A common wild rice-derived BOC1 allele reduces callus browning in indica rice transformation

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Callus browning, a common trait derived from the indica rice cultivar (Oryza sativa L.), is a challenge to transformation regeneration. Here, we report the map-based cloning of BROWNING OF CALLUS1 (BOC1) using a population derived from crossing Teqing, an elite indica subspecies exhibiting callus browning, and Yuanjiang, a common wild rice accession (Oryza rufipogon Griff.) that is less susceptible to callus browning. We show that BOC1 encodes a SIMILAR TO RADICAL-INDUCED CELL DEATH ONE (SRO) protein. Callus browning can be reduced by appropriate upregulation of BOC1, which consequently improves the genetic transformation efficiency. The presence of a Tourist-like miniature inverted-repeat transposable element (Tourist MITE) specific to wild rice in the promoter of BOC1 increases the expression of BOC1 in callus. BOC1 may decrease cell senescence and death caused by oxidative stress. Our study provides a gene target for improving tissue culturability and genetic transformation.

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In contrast to japonica rice (Oryza sativa ssp. japonica), Agrobacterium tumefaciens-mediated transformation of indica rice (Oryza sativa ssp. indica) is hindered by callus browning. Callus browning is a common feature of many plant species that presents problems for in vitro culture, a process required for transgenic breeding, resulting in decreased regenerability, poor growth, and even death. The use of antioxidants, adsorbing agents, low salt concentrations, and growth regulators may lessen the effects of callus browning to a certain degree, but because some species do not tolerate these treatments, there is no universal solution to this problem.

The physiological and biochemical mechanisms of callus browning have been extensively studied. For instance, reducing the activity of polyphenol oxidase (PPO), which catalyzes the oxidation of phenols to quinones that in turn polymerize to form brown pigments, can mitigate the browning of callus. Browning calli have lower antioxidant enzyme activity than non-browning calli and oxidative stress is associated with callus browning. Endogenous and exogenous ethylene (ET) cause browning in plant cell cultures. In addition, reduced nitrite reductase activity leads to callus browning.

Different periods of in vitro rice tissue culture are controlled by many complex genes, respectively. Several quantitative trait loci (QTLs) for culturability traits in rice have been identified. Using positional cloning, a major QTL gene encoding ferredoxin-nitrite reductase (NIR) that determines regeneration ability in rice was identified. QTLs involved in callus browning have been identified on all 12 rice chromosomes. However, to our knowledge, there have been no reports on the isolation of genes related to callus browning in any plant species. The molecular mechanisms underlying physiological processes inducing browning in plant cell culture still remain unclear.

Genetic analyses of rice culturability have mainly been performed on cultivated rice, and few studies have employed common wild rice (Oryza rufipogon Griff.). In this study, we isolate and characterize a gene responsible for mitigating callus browning in rice, named BROWNING OF CALLUS1 (BOC1). The BOC1 allele is specific to common wild rice. BOC1 is a allele of a rice homolog of SRO (SIMILAR TO RCD ONE), termed OsSRO1c. The appropriate upregulation of BOC1 significantly reduces callus browning during callus proliferation. BOC1 is upregulated in common wild rice callus due to the presence of the Tourist MITE in the promoter region of BOC1. Additionally, BOC1 improves the genetic transformation efficiency and frequency of Hygromycin (Hyg)-resistant calli following gene transfer. BOC1 may regulate oxidative stress and programmed cell death (PCD), which may repress the callus browning. Our findings not only elucidate the mechanisms of callus browning during tissue culture, but also provide important insights into establishing a suitable genetic transformation system for the molecular breeding of rice.

Results

Phenotypic analysis of introgression line YIL25. To identify the gene responsible for callus browning in rice, we constructed a set of introgression lines using a Yuanjiang common wild rice (YJCWR, O. rufipogon) accession that is relatively resistant to browning (Fig. 1b) as a donor and an elite indica cultivar Teqing (O. sativa) that is more susceptible to callus browning (Fig. 1a) as the recipient. We screened the introgression line YIL25 by inoculating mature seeds on unimproved NB (UNIB) medium (NB basal medium without additives) for 7 days, and transferred the resulting scutellum-derived calli to UNIB medium for 3 weeks of subculture (Fig. 1c). The callus browning rate (CBR) and callus browning index (CBI) were lower in YIL25 than Teqing (Fig. 1j, k). YIL25 contained three YJCWR chromosomal segments on the short arm of chromosomes 2, 3, and 5 (Supplementary Fig. 1). We, therefore, chose YIL25 as a reduced-browning line to cross with Teqing. The F1 plants showed similar phenotypes of CBR and CBI to YIL25 (Supplementary Fig. 2). These results indicated that the reduced-browning trait is controlled by a dominant gene. The histological examination indicated that the cell arrangement of YIL25 calli was more even and the cells were smaller and denser compared to Teqing (Fig. 1d, e). Observations using scanning electron microscopy (SEM) revealed that YIL25 calli consisted of many globular nodules (Fig. 1h) and the cells of YIL25 were turgid (Fig. 1i), whereas Teqing calli had fewer globular nodules (Fig. 1f) and the cells of Teqing were accid...
RT-qPCR analysis showed that the relative expression levels of LOC_Os03g12820 were significantly elevated in the pCPL-BOC1-Teqing positive plants compared to the negative plants (Teqing) (Fig. 3j). The CBI was reduced in these positive lines when the expression level of LOC_Os03g12820 was elevated at least 1.8 fold compared with the negative plants (Fig. 3f–j). This finding indicates that LOC_Os03g12820 mitigates the callus browning phenotype. These results suggest that LOC_Os03g12820 is BOC1 regulates callus browning in rice.

Next, we generated transgenic Teqing plants overexpressing the LOC_Os03g12820 coding sequence of YIL25 (as shown in Supplementary Fig. 3, the rose red and red underline represent the nucleotide sequence of the overexpression construct, designated as pOE-BOC1-Teqing) and obtained 22 positive transgenic lines. RT-qPCR indicated that BOC1 was strongly upregulated in the pOE-BOC1-Teqing positive transgenic lines compared to the negative plants (Fig. 3o). Nevertheless, when BOC1 expression levels were 8.8-times higher in the tested pOE-BOC1-Teqing positive lines compared to the negative plants, callus browning was more severe (Fig. 3k–o). This result indicates that only the appropriate expression of BOC1 reduces callus browning.

BOC1 encodes an SRO protein of 463 amino acids, which includes a putative N-terminal RNA Recognition Motif (RRM) domain, a poly (ADP-ribose) polymerase (PARP)-like ADP-ribose transferase catalytic domain, and a C-terminal RST (RCD1, RADICAL-INDUCED CELL DEATH1—SRO [SIMILAR TO RCD ONE]—TAF4 [TBP-ASSOCIATED FACTOR4] (RST) domain (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi; Supplementary Fig. 4a). To evaluate the conservation of BOC1

Fig. 1 Phenotypic analysis of calli subcultured for 21 days. a–c Callus browning phenotypes observed in the calli after subculture for 21 days including a indica variety Teqing, b Yuanjiang wild rice (Oryza rufipogon, YJCWR), and c the introgression line YIL25. Scale bars, 2 cm. d, e Paraffin sections of d Teqing and e YIL25. Scale bars, 100 μm. f–i Scanning electron microscopy of Teqing and YIL25 calli. f Teqing calli. Thirty calli each from Teqing and YIL25 were collected for analysis of paraffin sections and scanning electron microscopy. Scale bars, 300 μm. g Enlarged from (f). Scale bars, 40 μm. h YIL25 calli. Scale bars, 300 μm. i Enlarged from (h). Scale bars, 40 μm. j, k Comparison of the CBR and CBI values of various calli. Values in (j, l) are means ± SE (n = 3 biologically independent samples). Two-tailed Student’s t-tests were performed to determine significant differences. Source data underlying Figs. j, k are provided as a Source Data file.
in other plant species, we performed protein sequence alignment using the full-length protein sequence of BOC1 as a query in BLAST analysis and selected 24 putative homologs (sharing > 80% sequence identity with BOC1 at the nucleotide level) to construct a phylogenetic tree. Phylogenetic analysis indicated that BOC1 is closely related to other monocots, such as *Oryza brachyantha*, *Brachypodium distachyon*, *Triticum urartu*, and *Aegilops tauschii* (Supplementary Fig. 4b).

**Transcriptional characterization of BOC1.** To elucidate the expression profiles of BOC1 in different organs by RT-qPCR, the expression level of BOC1 was highest in YIL25 calli after 21 days of subculture, followed by the tiller base, roots, culms, and leaves, and it was lowest in young panicles (Supplementary Fig. 5). BOC1 expression increased gradually in Teqing and YIL25 throughout tissue culture. BOC1 expression did not significantly differ between Teqing and YIL25 during the early stage of tissue culture, but significant differences were observed during the later stage; BOC1 expression was notably higher in YIL25 than in Teqing in callus after 21 days of subculture (Fig. 4a). We further examined the expression of BOC1 in calli after 21 days of subculture by RNA in situ hybridization and observed that the hybridization signals were stronger in YIL25 than in Teqing, confirming the notion that BOC1 is expressed at higher levels in YIL25 callus (Fig. 4b).

Sequencing analysis revealed one 337 bp deletion, one 1 bp deletion, and three SNPs in the promoter region of BOC1 between Teqing and YIL25 (Fig. 4c). To determine whether these deletions and SNPs in the BOC1 promoter region directly
inverted-repeat transposable element (Tourist) deletion is responsible for the reduced expression level of that of Teqing (Fig. 4c). These results suggest that the 337 bp decreased the promoter activity of BOC1 However, the 337 bp deletion (construct M1) significantly

query and determined that it is the Tourist rice transposable element database using the 337 bp sequence as a

repeatmasker.org/cgi-bin/WEBRepeatMasker), we examined the Teqing calli. Using the RepeatMasker Web Server (http://www.

of YIL25 into binary vector pGreenII 0800-

inserting different combinations of mutant promoter fragments contribute to its expression, we generated various constructs by inserting different combinations of mutant promoter fragments of YIL25 into binary vector pGreenII 0800-LUC. We introduced these constructs into rice protoplasts produced from callus that had been cultured for 21 days and examined the expression of the luciferase reporter gene (LUC). As shown in Fig. 4c, no significant differences in activity were observed between the YIL25 promoter fragment and mutated promoter fragments containing three

SNPs (constructs M2–M4) and one 1 bp deletion (construct M5). However, the 337 bp deletion (construct M1) significantly decreased the promoter activity of BOC1 to a level similar to that of Teqing (Fig. 4c). These results suggest that the 337 bp deletion is responsible for the reduced expression level of BOC1 in Teqing calli. Using the RepeatMasker Web Server (http://www. repeatmasker.org/cgi-bin/WEBRepeatMasker), we examined the rice transposable element database using the 337 bp sequence as a query and determined that it is the Tourist-like miniature inverted-repeat transposable element (Tourist MITE), which belongs to the PIF/Harbinger superfamily. In addition, using

PLACE (Plant cis-acting Regulatory DNA Elements; http://www. dna.afrc.go.jp/htdocs/PLACE/), we identified three DOFCOREZM (AAAG, DOF TFBS)\textsuperscript{25}, one MYB1AT (TAACCA, MYB TFBS)\textsuperscript{26}, one MYCCONSensusAT (CAGATG, MYC TFBS)\textsuperscript{26}, and one ACGTATERD1 (ACGT, ACGT TFBS)\textsuperscript{28} in this sequence; these elements are associated with plant stress responses (Supplementary Fig. 6). Subcellular localization analysis indicated that the BOC1-GFP fusion protein was specifically targeted to the nucleus in rice protoplasts (Fig. 4d).

BOC1 influences genetic transformation efficiency. To determine if BOC1 enhances the transformation efficiency of rice due to decreased callus browning, we transformed the binary vector pCAMBIA1300 into YIL25 (YIL25 1300), Teqing (Teqing 1300), and Teqing 1300 as a control. The pCAMBIA1300-BOC1 complementation construct from YIL25 was transformed into Teqing (Teqing pCPL) by Agrobacterium tumefaciens-mediated transformation and cultured on UINB medium for hygromycin (Hyg)
**Fig. 4 Transcriptional characterization of BOC1.**

**a** Expression analysis of BOC1 in calli at different stages of subculture. Data are means, with bars showing SE, \( n = 3 \) biological replicates. Two-tailed Student’s t-tests were performed to determine significant differences.

**b** BOC1 expression patterns in Teqing and YIL25 calli subcultured for 21 days, as revealed by mRNA in situ hybridization. Thirty calli each from Teqing and YIL25 were collected for mRNA in situ hybridization. Scale bars, 100 \( \mu \)m.

**c** Transient expression assay constructs and expression levels of Luc relative to Ren. Data are presented as mean ± SE. Horizontal bars show the SE for all constructs (\( n = 10 \) biological replicates, two-tailed Student's t-tests). P-values of (a, c) were calculated with two-tailed Student’s t-tests. Subcellular localization assays were performed with three biologically independent experiments. Source data underlying Figs. a, c are provided as a Source Data file.
Physiological indices related to oxidative stress. To further explore the molecular mechanisms of callus browning, we measured various physiological and biochemical indices in calli at 21 days of subculture. Compared to Teqing, the $\text{H}_2\text{O}_2$ contents were lower in both YIL25 and pCPL calli and higher in pOE (pOE-BOC1-Teqing) calli (Fig. 6a; Supplementary Fig. 7a). In addition, we used Noninvasive Microtest Technology (NMT) on rice callus to detect intercellular $\text{H}_2\text{O}_2$ fluxes. There was a significant efflux of intracellular $\text{H}_2\text{O}_2$ in YIL25 and pCPL calli, whereas Teqing calli exhibited a significant influx of extracellular $\text{H}_2\text{O}_2$, suggesting that $\text{H}_2\text{O}_2$ was expelled from YIL25 and pCPL cells to alleviate cellular damage (Fig. 6b). MDA (malondialdehyde) is a biomarker of lipid peroxidation and an indicator of reactive oxygen species (ROS) production. Compared to Teqing, MDA contents were notably lower in the YIL25 and pCPL lines but higher contents in the pOE lines (Fig. 6c; Supplementary Fig. 7b), reflecting less damage from $\text{H}_2\text{O}_2$ in YIL25 and pCPL plants. Moreover, catalase (CAT), glutathione reductase (GR), and glutathione S-transferase (GST) activity and antioxidant glutathione (GSH) contents were higher in YIL25 and pCPL vs Teqing callus (Fig. 6d–g). However, GST activity and GSH contents were lower in pOE lines than in Teqing callus although CAT and GR activity did not significantly differ among lines (Supplementary Fig. 7c–f).

Finally, the levels of the plant hormones salicylic acid (SA) and ethylene (ET), as well as the direct ET precursor, 1-amincyclobutanolic acid (ACC), were significantly lower in YIL25 compared to Teqing (Fig. 6h–j). Collectively, these findings suggest that BOC1 may inhibit PCD caused by oxidative stress, thereby reducing callus browning.

RNA-seq analysis of BOC1. To analyze the molecular function of BOC1, we performed RNA-seq experiments using callus that had been subcultured for 21 days from Teqing and YIL25, Teqing and the complementation line pCPL-BOC1-Teqing (pCPL), and also Teqing and the overexpression line pOE-BOC1-Teqing (pOE). We identified 700 differentially expressed genes (DEGs), including 410 DEGs that were upregulated in YIL25 versus Teqing callus and pCPL versus Teqing callus and downregulated in pOE versus Teqing callus (UUD, Fig. 7a, Supplementary Data 1). The 290 remaining DEGs were downregulated in YIL25 versus Teqing callus and pCPL versus Teqing callus and upregulated in pOE versus Teqing callus (DDU, fold change $\geq 2$, FDR < 0.001) (Fig. 7b, Supplementary Data 2).

Among the UUD genes, 98 genes were involved in stress responses and cell death (Fig. 7c). Notably, these included 26 pentatricopeptide repeat protein genes, whose dysfunction leads to ROS accumulation. These genes were upregulated in YIL25

Fig. 5 BOC1 influences genetic transformation efficiency. a–c Transformed calli after three rounds of selection on Hyg-resistant UINB medium. Scale bars, 1 cm. d Comparison of the selection frequency of Hyg-resistant callus. Each dot represents the selective frequency of Hyg-resistant callus of 10 independent experiments. (e) Comparison of the transformation efficiency. Each diamond represents the transformation efficiency of 10 independent experiments. Teqing 1300, Teqing calli infected with Agrobacterium harboring the binary pCAMBIA1300 empty vector as a control. YIL25 1300, YIL25 calli transformed with the binary vector pCAMBIA1300. Teqing pCPL, Teqing calli infected with Agrobacterium harboring the pCAMBIA1300-BOC1 construct. The hollow box in each column in (d, e) represents the means of the selective frequency of Hyg-resistant callus and the transformation efficiency of 10 replicates, respectively. Box edges represent the 0.25 and 0.75 quantiles, with the median values shown by red, blue and yellow lines, respectively. Whiskers extend to data no more than 1.5 times the interquartile range, and the remaining data are indicated by dots. P-values of (d, e) were determined by two-tailed Student's t-tests. Source data are provided as a Source Data file.
and pCPL but downregulated in pOE (Fig. 7c; Supplementary Fig. 8a). GST conjugates with GSH to scavenge ROS in order to protect cellular components from damage. In the current study, 3 GST genes present in the UUD DEG set and absent in the DDU DEG set (Fig. 7c; Supplementary Fig. 8c). In addition, compared to Teqing, GST activity and GSH contents were higher in YIL25 and pCPL callus but lower in pOE callus (Fig. 6f, g; Supplementary Fig. 7d,f). Among the UUD genes were four aldehyde dehydrogenase genes; aldehyde dehydrogenase enhances tolerance to various abiotic stresses by scavenging cytotoxic aldehydes (Supplementary Fig. 8d), and MDA contents were higher in of Teqing and pOE callus compared to YIL25 and pCPL callus (Fig. 6c; Supplementary Fig. 7b).

\[ \text{LOC_Os01g67980} \]
which negatively regulates the senescence process, encodes senescence-associated protein OsSAG12-1. RNAi transgenic lines declined the expression of \( \text{OsSAG12-1} \) and developed early senescence31 (Fig. 7c; Supplementary Fig. 8f). In addition, the UUD genes included transcription factor genes associated with stress and defense responses, which might function in maintaining ROS homeostasis (Fig. 7c; Supplementary Fig. 8b, g).

Twelve DDU genes were associated with oxygenase and oxidase; cytochrome P450 has monooxygenase activity as a source of ROS32 (Supplementary Fig. 8h). The DDU group also included genes encoding aspartic proteinases, peptidases, and nuclease (involved in cell degradation), as well as genes encoding polygalacturonase, endoglucanase, and glycosyl hydrolases (involved in cell wall degradation) (Fig. 7c; Supplementary Fig. 8i). Emerging evidence suggests that aspartic proteases are involved in plant development and cell death33. Finally, five DDU DEGs encode positive regulators of the senescence process (Fig. 7c). These DEGs include \( \text{LOC_Os01g53260} \), encoding WRKY transcription factor OsWRKY23, which positively...
regulates the senescence process\textsuperscript{34}, and $\text{LOC}_{\text{Os}}12\text{g}36\text{h}5\text{O}$, encoding a pathogenesis-related bet v I family protein similar to probenazole-inducible protein PBZ1, which induces cell death in rice, tobacco, and \textit{Arabidopsis} via its RNase activity inside the cell\textsuperscript{35} (Supplementary Fig. 8j). When we treated callus that was subcultured for 7 days with 0.5\% H$_2$O$_2$ for 18 h, PBZ ($\text{LOC}_{\text{Os}}12\text{g}36\text{h}5\text{O}$) was upregulated compared to the untreated control (Supplementary Fig. 8k).

Taken together, these findings suggest that the appropriate expression of \textit{BOC1} may be beneficial for rice cells to be in reducing state, prevented various stress, and then inhibited cell death.

\textit{BOC1} promoter sequence variation affects callus browning. To investigate the relationships between mutations in the \textit{BOC1} locus and callus browning, we sequenced a ~4.6 kb genomic fragment covering the entire \textit{BOC1} gene (2740 bp), the 1452 bp 5′-flanking region, and the 380 bp 3′-flanking region, from 50 \textit{O. rufigogon} accessions (Supplementary Data 3) and 74 Asian rice cultivars (28 \textit{japonica} and 46 \textit{indica} cultivars, Supplementary Data 4). Nucleotide alignment showed that only 19 \textit{O. rufigogon} accessions contained the \textit{Tourist} MITE (Supplementary Data 3); however, the element at this locus was absent in all Asian rice cultivars examined (Supplementary Data 4). Therefore, we performed an association test of callus browning after 21 d of subculture vs. sequence variations in the \textit{O. rufigogon} accessions and Asian rice cultivars. The association test of the common wild rice revealed the strongest association signals at the \textit{Tourist} MITE and the four SNPs (Fig. 8a). Furthermore, we investigated the CBI of the 50 accessions of common wild rice and determined that this value was lower in the 19 accessions with the \textit{Tourist} MITE than in the 31 accessions without this insertion (Fig. 8b). Moreover, the \textit{BOC1} gene was expressed at higher levels in the 19 accessions with the \textit{Tourist} MITE than in the 31 accessions without the \textit{Tourist} MITE (Fig. 8c; Supplementary Data 3). In addition, we obtained previously reported the genome resequencing data for 446 \textit{Oryza rufigogon} accessions\textsuperscript{36} and recovered the reads that mapped to the \textit{Tourist} MITE flanking sequence in these accessions. We detected this site in 226 accessions (48 accessions with this MITE insertion and 178 accessions without this insertion). Forty-one of the 48 accessions with this insertion were in group

![Diagram](https://example.com/diagram.png)
III (closer to japonica) and 7 were in group II (the intermedia group between indica and japonica) (Supplementary Fig. 9a). By contrast, the 178 accessions without this MITE insertion were distributed among all the three groups including 65 in group I (closer to indica), 49 in group II, and 64 in group III (Supplementary Fig. 9a).

Nucleotide alignment of the 74 Asian rice cultivars revealed 9 Indels and 27 SNPs (Fig. 8d; Supplementary Data 4). To ascertain whether BOC1 was responsible for callus browning in Asian rice cultivars, firstly, an association analysis was carried out between callus browning and sequence variations of 74 Asian rice cultivars. The result indicated that the Indel8 (−433, T indel)
in the promoter of BOC1 was the strongest signals (Fig. 8d). However, this site was no polymorphic in the 50 accessions of O. rufipogon (Supplementary Data 3, 4). We further found that the CBI in 15 varieties with T insertion in the promoter of BOC1 was lower than that in 59 varieties without this insertion (Fig. 8e; Supplementary Data 4). We also analyzed the expression levels in these varieties and the result showed that the expression levels of BOC1 was higher in the 15 varieties with T insertion in the promoter of BOC1 than that in 59 varieties without this insertion (Fig. 8f; Supplementary Data 4). The above results suggest that the sequence variation in the BOC1 promoter influence callus browning by affecting the expression level of BOC1.

Finally, we analyzed the fixation index (FST, the level of population differentiation), on chromosome 3 in 1083 cultivated indica and japonica rice varieties. The FST level in BOC1 was 0.21, indicating that no significant differences in BOC1 exist between the indica and japonica subspecies (Supplementary Fig. 9b). Therefore, we propose that the effect of BOC1 on callus browning is not related to the differences between the indica and japonica subspecies.

Discussion

Here, we demonstrated that the proper expression of BOC1 decreases callus browning and increases the transformation efficiency of rice. BOC1 encodes an SRO (SIMILAR TO RCD ONE) protein that is allelic to OsSRO1c, a regulator of oxidative stress responses. RCD1 plays an important role in regulating cell death by controlling oxidative stress responses in Arabidopsis. OsSRO1c is the major stress-responsive gene in the SRO family. Both osso1c-1 mutants and amiRNA (artificial microRNA)-OsSRO1c plants are hypersensitive to drought stress at the seedling stage. In addition, OsSRO1c-overexpressing rice plants showed more severe damage under drought stress than wild-type plants, whereas plants overexpressing SNAC1, encoding a NAC transcription factor that directly regulates OsSRO1c expression, showed increased drought resistance. In the current study, RNA interference and BOC1 overexpression plants showed a callus-browning phenotype similar to or more severe than that of Teqing, whereas lines harboring the complementation vector exhibited reduced callus browning, indicating that the appropriate upregulation BOC1 reduces callus browning.

Transposable elements, among the most variable components of the genome, can replicate and integrate into positions, which affects the expression levels of adjacent genes, thereby resulting in genetic diversity. For example, the terminal-repeat retrotransposon in miniature (TRIM) elements insertion in the promoter of MS2 allele in the male-sterile ms2 mutant resulted in an anther-specific expression pattern in wheat. In maize, an 82 bp miniature inverted-repeat transposable element (MITE) into the promoter of ZmNAC111 represses its expression; this MITE is significantly associated with natural variation. In the current study, the presence of Tourist MITE in the BOC1 promoter significantly increased BOC1 expression in rice callus. However, the excessive accumulation of H2O2 might be involved in regulating PCD. Moreover, high levels of H2O2 induce the accumulation of SA, resulting in severe oxidative damage. In turn, SA pretreatment mediates the accumulation of H2O2. Endogenous ET in in vitro culture may inhibit the synthesis of protective enzymes, such as CAT, for scavenging H2O2, which is involved in senescence, browning, and necrosis of callus. In the current study, YIL25 callus contained decreased levels of H2O2 and the phytohormones ET and SA, as well as increased levels of CAT, GR, and GST activity compared to Teqing. The RNA-seq data analysis revealed that BOC1 influences the expression of genes associated with ROS production and scavenging, stress response and cell death. In recently report, RCD1 coordinates chloroplast and mitochondrial functions through interaction with transcription factors. RCD1 participates in regulation of mitochondrial respiration and chloroplast ROS processing and may contribute to the survival of plant under a changing environment. However, as shown in Supplementary Fig. 4b, RCD1 and OsSRO1c have different evolutionary histories which may have contrasting functions in the regulation of ROS related processes. In the current study, we use the calli subcultured for 21 d in the dark and is not necessary for photosynthesis by origin of carbon from medium. Therefore, the interaction and phenotype of BOC1 may be different from seedling stage, which will be an interesting research topic.

Common wild rice, the wild progenitor of cultivated rice, has an important gene pool that includes some alleles no longer present in cultivated rice. The insertion of the 337 bp Tourist MITE in the BOC1 promoter increases BOC1 expression. This insertion is only detected in certain accessions of wild rice, but not in cultivated rice. Therefore, wild rice could be used to provide alleles for improving the tissue culture characteristics and genetic transformation efficiency of cultivated rice. Moreover, the genetic manipulation of the BOC1 promoter in cultivated rice might also improve tissue culture ability. Perhaps the BOC1 allele of wild rice and even similar alleles in other crops of the Gramineae family could be used to create recipient varieties more amenable to genetic transformation. The use of BOC1 in in vitro rice callus propagation might help improve genetic transformation efficiency and selection to facilitate the molecular breeding of rice.

Methods

Plant materials. The wild rice introgression line YIL25, with reduced levels of callus browning, was derived from a cross between an O. rufipogon accession (YCWR) as the donor and the elite indica variety Teqing (O. sativa) as the recipient. The mapping population was developed from a cross between introgression line YIL25 and Teqing. The 50 common wild rice accessions and 74 O. sativa
varieties (28 japonica and 46 indica varieties) used in this study are listed in Supplementary Data 3 and Supplementary Data 4.

**Tissue culture procedure.** Mature, healthy, dehusked seeds were sterilized by immersion in 70% ethanol for ~2 min, followed by 15% sodium hypochlorite solution for 15 min with shaking, and rinsed three or four times with sterile water on an ultrasonic workbench. Unimproved NB (UNB) medium (NB medium containing N6 macronutrient components, B5 micronutrient components, and organic component supplemented with 2 mL of 2,4-D and 30 g/L of sucrose) was used for callus induction and subculture. The pH of the medium was adjusted to 5.8 with 1 N KOH, and 3 g/L of Phytagel was added before autoclaving at 121 °C for 20 min. Approximately 90 mature seeds per line, evenly distributed among three Petri dishes, were incubated in induction medium for 7 days at 28 °C in the dark. The induced calli were transferred to subculture (UNB) medium and incubated in the dark at 28 °C for 3 weeks. The traits of the calli in each Petri dish were recorded.

**Phenotype observations.** The tendency for callus browning was categorized into four levels: (0) less than 1/10 of the callus tissue was brown (recorded as no browning); (1) 1/10 – 1/3 of the callus tissue was brown, considered light browning; (2) 1/3 – 2/3 of the callus tissue was brown, considered medium browning; (3) 2/3 – 1 of the callus tissue was brown, considered deep browning; and (4) the callus was completely brown. The tendency for callus browning, selection frequency of Hgy-resistant calli and transformation efficiency were calculated according to the following Eqs. (1, 2, 3, and 4):

\[
\begin{align*}
\text{Callus browning rate (CBR)} & = \frac{\text{(number of calli showing browning)} \times \text{(number of transferred calli)}}{100} \\
\text{Callus browning index (CBI)} & = \left(\frac{\text{(number of calli at each browning level)}}{\text{(highest browning level)}}\right) \times 100 \\
\text{Selection frequency of Hgy-resistant calli} & = \left(\frac{\text{(number of infected calli)}}{\text{(number of transferred calli)}}\right) \times 100 \\
\text{Transformation efficiency} & = \left(\frac{\text{(number of independent transgenic events regenerating plants)}}{\text{(number of transferred calli)}}\right) \times 100
\end{align*}
\]

**Scanning electron microscopy.** To prepare histological sections, calli that had been subcultured for 21 days were fixed in 2.5% glutaraldehyde-phosphate buffer saline fixative solution (pH 7.2) for more than 2 h and postfixed in the same buffer containing 1% OsO4. After being dehydrated through an ethanol series and dried using a critical point dryer, the calli were coated with a thin layer of gold and dried at 45 °C. The dry calli were gold plated and photographed under a Hitachi S3400 scanning electron microscope (Japan).

**Primers.** The primers used in this study are listed in Supplementary Data 5.

**Genetic confirmation.** The RNA interference construct was generated by inserting a hairpin sequence with a 350 bp and a 346 bp cDNA inverted repeat fragment of BOCI into the pTCK303 vector driven by the maize Ubiquitin promoter and transformed into YIL25 (pRl-BOCI-YIL25). A 4572 bp genomic fragment from YIL25 harboring the entire BOCI gene sequence with the 1452 bp 5′-flanking region and the 380 bp 3′-flanking region was amplified and inserted into binary vector pCAMBIA1300 (http://www.cambia.org) to form the complementary construct (pCPL-BOC1-ORF), which was amplified and inserted into the pCAMBIA1301 vector (http://www.cambia.org) driven by the Ubiquitin promoter. Both the pCPL and pOE constructs were transferred into Teqing and designated as pCPL-BOC1-Teqing and pOE-BOC1-Teqing, respectively. Relevant primer sequences are listed in Supplementary Data 5.

**RT-qPCR.** Total RNA was extracted from various samples using TRIzol reagent (Life Technologies) and purified using an RNaseasy Mini Kit (Qiagen) following the manufacturer’s instructions. First-strand cDNA was synthesized using Oligo(dT)15 primers (TaKaRa) and SuperScript III Reverse Transcriptase (Invitrogen) with 3 μg of total RNA. The qPCR was performed using a CF96 Real-Time System (Bio-Rad), with each reaction containing 5 μL of first-strand cDNAs, 4 μM of gene-specific primers, and 5 μL of real-time PCR SYBR MIX (iQ® SYBR® Green Supermix, Bio-Rad) under the following conditions: initial denaturation at 95 °C for 3 min followed by 40 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. The rice housekeeping gene Actin (LOC_Os05g50885) was used as an internal control to normalize the gene expression data using the relative quantification method (2-ΔΔCT)60. Each set of experiments was repeated three times. The primers used for RT-qPCR and cDNA amplification are listed in Supplementary Data 5.

**mRNA in situ hybridization.** mRNA in situ hybridization was performed with the method of Javleel et al.,21 with minor modifications. YIL25 and Teqing calli that had been subcultured for 21 days were fixed in 3.7% FA solution at 4 °C overnight, dehydrated, and embedded in paraffin (Paraplast Plus, Fisher Scientific). The calli were sliced into 10-μm sections with a microtome (Leica RM2145). A 344 bp fragment of BOCI cDNA was amplified and used as the template to generate sense and antisense RNA probes, which were labeled using a DIG RNA labeling Kit (Roche). According to the manufacturer’s instructions, slides were observed under a microscope (Leica DMR). Relevant primer sequences are listed in Supplementary Data 5.

**Luciferase assays.** The promoter sequences upstream of the BOCI promoter start site of YIL25 and Teqing were amplified using specific primers designed with site-directed mutations at the InDels and SNPs. The fragments were inserted into the pGreen 0800-LUC vector to drive the expression of the firefly luciferase (LUC) gene. The effector plasmid DNA was transformed into rice protoplasts prepared from rice calli that had been subcultured for 21 days in the dark using the polyethylene glycol-mediated method.22 The enzyme solution contained the following: 1.5% (w/v) “Onozuka” R-10 cellulase (Yakult Pharmaceutical Industry), 0.75% (w/v) macerozyme R-10 (Yakult Pharmaceutical Industry), 0.5 M D-mannitol, 2 mM 2-(N-morpholino)ethanesulfonic acid (MES; Sigma-Åldrich, cat. no. M3671), and 0.1 × MS medium, pH 5.7. The protoplasts were lysed with Passive Lysis Buffer (PLB, Promega) and assayed using the Dual-Luciferase Reporter Assay System (Promega). Relevant primer sequences are listed in Supplementary Data 5.

**Subcellular localization.** The entire coding sequence of BOCI was cloned into a GFP vector under the control of the 35S promoter to generate 35S-BOCI-GFP. The GFP coding sequence was fused to the C terminus of OsMADS15 to generate OsMADS15-RFP and was used as a nuclear marker. The two constructs were transformed into protoplasts and incubated at 28 °C in the dark for 16 h. Fluorescence was examined under a confocal laser scanning microscope.

**Agrobacterium-mediated genetic transformation.** The binary plasmid vectors described above were transferred into Agrobacterium tumefaciens strain EHA105. Sterilized mature seeds were inoculated on UNB medium and cultured at 28 °C in the dark. After 7 days of incubating, the calli were collected and used for transformation. After 3 days of cocultivation with Agrobacterium at 25 °C in constant darkness on NB-Acetosyringone (AS) medium (30 g/L sucrose, 0.3 g/L casamino acids, NB medium salt mixture [Duchefa Biochemical], 0.5 mg/L glucose, and 2.5 mg/L 2,4-D, 20 mg/L AS, pH 5.3), the calli were washed two or three times with sterile water and rinsed once with sterile water containing 200 mg/L Timentin (Caisson Labs, USA) and 200 mg/L cephalosporin (Wako Pure Chemical Industries). The calli were cultured on UNB medium containing 50 mg/L hygromycin B (Hyg, Merck), 200 mg/L Timentin, and 200 mg/L cephalosporin for three rounds of 15 days each at 28 °C in the dark. For regeneration, vigorously growing calli were transferred to MS regeneration medium (30 g/L sucrose, 30 g/L sorbitol, 2 g/L casamino acids, MS Medium Salt Mixture [Duchefa Biochemical], 1.1 g/L MES, 2 mg/L 1-naphthalene acetic acid, and 1 mg/L kinetin, pH 5.8) and cultured for 15 to 30 days at 28 °C (16 h light/8 h dark). The means in the genetic transformation efficiency test represent data from 10 groups of independent experiments, with ~80 to ~120 transformed calli per group.

**Quantitative determination of H2O2 contents and H2O2 flux.** H2O2 concentrations were measured in calli subcultured for 21 days26. The calli (100 mg) were homogenized in 1 mL 0.1% (w/v) TCA in an ice bath. The homogenate was centrifuged at 12,000 × g for 15 min, and 1 mL of the supernatant was added to 1 mL 100 mM potassium phosphate buffer (pH 7.0) and 2 mL 1 M KI. The absorbance of the supernatant was read at 390 nm based on the fresh weight of the callus. H2O2 flux was measured using the Non-invasive Micro-test Technology (NMT) System (NTMT100-SIM-X, Younger USA Sci. & Tech. Corp., Amherst, MA, USA) at the Xuyue Beijing NMT Service Center (Xuyue Beijing Sci. and Tech. Co., Ltd., Beijing, China). Callus that had been subcultured for 21 d was fixed onto the bottom of a Petri using a filter paper strip and resin block. The callus in the Petri dish was incubated in measuring solution (0.1 mM KCl, 0.1 mM CaCl2, 0.1 mM MgCl2, 0.5 mM NaCl, 0.3 mM MES, 0.2 mM NaSO4, pH 6.5) for ~20 min. The H2O2 concentration was measured in the same buffer, and 5–10 mL of fresh measuring solution. The H2O2 flux microsensor was placed ~10 μm from the calli, and H2O2 flux at each site was measured for 10 min; each measurement repeated four times. Flux data were obtained using imFluxes V2.0 software (Younger USA LLC, Amherst, MA, USA) according to the following equation: a positive value represents influx and a negative value represents efflux. The H2O2 flux was calculated based on Fick’s law of diffusion (5):

\[
J = \frac{Dc}{dx} = -D_s \frac{dc}{dx}
\]
Measurement of lipid peroxidation. MDA was measured using the thiobarbituric acid (TBA) assay\(^6\). The calli (100 mg) were homogenized in 1 ml 0.1% (w/v) TCA in an ice bath. After centrifuging the homogenate at 12,000 × g for 10 min, 0.5 ml of the supernatant was combined with 0.5 ml 0.6% thiobarbituric acid (TBA) and incubated at 95 °C for 15 min. The samples were cooled immediately in an ice bath and the absorbance of the supernatant measured at 450, 532, and 600 nm.

**Assay of GSH content.** GSH content was assayed by Shanghai Sanshu Biotechnology Co., Ltd (Shanghai, China). The reaction mixture contained plant extract, 0.1 M NaH₂PO₄ (pH 7.7), and DTNB (5,5-dithiobis-2-nitrobenzoic acid). Absorbance was recorded at 412 nm\(^6\).

**Enzyme activity assays.** The enzyme activity assays were carried out by Shanghai Sanshu Biotechnology Co., Ltd (Shanghai, China). Catalase (CAT) activity was measured based on the consumption of H₂O₂ and the decrease in absorbance at 240 nm\(^6\). Glutathione reductase (GR) was assayed based on the decrease in Aso due to nicotinamide adenine dinucleotide phosphate (NADPH) oxidation. The reaction mixture of GR contained 1.0 ml of 0.1 M potassium phosphate buffer (pH 8.0) including 0.5 mM EDTA, 0.5 mM MgCl₂, 1 mM NADPH, 10 mM oxidized glutathione (GSSG), and 0.1 ml enzyme extract\(^6\). The glutathione S-transferase (GST) was assayed using the substrate 1-chloro 2,4-dinitrobenzene (CDNB), and the activity was detected spectrophotometrically based on the change in absorbance at 340 nm\(^6\).

**Endogenous hormone level measurements.** The calli were ground into a powder in liquid nitrogen in a mortar and each sample (0.05 g) was transferred to a 1.5 ml screw-cap tube. After adding 50 μl of internal standards and 500 μl of extraction solvent (2-propanol: H₂O: HCl = 2:1:0.002) to each tube, the sample was incubated on a shaker at 900 × g for 30 min at 4 °C. After adding 1 ml trichloromethane, the sample was shaken for 30 min and centrifuged at 14,000 × g for 5 min at 4 °C to form two phases. A 1.2 ml sample of solvent from the lower phase was dried in a nitrogen evaporator, dissolved in 0.1 ml methanol and endogenous SA levels quantified by high-performance liquid chromatography-mass spectrometry (HPLC-MS/MS\(^6\)).

Ethylene was analyzed according to a published method\(^7\) with minor modifications. The calli were placed into 100-ml vials that were promptly sealed with a rubber plug and incubated at 28 °C for 48 h in the dark. Ethylene content in 2 ml of the headspace gas was analyzed with a gas chromatograph (GC-17A, Shimadzu, Japan).

**RNA-seq analysis.** Total RNA was isolated from Teqing and YIL25, pCPL and pOE and Teqing calli (subcultured for 21 days) with three biological replicates. Paired-end libraries were constructed and sequenced on the Illumina HiSeq 2500 platform at the Novogene Company (China). The RNA-seq reads were aligned to the reference genome of N. tabacum ssp. orientale cv. Nipponbare in rice (Oryza sativa L.)\(^8\). MDA was measured using the thiobarbituric acid (TBA) assay\(^6\). The calli (100 mg) were homogenized in 1 ml 0.1% (w/v) TCA in an ice bath. After centrifuging the homogenate at 12,000 × g for 10 min, 0.5 ml of the supernatant was combined with 0.5 ml 0.6% thiobarbituric acid (TBA) and incubated at 95 °C for 15 min. The samples were cooled immediately in an ice bath and the absorbance of the supernatant measured at 450, 532, and 600 nm.

**Data availability.** Data supporting the findings of this work are available within the paper and its Supplementary Information files. A reporting summary for this Article is available as a Supplementary Information file. The datasets generated and analyzed during the current study are available from the corresponding author upon request. RNA-seq data for callus that had been subcultured for 21 days from Teqing and YIL25, Teqing and the complementation line pCPL-BOC1-Teqing, and Teqing and the overexpression line pOE-BOC1-Teqing was deposited in the National Center for Biotechnology Information (NCBI) under GEO accession number GSE142094. The source data underlying Figs. 1j, 1k, 2a, 2b, 3c, 3i, 3o, 4c, 4e, 5, 6, 8c, 8e, and 8f, as well as Supplementary Figs. 2, 5, 7, 8k, and 9 are provided as a Source Data file.
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