T Easy (Promega, Madison, WI, USA). For in plant expression of *CIGR2*, the resultant pGEM-CIGR2 plasmid was digested with *Spe*I and the resultant fragment was ligated into the *Spe*I site of pTA7001.

For the generation of *CIGR2*-RNAi rice lines, the 956 bp loop sequence of *GUS* was amplified by PCR using the primers shown in Table S1 and cloned into the *Xba*I/*Sac*I site of pBI333, to yield pBI333/GUS-Loop. The trigger region (sense- and antisense-strand) of *CIGR2* was amplified by PCR (496 bp) and cloned into pBI333/GUS-Loop at the *Xba*I/*Kpn*I (antisense-strand) and *Sal*I/*Sac*I (sense-strand). For the construction of *OsHsf23*-RNAi, the trigger sequence was amplified by PCR (256 bp) and cloned into pANDA,[22] using the Gateway system (Invitrogen). The resultant vector was introduced into rice via *R. radiobacter* strain EHA105-mediated transformation, as previously described.[19]

Reporter assay for transcriptional activation by CIGR2. A DNA fragment containing ORF of *CIGR2* was amplified by PCR. The coding sequence for the *GAL4* DNA binding domain (GAL4-BD) was fused with the *CIGR2* ORF-coding sequence, as amplified by PCR, noted above. The resultant chimeric gene was cloned into pBI221 and used as an effector. The reporter plasmid was kindly supplied by Dr M. Ohme-Takagi of The National Institute of Advanced Industrial Science and Technology (Japan). Plasmid pPTRL, which contains a *Renilla luciferase* gene under the control of the CaMV 35S promoter, was used as an internal control. In the transient assays, plasmids of reporter (2 µg), effector (2 µg), and internal control (0.04 µg)

were mixed and introduced into suspension-cultured rice cells at four days after sub-culture by particle bombardment method using the PDS-1000 He biolistic particle delivery system (Bio-Rad, Hercules, CA, USA). Transformed rice cells were further incubated for 24 h at 24 °C in the dark. LUC assay was performed with the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions. Luminescence was measured using a TD20/20 luminometer (Turner Designs, Sunnyvale, CA, USA).

Transactivation of OsHsf23 *by* CIGR2. The open reading frame of *GUS* in pBI221 was replaced with the DNA fragment, including entire ORF and 3′-UTR, of *CIGR2* (1946 bp) amplified by PCR. A DNA fragment containing entire ORF of *EL2* was prepared by digestion of the previously isolated cDNA clone[23] with restriction enzymes and substituted for *GUS* in pBI221. These two plasmids were used as effectors. For construction of reporter plasmid, 35S promoter of pBI221 was replaced with the 5′-upstream sequence of *OsHsf23* (2086 bp) amplified by PCR. Transient assays in cultured rice cells by particle bombardment were performed as described above. GUS activity was measured as previously reported.[24]

RNA isolation and RT-PCR. Total RNA was extracted from the suspension-cultured cells or leaves of rice using Sepasol RNA I Super (Nacalai Tesque, Kyoto, Japan), and 1 µg was used for first-strand cDNA synthesis using the PrimeScript RT Reagent Kit (Takara Co., Ltd.). PCR analysis was performed as follows: denaturation a 98 °C (2 min), followed by 28 cycles of 98 °C (30 s), 55 °C (30 s), 72 °C (30 s), with a final extension of 72 °C (2 min). In some experiments, the relative levels of mRNAs were quantified by quantitative PCR using MX3000P (Stratagene), with *OsUBI1* (Os06g0681400) as an internal standard. The sequences of the PCR primers are listed in Table S1.

Microarray and northern blot analysis. Rice plants transformed with pTA7001/*CIGR2* or vector were grown as described above and sprayed with dexamethasone (DEX) in ethanol at a final concentration of 30 µM. At 24 and 30 h after treatment, total RNAs were isolated, mixed at 1:1, and subjected to microarray analysis.[25] In brief, total RNAs from the pTA7001/*CIGR2*- and vector control-line were labeled with cy5 and cy3, respectively, and probed with rice 22k oligoarray (Agilent technologies, G4138A), according to the manufacturer's protocol. Signal data were log transformed and analyzed by two-way analysis of variance using Subio platform software (Subio Inc. Japan, http://www.subio.jp/). Experiments were carried out twice and clones with a fold change of 2< in average were scored as up-regulated genes.

Results

CIGR2 is a transcriptional activator

We previously demonstrated that CIGR2 is localized within the nucleus of onion epidermal cells,[8] supporting our hypothesis that it functions as a transcription factor. To further test this, we constructed an effector plasmid encoding a fusion protein of *CIGR2* with the DNA binding domain of yeast *GAL4* under the control of a constitutive *35S* promoter, and co-introduced this fusion with a reporter plasmid containing a GAL4-binding sequence (*UAS*) into cultured cells of rice by particle bombardment. As shown in Fig. 1, we observed an approximate 4.5-fold induction in LUC activity induced by the effector plasmid, compared to the induction by our control plasmid in cultured cells at 48 h after injection. Based on this, we conclude that CIGR2 possesses a high likelihood to function as a transcriptional activator.

The heat shock transcription factor OsHsf23 *is regulated by CIGR2*

Based on our observation of the transcriptional activity of CIGR2, we hypothesized that we could use this function to identify genes that were regulated in response to the chitin-inducible expression of *CIGR2*. To this end, the full-length cDNA of *CIGR2* was cloned into a DEX-inducible binary vector, pTA7001[26] and introduced into rice for the generation of stable homozygous expression lines. In total, three independent transgenic lines of rice were generated, and our analysis (data not shown) showed that one line (i.e. Line #7) showed an induction of *CIGR2* following treatment with DEX, while the additional two lines did not. The accumulation of the *CIGR2* mRNA was visible as detected by northern blot hybridization at 4–72 h after treatment. In contrast, when "vector only"-control rice plants (vector control-line) were treated with DEX, no such accumulation of *CIGR2* mRNA was observed (data not shown).

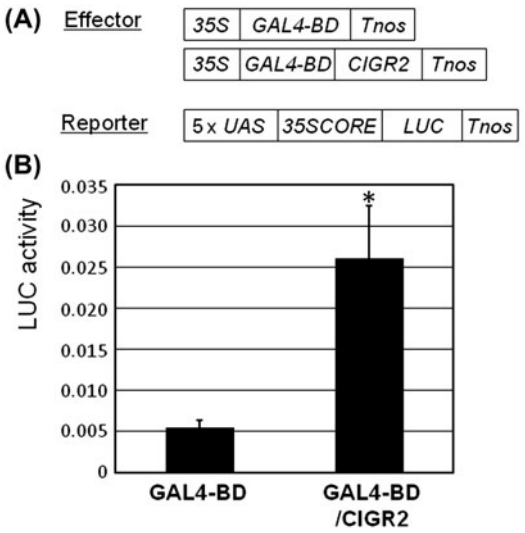

Fig. 1. Transactivation of *5xGAL4-UAS::LUC* reporter gene by GAL4-BD/CIGR2 in suspension-cultured rice cells.

Notes: (A) Structure of effector and reporter plasmids. (B) Transient LUC reporter assay in cultured cells of rice cv Nipponbare. The cDNA for GAL4-BD or CIGR2 fused with GAL4-BD was co-introduced with the reporter plasmid into rice cells. At 48 h, GUS activity per mg protein was measured, and presented as a relative value to the activity by GAL4-BD. An asterisk and error bars indicate significant difference in a *t*-test at $p < 0.05$ and SD, respectively.

To identify CIGR2-regulated genes, total RNA isolated at 24–30 h after treatment with DEX were pooled and probed with the rice 22k oligoarray; three biological replicates for pTA7001/*CIGR2*-transformed, as well as vector control-line, were used as oligoarray substrates. Using this approach, we identified 153 genes whose mRNA levels were at least twofold higher in pTA7001/*CIGR2*-expressing rice than those in vector control-line (Table S2). However, it was considered that the genes responsive to DEX both in pTA7001/*CIGR2*- and vector control-line were also included in these candidates. In fact, RT-PCR analysis of randomly selected five genes indicated that four genes were weakly activated in vector control-line (data not shown), and one for OsHsf23 was specifically activated in pTA7001/*CIGR2*-line treated with DEX (Fig. 2(A)).

To further confirm the induced expression of *OsHsf23* by CIGR2, we carried out an effector/reporter assay whereby the full-length cDNA of *CIGR2* under the control of 35S promoter (effector), and 5′-upstream region of *OsHsf23* (2086 bp) fused with *GUS* (reporter), were co-introduced into cultured rice cells by particle bombardment. GUS activity was measured in order to represent the activity of CIGR2 as transcriptional factor. As a control, *EL2*, previously isolated as an elicitor-responsive gene,[23] was substituted for *CIGR2* (Fig. 2(B)). As shown in Fig. 2(C), *OsHsf23* was significantly activated by *CIGR2* compared to *EL2*, indicating that *OsHsf23* is regulated by *CIGR2*.

Construction of the knock-down lines of CIGR2 *and* OsHsf23

To define the biological function(s) of *CIGR2* and *OsHsf23*, we constructed RNAi-based binary vectors to suppress the expression of these two genes, and constructed transgenic rice plants expressing the *CIGR2*- and *OsHsf23*-RNAi derivatives. As shown in Fig. S1, qRT-PCR analysis demonstrated that we successfully isolated transformed rice plants in which *CIGR2* and *OsHsf23* expression was suppressed in their respective stable lines. In the experiments described below, we used transformed lines of rice in which the expression of *CIGR2* or *OsHsf23* was not suppressed (i.e. negative control).

Suppression of CIGR2 *and* OsHsf23 *leads to increased level of granulated cells during an incompatible interaction with* M. oryzae

During incompatible rice-*M. oryzae* interactions, hypersensitive cell death (HCD) is considered to occur in the primarily invaded epidermal cells of the leaf sheath as they exhibit cytoplasmic granulation accompanied by the loss of plasmolysis.[16] The observation that the level of cell death evaluated by Evans blue staining increases in the suspension-cultured cells in the *CIGR2*- and *OsHsf23*-RNAi knock-down lines (Fig. S2) prompted us to examine the ratio of granulated in the leaf sheath of the RNAi lines inoculated with an avirulent isolate of *M. oryzae*, P91-15B. As shown in Fig. S3, we could detect the cytoplasmic granulation in epidermal cells from rice leaf sheath cells following pathogen inoculation. By microscopic observations, we found that the suppression of *CIGR2* or *OsHsf23* significantly increased the ratio granulated cells (Fig. 3). In these experiments, responses by the null RNAi lines were indistinguishable from those by non-transgenic rice.

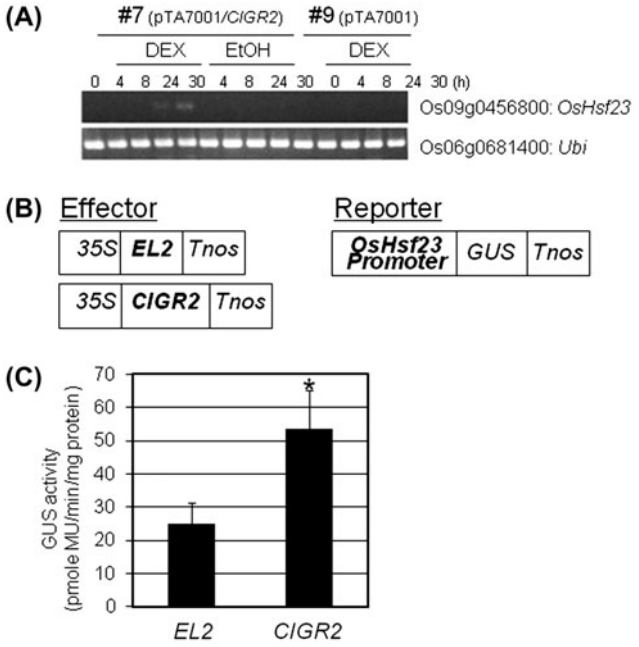

Fig. 2. Activation of *OsHsf23* by *CIGR2*.

Notes: (A) Leaves of sibling plants (T1) of a pTA7001/*CIGR2* line (#7) were sprayed with DEX or ethanol (EtOH), total RNA was isolated according to the time course indicated at the top of the photograph and expression of *OsHsf23* was monitored by RT-PCR. As a control, total RNA isolated from a vector control-line (#9) treated with DEX was processed in the same way. (B) Schematic presentation of effecter and reporter plasmid. (C) Transient GUS reporter assay in cultured cells of rice cv Nipponbare. Two kinds of effecter plasmid (EL2 or CIGR2) were co-introduced with the reporter plasmid into cultured cells of rice by particle bombardment. Horizontal axis indicates GUS activity at 48 h after injection. An asterisk and error bars indicate significant difference against the control effecter, EL2, in a *t*-test at $p < 0.05$ and SD, respectively.

Unchanged resistance of the CIGR2-*RNAi lines to* M. oryzae

HCD is generally considered to be a key component of host resistance in rice in response to *M. oryzae* infection. To define the function of *CIGR2* and *OsHsf23*-RNAi lines to *M. oryzae* infection, we examined the response during infection. As shown in Figs. 4 and S4, following spray-inoculation of the RNAi lines with avirulent *M. oryzae*, lesion development in the RNAi lines tended to be larger than those in non-transformed plants, or in null RNAi lines, implying a compromise in resistance. To extend this, we next assessed the hyphal growth of *M. oryzae* in the RNAi-lines. As shown in Fig. 5, contrary to our prediction, we did not observe a significant difference in the extent of hyphal invasion of P91-15B in the leaf sheath of *CIGR2*-RNAi lines. Interestingly, these lines also exhibited similar susceptibility to a virulent isolate, Ai79-142, when spot-inoculated on the leaf (Fig. S5). These observations suggest that the increase in the levels of HCD found in *CIGR2*-RNAi is not tightly coupled with host resistance.

Discussion

To the best of our knowledge, this is the first report demonstrating that a GRAS family protein functions as a transcriptional activator during plant immune signaling. Among the GRAS family of proteins, NSP1 of *M. truncatula* has been shown to directly bind its target gene, *ENOD11*, through the formation of heterodimers with another GRAS family protein, NSP2.[14)]

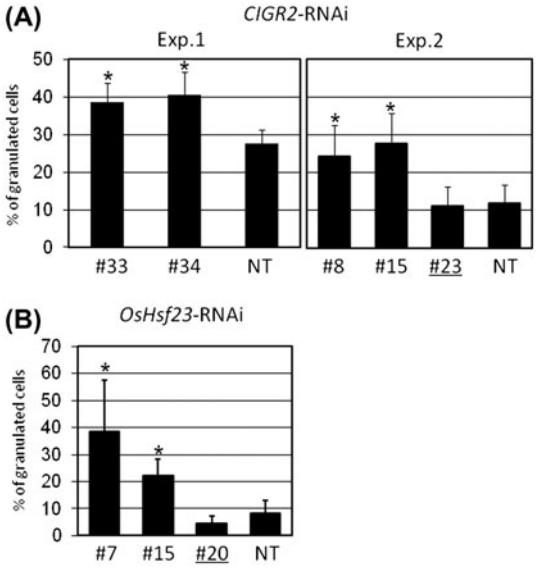

Fig. 3. Ratio of granulated cells in the epidermis of leaf sheath of *CIGR2*-RNAi and *OsHsf23*-RNAi lines inoculated with an avirulent isolate of *M. oryzae*, P91-15B.

Notes: (A) Ratio of appressoria that induced granulation in the epidermal cells of *CIGR2*-RNAi at 48 hpi. Experiments were carried out two times using different lines of transgenic rice. Asterisks indicate the significant difference against non-transgenic rice (NT) in each experiment in Dunnett's test at $p < 0.05$. #23 (underlined) indicates a null RNAi line. (B) Same as (A) except that *OsHsf23*-RNAi lines were tested. #20 (underlined) indicates a null RNAi line. Error bars indicate SD.

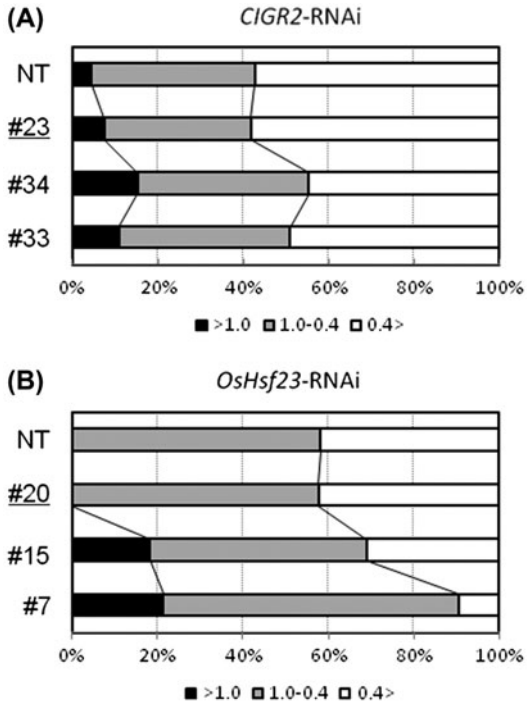

Fig. 4. Length of lesions formed on the leaves of *CIGR2*-RNAi lines.

Notes: The fourth leaves of intact seedlings of *CIGR2*-RNAi (A) and *OsHsf23*-RNAi (B) rice plants were spray-inoculated with an avirulent strain of *M. oryzae* (P91–15B) and lesions at 5 dpi were visualized by lacto-phenol/trypan blue staining. Ratios of lesion numbers whose longitudinal length were >1.0, 1.0–0.4 or <0.4 mm were indicated by black, gray, and white bars, respectively. #23 and #20 (underlined) indicate null RNAi lines of *CIGR2* and *OsHsf23*, respectively.

This co-factor binding function is similar to the DELLA family of proteins, which are required for GA signaling.[27)] It remains to be determined if CIGR2 functions through co-factor binding to activate the transcription of target genes, including the activation of *OsHsf23*, through which the molecular mode of activation of target genes by CIGR2 will be clarified.

In the present study, we showed that suppression of either *CIGR2* or *OsHsf23* by RNAi resulted in increased cell death in rice following pathogen infection (Fig. 3), suggesting that the activity of these two genes may function as suppressors of cell death. This is significant, as both *CIGR2* and *OsHsf23* are expressed and responsive to GNn in suspension-cultured rice cells,[6,7)] and in general, oligosaccharide elicitors, including GNn, are not potent inducers of cell death.[28)] Based on our observations, coupled with the elicitor activity of GNn, we posit that the activity of CIGR2 and OsHsf23 may function, in part, by suppressing the excessive cell death activity during pathogen infection. Indeed, this hypothesis is supported by our observation that in *CIGR2*-RNAi lines, we did not observe enhanced resistance to virulent or avirulent isolates of *M. oryzae* (Figs. 5 and S5). In fact, in leaves sprayed with an avirulent isolate, lesions appeared as brown-colored spots, and in general, were larger than those in control plants (Fig. 4). These data are in favor of a mechanism through which the development of larger lesions is mediated through the induction of cell

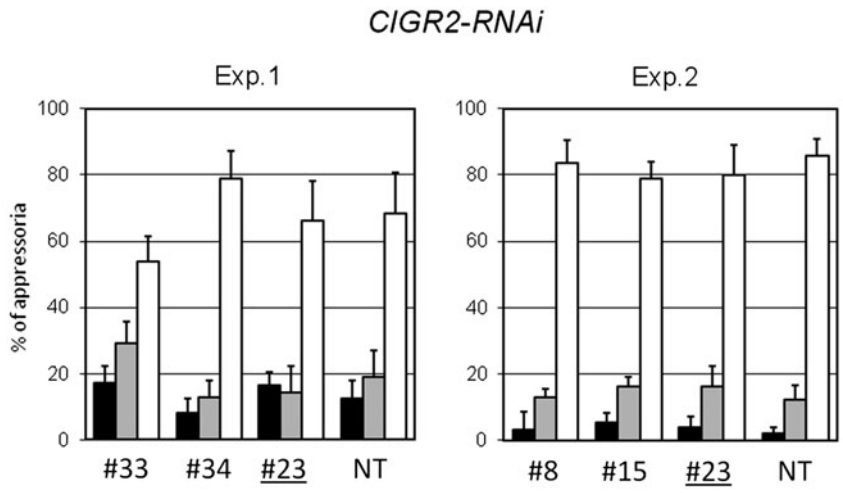

Fig. 5. Leaf sheath assay of *CIGR2*-RNAi lines.

Notes: Leaf sheath of the lines were inoculated with an avirulent isolate of *M. oryzae*, P91-15B. Ratio of appressoria which penetrate ≧2, 1 or 0 cells of epidermis of leaf sheath was shown by black, gray, and white bars, respectively. Error bars indicate SD. No significant differences were found against results in non-transgenic rice (NT) in Dunnet's test at $p < 0.05$. #23 (underlined) indicates a null RNAi line.

death in the cells surrounding the pathogen-invaded cells. Similar, confirmatory, results were obtained using the *OsHsf23*-RNAi lines. In total, based on these observations, we conclude that CIGR2 functions in the suppression of excessive cell death triggered by the activation of R-gene mediated resistance. Additionally, as shown, this activity is mediated in part by the expression and activity of *OsHsf23*. We previously reported that suppression of *CEBiP* or *OsCERK1*, involved in GNn recognition in rice, results in the enhanced susceptibility of rice to *M. oryzae*, indicating that low level of GNn detectable by these receptors is generated during rice-*M. oryzae* interactions.[29,30] It is, therefore, tempting to speculate that the locally produced GNn induces *CIGR2* and *OsHsf23*, leading to the suppression of excessive cell death during incompatible interactions.

In plants, HSTs are divided into three primary classes: A, B, and C.[31] The HST characterized herein, *OsHsf23*, encodes a B-type HST, identifiable by the absence of a trans-activation domain localized within the C-terminal region of the protein. To date, two genes encoding for B-type HSTs from *Arabidopsis*, *AtHsfB1*, and *AtHsfB2b*, have been shown to encode transcriptional repressors of a suite of genes associated with response to heat shock.[32] By analogy, we posit that OsHsf23 plays a role as a repressor of transcription of genes associated with HCD. To this end, the work described in the present study represents the first report demonstrating the role of a B-type HST in cell death. In *Arabidopsis*, *AtHsfA4a*, encoding an A-type HST, is a prominent factor in the tolerance to oxidative stress, likely through the regulation of the gene for ascorbate peroxidase, a key enzyme in the quenching system of reactive oxygen species.[33] The orthologue of *AtHsfA4a* in rice, *Spl7*, which was identified as a causal gene of lesion-mimic phenotype of *spl7*, is the only HST reported to be involved in cell death.[34] In this study, an analysis of the nucleotide coding sequence revealed that *spl7* encodes a dominant negative form which lacks DNA-binding activity. Thus, the target gene of Spl7 is considered to be involved in cell death signaling via the regulation of reactive oxygen species. Because *OsHsf23*-RNAi lines did not exhibit any growth retardation or lesion-mimic phenotype under normal growth conditions (data not shown), we posit that the mode of action of OsHsf23 is distinct from that of Spl7.

Author contributions

E. Minami, N. Shibuya, R.B. Day, and Y. Nishizawa designed the research. S. Tanabe, N. Hara, H. Onodera, S. Toki, N. Ishii-Minami, T. Hagio, Y. Fujisawa, and Y. Nishizawa performed experiments. E. Minami, N. Shibuya, Y. Nishizawa, and B. Day wrote the paper.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Supplemental material

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