ADDENDUM

Knock-down of a RING finger gene confers cold tolerance

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ABSTRACT
The plant-specific RING-domain finger proteins play important roles in plant development and stress responses. We recently identified and functionally characterized a stress-induced gene OsSRFP1 (Oryza sativa Stress-related RING Finger Protein 1) from rice.1 We showed evidences of the biotechnological potential of the suppression of OsSRFP1 expression in conferring cold tolerance. The increased cold tolerance of OsSRFP1 knock-down plants was associated with higher amounts of free proline and activities of antioxidant enzymes. In vitro ubiquitination assays showed that OsSRFP1 possessed E3 ubiquitin ligase activity. Some predicted interacting partners of OsSRFP1 might be the substrates for OsSRFP1-mediated protein degradation. Interestingly, OsSRFP1 had trans-activation activity, suggesting the dual roles of OsSRFP1 in post-translational and transcriptional regulations in stress responses.

During its entire life, crop is frequently encountered with various abiotic stresses, such as cold, high salinity, and drought. These stresses have become the major worldwide agricultural problems limiting crop productivity. Thus, molecular dissection of the complex mechanism plants in response to abiotic stresses is of significant importance and represents one of the major research topics in crop today.2-7 Crops can recognize stress and trigger appropriate responses involving the changes in metabolism, growth and development.8,9

One major regulatory network that permits plants to sense external environment changes within short time into a cellular response is the ubiquitination pathway.10,11 The ubiquitination pathway is highly conserved in all eukaryotes and involves the concerted activities of 3 consecutive enzymes, E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase) ligases.12,13 Ubiquitination mediated by E1, E2, and E3 conjugates either single- or multiple-ubiquitin molecules to the target protein, thus enabling the ubiquitin-labeled protein to be recognized by the 26S proteasome and targeted for degradation. In the process, E3 is the last step in the transfer of ubiquitin but is also responsible for recruiting the target protein for ubiquitination. E3 is considered the major substrate recognition component in the pathway. The ubiquitin proteasome pathway represents a fast-responding, flexible, and complex regulatory network for cellular communication to response to external stress, making it an ideal tool for plants to control their development and physiology in concert with their environments.

Plants generally encode for only 1 or 2 E1 proteins,14 while with significantly higher number of E2 proteins, for example, including 36 E2 isoforms in Arabidopsis that cluster into 12 groups. The greatest diversity was found for E3 ligases and is estimated to range above 1000 in plants. The large number and diversity of E3 ligases confer specificity upon the ubiquitination pathway. E3 ligases are subdivided into the HECT, U-box, and RING domain classes in Arabidopsis.15 This diversity of the pathway and its rapid reaction rate are highly suitable for plants to respond to changes in the environment and to allow precise timing of developmental steps and growth.10,16
Based on the large number of different RING-finger proteins, it is not surprising that a multitude of work has been done on RING-type E3s in recent years, emphasizing the broad impact they have in plant development, hormonal control, and defense against biotic and abiotic stresses.\(^{11}\) A number of stress-inducible RING proteins can enhance the plant tolerance to salinity, drought, and low-temperature stresses.\(^{17}\) In rice, many RING finger proteins have been reported with roles involved in stress responses. For instance, overexpression of RING finger genes OsRDCP1 and OsCTR1 which encode E3-ubiquitin ligases, improved drought tolerance of the transgenic plants,\(^ {18,19} \) whereas OsHCI1 overexpression conferred high temperature tolerance.\(^ {20} \) E3-ubiquitin ligase OsHOS1 functioned in the modulation of cold stress response,\(^ {21} \) while the SINA E3 ligase OsDIS1 played a negative role in drought-stress tolerance through the regulation of stress-related genes by post-translational regulation of OsNek6 in rice.\(^ {22} \)

Thus, RING proteins seem to be key regulatory components of stress tolerance and therefore can be considered as candidate genes for enhancing stress tolerance in crops. RING-finger proteins, most belonging to the E3 ubiquitin ligases, play critical roles in response to abiotic stresses in plants. Knocking-down a stress-induced RING finger gene OsSRFP1 (MSU ID LOC_Os03g22680) encoding an E3 ubiquitin ligase, confers cold tolerance through enhancing antioxidant protection in rice, which indicates that suppression of OsSRFP1 is a powerful tool for breeding high-yield crops under unfavorable growth conditions. The objective of this work was to explore the molecular mechanisms of OsSRFP1 involved in abiotic stress tolerance in rice, and, at the same time, to present potential applications of OsSRFP1 in improving tolerance of rice against abiotic stresses, such as cold.

We undertook a preliminary approach to study the role of OsSRFP1 in stress tolerance by measuring its transcript levels in response to several kinds of abiotic stresses and growing transgenic plants in the presence and absence of stress. The expression of endogenous OsSRFP1 was found to be induced by salt, drought, cold, H\(_2\)O\(_2\), and abscisic acid treatments. OsSRFP1 is ubiquitously expressed in various rice tissues, with the higher expression levels in roots, panicles and culm nodes.\(^ {1} \) The constitutive expression of OsSRFP1 seems to be essential for plant growth and development.

**Figure 1.** Silencing of OsSRFP1 confers cold tolerance in rice. Phenotypes of 2-week-old transgenic rice plants and the wild type before and after cold treatment, followed by recovery for 2 weeks.

**Figure 2.** Distribution of stress-related cis-acting elements in the 2,000 bp promoter region of OsSRFP1. The 2,000-bp upstream sequence of OsSRFP1 was analyzed by Matinspector program at Genomatix website (http://www.genomatix.de/). Different cis-elements for TFs were labeled with different colors.
under normal growth conditions through maintaining the low levels of the proteins affecting plant growth. The higher cold tolerance was observed in OsSRFP1 RNAi plants (Fig. 1) suggests that the proteins involved in stress tolerance were released due to the repression of OsSRFP1 and these proteins may directly help in stress tolerance in rice. The increased cold tolerance of transgenic plants was linked to higher amounts of free proline and lower levels of electrolyte leakage in OsSRFP1 knock-down transgenic plants, suggesting that silencing of OsSRFP1 maintains the stability of membrane systems, which is the typical index of cold tolerance. Moreover, it was found that the antioxidant system was more efficient in RNAi plants and this could be the foundation of the improved cold tolerance as many genes involved in antioxidative responses were observed by microarray analysis. The changes of gene expression may result in the changes in protein expression and activity. Overall, under normal growth conditions, activities of POD (Peroxidase), T-SOD (Total Superoxide dismutase), and CAT (Catalase) were comparable between wild type and OsSRFP1-RNAi plants. There were marked increases in activities of these enzymes for the rice seedlings upon exposure to cold stress. The cold stress-induced increases in activities of POD, T-SOD, and CT were higher in the OsSRFP1-RNAi plants than in wild type plants. The study of antioxidant enzymes activities between wild type and OsSRFP1-RNAi plants could be critical to assess the role of OsSRFP1 in response to cold stress.

Transcriptional activities of plants are also critical in responses to environmental stresses. In plant stress responses, transcriptional regulatory networks affecting stress-responsive gene expression play central roles in conferring stress tolerance and protecting plants from adverse environmental conditions. Plant stress responses are regulated by multiple signaling pathways that activate gene transcription and the downstream machinery. In the signal transduction network from the perception of stress signals to stress-responsive gene expression, various transcription factors (TFs) and cis-acting elements in stress-responsive promoters function in the plant’s adaptation to environmental stresses. In this sense, we have previously shown that besides its E3 ubiquitin ligase activity, OsSRFP1 possessed the trans-activation activity. Moreover, OsSRFP1 was localized in the nucleus. In accordance with these findings, the observation that OsSRFP1 overexpression has a high impact on the rice transcriptome favors that OsSRFP1 may act at the transcriptional level for regulation of stress-related genes. It is interesting that an E3 ubiquitin ligase activity of OsSRFP1 may involve in the changes in protein expression and activity between wild type and OsSRFP1-RNAi plants. The study of antioxidant enzymes activities between wild type and OsSRFP1-RNAi plants could be critical to assess the role of OsSRFP1 in response to cold stress.

| Table 1. Distribution and sequences of stress-related cis-acting elements in the promoter region of OsSRFP1. |
| --- | --- |
| Position | Sequence |
| ABRE | 1346–1362| cgcATTCtccatt |
| CNAC | 216–236| tgcGCTTtcattgaaat |
| NACF | 408–428| attgCCTtcaggagagtgt |
| DREB | 792–812| ccttCCTTtcgccgcatctc |
| OsSRFP1 | 558–584| cttaaaggtctcagacACGctcattta |
| NACF | 560–586| cttcaaaaggtcagacACGctcattat |
| DREB | 860–886| tcatcgttgactacgtCACGCCactct |
| OsSRFP1 | 862–888| accttgctacctgcACGCCacact |
| NACF | 1603–1629| aqctgatcatactcCAAGaaagtga |
| DREB | 1720–1746| acaatatttttACTGgttatcttt |
| OsSRFP1 | 1811–1837| tggtaacctgactACGctcactta |
| MYBL | 311–331| gatgACGCGcGacca |
| NACF | 1980–2000| tgggccGGGgcgcacgcac |
| ERRE | 797–815| fGGGagaacggaagaat |
| EPFF | 1659–1677| aACGGGgtcaactagact |
| DREB | 783–801| cgggagctaccttcCACG |
| GBOX | 1659–1677| aagtttaaaaaAGTcactac |
| HEAT | 968–990| tcagaTGAGcatcttcgc |
| ATAT | 1343–1363| tttatatACTGgttcactag |
| DREB | 1810–1830| tgaagatACGCGactcctca |
| WBXF | 13–29| aagagagtctcGGAaga |
| SALT | 468–484| tttataATTCTcattt |
| MYBL | 481–497| taaatatttAAGAagg |
| MIG | 521–537| tggtaaatttAAGAAc |
| MYBL | 917–933| catagcccACATGctcatt |
| MYBL | 1607–1623| tccaaatcttcACGAAaa |
| WBXF | 1752–1768| tggatcataaAGAtctc |
| SALT | 316–332| agcattTGACgcaaca |
| MIG | 1249–1265| taattTTGTacataaag |
| MYBL | 1903–1917| gtttgtGGGggggt |
| MYBL | 286–279| caaaatTGGGgagat |
| MYBL | 318–311| caaaatTGGGgagat |
| SALT | 841–834| atacagTGGGgagat |
| MYBL | 1522–1515| ataatTTGTgacttc |
| MYBL | 62–78| aatcctcaAGTTAggca |
| SALT | 252–268| agttgttGGGGgcgtcaca |
| MYBL | 746–762| taagatgtGTGtctagg |
| MYBL | 750–766| atcggtTATGtctgat |
| MYBL | 754–770| tggatTTGTgacttg |
| MYBL | 824–840| tgaatcACGTGctg |
| MYBL | 1192–1208| aagattTTCGgtatctg |
| MYBL | 1336–1352| tgttcttACGTatcaaa |
| MYBL | 1354–1370| ataatTTTATaac |
| MYBL | 1394–1410| aatataCTGTtctgta |
| MYBL | 1480–1496| ttaaaatCTTAAaggt |
| MYBL | 1485–1501| ttaatttTTGTTactaa |
| MYBL | 1696–1712| acaggtTAAGCagagac |
| MYBL | 1836–1852| ctagggATGTTact |
| MYBL | 8–24| cagcATCTtcttcataat |
| MYBL | 13–29| aagagATGTTgaggaag |
| MYBL | 514–700| gcccccATCCTcctgg |
| MYBL | 681–697| gftataATTTTttataat |
| MYBL | 899–915| tagATATcagacagac |
| MYBL | 904–920| tggTATATcataaat |
| MYCL | 1319–1335| aacgAAAACTtctgaataaa |
| MYCL | 1324–1340| cagtATTGtcttttataat |
| MYCL | 48–66| gcgaatCATGgttataaag |
| MYCL | 371–389| ttttcTACGtctgacatc |
| MYCL | 613–631| tttcgtATCAAGtattat |
| MYCL | 822–840| cttgaatACAGTggtgctg |
confers transcriptional activation activity, suggesting that OsSRFP1 have dual functions in the regulation of cold tolerance both in transcriptional level and post-translational level.

As OsSRFP1 may have dual functions in modulating stress responses in rice, we have analyzed the cis-elements within the promoter of OsSRFP1 and predicted functional partners in silico for the purpose to further understand the OsSRFP1 functions. The 2,000-bp promoter sequence of OsSRFP1 was cloned and analyzed in silico. The promoter sequence contains some putative stress-related cis-acting elements, such as, ABRE, NACF, DRE, EPFF (EPF-type zinc finger factors binding sites), and MYBS (MYB binding sites) (Fig. 2 and Table 1). These stress-related cis-acting elements may be responsive for stress-regulated expression of OsSRFP1. On the other hand, the results of predicted interactome analysis showed that OsSRFP1 interacts with a variety of different proteins, such as MYB family transcription factor, CHCH domain containing protein, coatomer subunit epsilon, FAD-linked sulfhydryl oxidase ALR, WD repeat-containing protein (Fig. 3). Among the 10 proteins with a score higher than 0.5 from the OsSRFP1 predicted functional partners data, the MYB family transcription factor, and CHCH domain containing protein which may also function as a transcription factor, exhibited an obvious expression induction (>2-fold) under at least one type of abiotic stresses (Fig. 4), suggesting that these predicted functional partners might be coordinately contributed to the stress responses in rice. The interactome analysis suggests that OsSRFP1 might be functioning in post-translational regulations of its interacting proteins to participate in the responses of rice to stress conditions. It will be interesting to characterize the interaction of OsSRFP1 and its partners in detail to understand its exact mechanism in imparting stress tolerance.

Through gene expression analysis, it was observed that the transcripts of numerous genes involved in protection against oxidative stress were reduced in OsSRFP1-overexpression rice plants under cold stress.
It is still unknown that expression changes of these antioxidative genes are due to the transcriptional regulation or indirectly post-translational regulation mediated by OsSRFP1. Nevertheless, identification substrate targets of OsSRFP1 and uncovering the signaling network may shed some light on the molecular mechanism underlying the OsSRFP1-dependent response to abiotic stress.

In this study, we cloned a new gene, characterized its function, and further identified the OsSRFP1-mediated pathway that facilitates rice to stress response. Our findings suggest a new mechanism for rice to overcome abiotic stresses through suppressing OsSRFP1 to enhance antioxidant protection, and provide new insight into the mechanisms underlying plant acclimation to abiotic stresses and thus improve crop tolerance. We knock-down the expression of OsSRFP1 encoding an E3 ubiquitin ligase to confer abiotic stress tolerance through enhancing antioxidant protection in rice, which indicates that suppression of

Figure 4. In Silico expression analysis of predicted OsSRFP1-interacting proteins under stress treatment in rice. Gene ID of predicted OsSRFP1 interacting proteins were analyzed on Affymetrix microarray datasets (http://ricearray.org). (A) Heatmap of expression analysis of predicted OsSRFP1 interacting proteins under stress treatment. Heatmap scale bar: 5, 9, 13. (B) Expression graph analysis of predicted OsSRFP1 interacting proteins under stress treatment. Array element ID: Os.49023.1.S1_x_at is representative of OsSRFP1, Os.10901.1. S1_a_at is representative of the gene coding the MYB family transcription factor, Os.53502.1.S1_at is representative of the gene coding the CHCH domain containing protein. The data in the graph indicate the log2 fold of the microarray data in rice seedlings under drought, salt, and cold stress relative to that under control condition. Data represent means and standard errors of 3 replicates.

Figure 5. Growth of OsSRFP1-RNAi transgenic rice. (A) qRT-PCR identification of OsSRFP1-RNAi transgenic rice lines. Data represent means and standard errors of 3 replicates. (B) The growth of OsSRFP1-RNAi transgenic rice lines at 6-leaf stage. Scale bars = 10cm.
OsSRFP1 is a powerful tool for breeding high-yield crops. There were no significant morphological alterations in RNA interference silencing of OsSRFP1 plants (Fig. 5). Most agronomic traits were unchanged in the transgenic plants (Table 2). That means down-regulation of OsSRFP1 does not affect rice grain yield. This characteristic shows the strong potential value of OsSRFP1 in abiotic stress tolerance engineering. Therefore, our findings not only provide new insights into the functional mechanisms underlying abiotic stress tolerance in plants, but also facilitate molecular breeding efforts to improve cold and salt tolerance in staple crops. The recently discovered RNA-based CRISPR/Cas9 system, offers a more precise route to crop improvement, and creates non-transgenic crops with pre-determined traits.30-32 We believe that this technique is among the most promising new biotechnology tools for plant breeding. Using the CRISPR/Cas9 system to knockdown OsSRFP1 to generate inheritable and “transgene free” targeted genome-modified rice may be a great advance we can expect.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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**Table 2.** Statistic data of agronomic traits of wild type and OsSRFP1 RNAi transgenic lines.

| Trait                    | WT              | RNAi1             | RNAi2             |
|-------------------------|-----------------|-------------------|-------------------|
| Plant height(cm)        | 101.80 ± 1.64   | 100.40 ± 1.34     | 100.00 ± 3.80     |
| Tilling number          | 7.60 ± 1.82     | 6.20 ± 1.30       | 6.40 ± 1.34       |
| Panicle length(cm)      | 20.70 ± 1.51    | 20.16 ± 0.68      | 21.22 ± 1.02      |
| Grain number per panicle| 137.80 ± 38.54  | 127.60 ± 10.21    | 132.80 ± 43.52    |
| 1000 Grains weight(g)   | 26.3 ± 0.16     | 26.1 ± 0.54       | 25.8 ± 0.60       |

WT: Wild type; RNAi1 and RNAi2: OsSRFP1 RNAi transgenic rice lines. Data were collected from samples at maturity. Data represent means and standard errors of 3 replicates (n = 10 individuals in each line).
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