Cytokinin oxidase/dehydrogenase OsCKX11 coordinates source and sink relationship in rice by simultaneous regulation of leaf senescence and grain number

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Summary

The flag leaf and grain belong to the source and sink, respectively, of cereals, and both have a bearing on final yield. Premature leaf senescence significantly reduces the photosynthetic rate and severely lowers crop yield. Cytokinins play important roles in leaf senescence and determine grain number. Here, we characterized the roles of the rice (Oryza sativa L.) cytokinin oxidase/dehydrogenase OsCKX11 in delaying leaf senescence, increasing grain number, and coordinately regulating source and sink. OsCKX11 was predominantly expressed in the roots, leaves, and panicles and was strongly induced by abscisic acid and leaf senescence. Recombinant OsCKX11 protein catalysed the degradation of various types of cytokinins but showed preference for transzeatin and cis-zeatin. Cytokinin levels were significantly increased in the flag leaves of osckx11 mutant compared to those of the wild type (WT). In the osckx11 mutant, the ABA-biosynthesizing genes were down-regulated and the ABA-degrading genes were up-regulated, thereby reducing the ABA levels relative to the WT. Thus, OsCKX11 functions antagonistically between cytokinins and ABA in leaf senescence. Moreover, osckx11 presented with significantly increased branch, tiller, and grain number compared with the WT. Collectively, our findings reveal that OsCKX11 simultaneously regulates photosynthesis and grain number, which may provide new insights into leaf senescence and crop molecular breeding.

Introduction

Leaf senescence is the final stage in the leaf life cycle, which is manifested by irreversible yellowing of the leaves arising from rapid breakdown of chlorophyll and the photosynthetic apparatus. During the process, the stored nutrients are then released and remobilized to the reproductive organs (Gan and Amasino, 1997; Hortensteiner and Feller, 2002; Lim et al., 2007). In a senescing leaf, chlorophyll degradation is sequentially catalysed by several chlorophyll catabolic enzymes (CCEs) (Gao et al., 2016) and senescence-associated genes (SAGs) are highly differentially expressed (Gan and Amasino, 1997; Hortensteiner, 2009). Delaying leaf senescence prolongs photosynthetic activity, increases biomass accumulation, improves energy capture and nitrogen utilization, and enhances grain filling and crop yield (Rivero et al., 2007). Thus, one prospective way to improve crop yield is to postpone leaf senescence. An effective approach is to identify mutants with the delayed leaf senescence (DLS) trait (Jiang et al., 2007; Thomas and Howarth, 2000).

Various endogenous and exogenous factors regulate leaf senescence in intricate crosstalk pathways (Lim et al., 2007). Cytokinins and abscisic acid (ABA) are antagonistic phytohormones suggested to control leaf senescence (Back and Richmond, 1971). An increase in the ABA content is accompanied by the altered expression levels of ABA-related genes in the leaves that undergo the senescence and ABA promotes leaf senescence (Liang et al., 2014; Lim et al., 2007; Zhang and Gan, 2012). Conversely, the cytokinins which decline during leaf senescence (Singh et al., 1992) are considered as the most effective phytohormones for retarding leaf senescence and decelerating photosynthetic deterioration (Gan and Amasino, 1995; Zwack and Rashotte, 2013). There are numerous reports regarding the regulation of the metabolism, and receptors of cytokinins have demonstrated the potential of these plant growth regulators for suppressing leaf senescence (Gregersen et al., 2013). For example, overexpression of an isopentenyl transferase (IPT), the rate-limiting enzyme in cytokinin biosynthesis, under SAG12 promoter in tobacco effectively delayed leaf senescence (Gan and Amasino, 1995). Cytokinin levels in the myb2 mutant of Arabidopsis thaliana (Arabidopsis) were elevated and whole-plant senescence was delayed (Guo and Gan, 2011). The HISTIDINE KINASE 3 (AHK3) gain-of-function mutant ore12-1 (Kim et al., 2006) and...
rock3 (Bartrina et al., 2017) presented with a comparatively enhanced cytokinin-mediated delay of leaf senescence. Arabidopsis type-B response regulator 2 (ARR2) is phosphorylated by AHK3 and regulates the cytokinin-mediated delay of leaf senescence (Kim et al., 2006). However, the mechanisms by which cytokinins regulate leaf senescence remain poorly understood.

Cultivated rice is a staple crop for more than half of the global population. Rice sink strength and source activity determine grain production, they were expected to be strongly correlated with DLS phenotype and grain yield (Jameson and Song, 2006). Meanwhile, cytokinins also regulate the transition from stem elongation to heading, which is vital to crop productivity (Kim et al., 2006). In numerous plant species, CKXs are encoded by multi-gene families (Chen et al., 2020), and more than eleven CKX homologs have been annotated in rice (Schmulling et al., 2003). Quantitative trait loci (QTL) analysis of a Gna1a mutant revealed that a mutation in OsCKX2 is responsible for enhanced cytokinin accumulation in the inflorescence meristems (Ashikari et al., 2005; Bartrina et al., 2011; Li et al., 2013a). By modulating cytokinin metabolism or exogenous cytokinin application at various plant developmental stages, rice panicle architecture and grain yield may be improved (Ashikari et al., 2005; Koprna et al., 2016; Li et al., 2011). In view of the substantial impacts of the endogenous cytokinins, manipulation of the cytokinin biosynthesis and degradation pathways is a feasible approach towards improving crop productivity (Werner et al., 2006).

Cytokinin oxidase/dehydrogenase (CKX) is the main enzyme for inactivating cytokinins by irreversibly cleaving their N6 side chains to generate adenine or adenosine. Directly or indirectly targeting CKX could influence cytokinin homeostasis (Schmulling et al., 2003). In numerous plant species, CKXs are encoded by multi-gene families (Chen et al., 2020); and more than eleven CKX homologs have been annotated in rice (Schmulling et al., 2003). Quantitative trait loci (QTL) analysis of a Gna1a mutant revealed that a mutation in OsCKX2 is responsible for enhanced cytokinin accumulation in the inflorescence meristems (Ashikari et al., 2005). It was also confirmed that Gna1a/OsCKX2 expression was regulated by the rice zinc finger protein DST whose activation augmented grain production (Li et al., 2013b). CKX members differ in terms of their biochemical properties, substrate specificities, and expression levels in various plant tissues, enabling them to coordinately contribute to plant development (Galuszka et al., 2007; Werner et al., 2006). Systematic characterization of the aforementioned multigene family may clarify the roles of each individual gene family member, especially those responsible for cytokinin decline during leaf senescence.

In this study, we used CRISPR/Cas9 gene editing to target eleven CKX gene family members for systematic characterization of the CKXs in rice. OsCKX11 was found to be simultaneously associated with leaf senescence and grain number. In situ hybridization and RT-qPCR indicated that OsCKX11 was highly expressed in panicles and was significantly induced by ABA and leaf senescence. The enzymatic assays of recombinant OsCKX11 protein indicated it catalysed multiple cytokinin substrates but preferentially trans-zeatin (tZ) and cis-zeatin (cZ). Disruption of OsCKX11 significantly increased cytokinin but decreased ABA contents in oscxk11 mutant leaves, resulting in a delayed leaf senescence phenotype. Meanwhile, the grain number of the oscxk11 mutants was significantly increased and the grain yield was enhanced compared to WT. Taken together, our study reveals that OsCKX11 regulates photosynthesis and grain number, thereby coordinating the source–sink relationship. The study provides new insights into cytokinin-inhibited leaf senescence and crop molecular breeding.

Results

Generation of CKX family mutants in rice by CRISPR/Cas9

A search of the entire rice genome disclosed eleven putative homologous CKXs identified as OsCKX1-OsCKX11 (Ashikari et al., 2005; Schmulling et al., 2003). Their open reading frame (ORF) lengths ranged from 1557 to 1719 bp. The deduced OsCKXs proteins had typical flavin adenine dinucleotide (FAD)-binding (PF01565) and cytokinin-binding (PF09265) domains. OsCKX1-OsCKX11 are distributed in seven chromosomes with a pair of tandem duplication genes OsCKX6/OsCKX7 and two pairs of segmental duplication genes OsCKX4/OsCKX9 and OsCKX3/OsCKX8 (Figure S1a). The phylogenetic tree which was constructed using the amino acid sequences of the CKX gene families in Oryza sativa L., Arabidopsis thaliana and Zea mays shows the eleven OsCKXs proteins were divided into four groups (Figure S1b).

To establish the roles of the eleven OsCKXs in plant growth and development, CRISPR/Cas9 technology was employed to generate knockout (KO) mutant lines of OsCKX1-OsCKX11. We designed the gRNA to target the exon, induced mutations in the coding regions and possibly inactivated the OsCKXs (Figure S2a). Transgenic lines were generated and the mutations were characterized by genomic DNA sequencing. They comprised nucleotide insertions and deletions near their respective sgRNA sequences and caused ORF shifts in the CKX genes (Figure S2b). Three independent homozygous mutant alleles per OsCKX were selected for further investigation (Figures S2b; S3).

Identification of a novel ckx mutant involved in leaf senescence

Endogenous cytokinin levels decrease prior to the onset of leaf senescence (San and Amasino, 1996; Singh et al., 1992). As the CKX enzyme catalyses cytokinin degradation, we suspected that OsCKX knockout would result in an increase of the endogenous cytokinin levels and a delay of leaf senescence. To identify the OsCKXs with dedicated roles in leaf senescence, we screened for loss-of-function OsCKX mutants with the delayed leaf senescence (DLS) phenotype. Dark-induced stress is an effective and widely used method of inducing leaf senescence. The rice leaf is amenable and conducive to this treatment (Brouwer et al., 2012). The adult flag leaves of three independent homozygous mutant lines were incubated in darkness and under normal light. The chlorophyll content index (CCI) and chlorophyll fluorescence parameter (Fv/Fm) quantified leaf senescence. The oscxk11 mutant presented with the DLS phenotype in darkness and under normal light whereas the oscxk10 mutants had normal phenotypes relative to the WT (Figure 1; Figures S4; S5). CCI and Fv/Fm were stable in all three independent homozygous oscxk11 lines but were significantly reduced in the WT after light incubation for ≥ 8 days (Figures S4; S5). Therefore, OsCKX11 might be the unique CKX involved in rice leaf senescence.
OsCKX11 expression pattern and subcellular localization

We used RT-qPCR to determine the spatiotemporal expression patterns of OsCKX11. OsCKX11 was widely expressed in rice leaves, panicles, roots and stems; its OsCKX11 expression was greater in the senescing than the young or adult leaves (Figure 2a). To understand the roles of OsCKX11 in leaf senescence, we separated the base, middle and tip of senescing rice leaves and measured the OsCKX11 expression levels. OsCKX11 expression was always higher in the middle (senescing) but lower in the tip (senescent) part of the leaf (Figure 2b). The expression of all eleven OsCKXs in the senescing leaves were measured and OsCKX11 was found to have the highest expression level of them all (Figure 2c). This finding was consistent with the function of OsCKX11 in rice leaf senescence.

As leaf senescence is regulated by various phytohormones, we applied RT-qPCR to verify OsCKX11 expression in rice seedlings treated with various phytohormones including 6-benzylaminopurine (6-BAP), tZ, auxin (indoleacetic acid; IAA), abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA) and 1-aminocyclopropane-1-carboxylic acid (ACC). OsCKX11 was dramatically induced by ABA but only slightly induced by the other plant growth regulators (Figure 2d). When rice seedlings were treated with ABA at different time intervals, OsCKX11 induction was detected at 0.5 h, increased to its highest level by 1.5 h, and declined by 3 h (Figure 2e). This expression pattern indicated that OsCKX11 was induced by ABA and could interact with it during leaf senescence.

Subcellular localization of OsCKX11 was explored by creating an OsCKX11-GFP fusion protein under the control of the CaMV 35S promoter and transiently expressing it in rice protoplasts. Free GFP served as a control. When GFP alone was expressed,
fluorescence signals were observed in the cytoplasm and the nucleus. In contrast, OsCKX11-GFP fusion fluorescence was detected exclusively in the cytosol (Figure 2f). Therefore, OsCKX11 is a cytosolic enzyme and governs cytosolic cytokinin degradation. Its closest homolog ZmCKX10 in maize (Smehilova et al., 2009). Recombinant OsCKX11 preferentially catalyses the degradation of cytokinins cZ and tZ.

CXK modulates cytokinin levels by catalysing their irreversible degradation and generating adenine or adenosine and an aldehyde (Brownlee et al., 1975). Here, we expressed and purified MBP-OsCKX11 fusion protein from Escherichia coli and used it in

Figure 2 OsCKX11 expression pattern and subcellular localization. (a) RT-qPCR analysis of OsCKX11 expression in leaves, panicles, roots and stems. YL, NS, OS and LS: young (3 days before heading), non-senescent (12 days after heading), onset of senescence (35 days after heading) and late senescence (50 days after heading), respectively. (b) Relative OsCKX11 expressions in tip, middle and basal part of senescing leaves detected by RT-qPCR. (c) Relative expression of rice OsCKX1–11 in senescing leaves determined by RT-qPCR. (d) OsCKX11 expression detected by RT-qPCR in rice seedlings treated with 6-BAP, iZ, IAA, ABA, JA, SA and ACC. (e) RT-qPCR analysis of OsCKX11 expression in rice seedlings treated with ABA for 0.5, 1 and 3 h. Data are means ± SD of three biological replicates. UBQ5 was the internal control. *, not detected or detected at very low levels. (f) Subcellular localizations of 35S: eGFP (top row) and 35S:OsCKX11-eGFP (bottom row) in rice protoplast. Bar = 10 μm. [Colour figure can be viewed at wileyonlinelibrary.com]
OsCKX11 regulates leaf senescence and grain number in rice

OsCKX11 presents with elevated chlorophyll and DLS phenotypes

To elucidate the physiological function of OsCKX11, we assessed the natural leaf senescence phenotype of field-grown osckx11 mutants. The three loss-of-function mutant alleles of osckx11 previously used in the DLS assay were selected for this phenotypic analysis. Relative to WT, the leaf chlorophyll content in the osckx11 mutants had increased at the elongation stage (Figure S7). However, there were no other obvious morphological differences between the WT and osckx11 at the young and adult stages. By the mature and senescing stages, though, the osckx11 rice turned yellow later than the WT (Figure 4a,b; Figure S7). Diaminobenzidine tetrahydrochloride (DAB) and nitroblue tetrazolium (NBT) staining revealed that the reactive oxygen species (ROS) levels (including H2O2 and O2-) were lower in the osckx11 than the WT leaves (Figure 4c). Therefore, we suggest that the OsCKX11 mutation delayed leaf senescence.

Chlorophyll content and Fv/Fm are broadly used to measure leaf senescence progression. We measured the flag leaf chlorophyll content and Fv/Fm at 18 to 60 DAH (day after heading), between the adult and senescing stages. The osckx11 consistently presented with higher chlorophyll content and Fv/Fm than the WT as the leaf aged. In fact, the discrepancy between the osckx11 and WT in terms of chlorophyll content increased with advancing plant age. The widest gaps between them were observed at 48 DAH when the chlorophyll content in osckx11 was on average 34% higher than that of WT (Figure 4d,e). Further, the leaf tip chlorophyll content was on average 59% higher in osckx11 than WT at 48 DAH (Figure S7c). The net photosynthetic rates at the grain-filling and grain-full maturity stages were 9.77% and 29.98% greater in osckx11 than WT, respectively (Figure 4f). Thus, the OsCKX11 mutation enhanced the photosynthesis rate by delaying leaf senescence and prolonging maximal photosynthetic activity.

osckx11 caused cytokinin overaccumulation in young and senescing rice leaves

The leaf cytokinin levels were quantified by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) to establish whether the endogenous cytokinin content was responsible for the DLS phenotype of osckx11. We quantified the leaf cytokinin levels in osckx11 and WT at the young and senescing leaf stages. At the young leaf stage, the IZ, C2 and iP levels showed 1.5-, 3.0- and 2.0-fold increase in osckx11 than WT, respectively. In contrast, the levels of the other cytokinins were nearly equal in both strains (Figure 4g). At the onset of senescence, the IZ, C2, ZR, iP, iP and DHZ levels were all significantly higher by 1.2-, 2.5-, 1.4-, 1.7-, 3.2-, 1.3- and 1.3-fold in osckx11 than in WT. However, Z was more abundant in the mutant than the WT (Figure 4h). Thus, OsCKX11 participates in cytokinin metabolism in vivo and C2, Z and iP are strongly associated with leaf senescence.

As osckx11 presented with significantly elevated endogenous cytokinin levels compared to WT, we evaluated rice type-A response regulator (OsRRs) expression to determine whether cytokinin signalling was altered in osckx11 relative to WT. Of the thirteen OsRRs, OsRR1, OsRR2, OsRR6, OsRR9 and OsRR10 were all up-regulated, whereas OsRR2 and OsRR4 were down-regulated in osckx11 (Figure S8). Therefore, we suggest that each OsRR may respond differently to the increased cytokinin content in rice.

OsCKX11 links the ABA metabolic and chlorophyll degradation pathways

As ABA plays a vital role in leaf senescence and OsCKX11 was induced by ABA (Figure 2d,e), we speculate that OsCKX11 may play roles in the crosstalk between ABA and cytokinins during leaf senescence. To elucidate the mechanism by which OsCKX11 functions in leaf senescence, we measured the ABA levels in osckx11. The ABA levels in osckx11 were significantly lower than those in the WT at the adult (NS) and senescence onset (OS) stages (Figure 5a,c). Therefore, an increase in cytokinin content may result in a corresponding decrease in ABA content. To determine how cytokinin affects the ABA level, we assessed the expression of several key ABA biosynthetic and degradation genes such as the OsNCEDs and the OsABA8oxs that regulate the ABA metabolic pathway. Compared with the WT, the transcription levels of the ABA biosynthetic genes OsNCED1 and OsNCED3 were significantly down-regulated and those of the ABA inactivating genes such as OsABA8ox1, OsABA8ox2 and OsABA8ox3 were up-regulated in the adult and early senescence flag leaves of osckx11 (Figure 5b,d,e). Thus, the observed reductions in the ABA levels may have been the consequence of alterations in the genes associated with ABA metabolism.

The DLS phenotype was evident in the flag leaves by the time of the onset of senescence. For this reason, we quantified the transcript levels of several chlorophyll-degrading genes (CDGs). The expression levels of the RED CHLOROPHYLL CATABOLIC REDUCTASE 1 (RCCR1), STAY-GREEN (SGR) and NON-YELLOW COLORING3 (NYC3) genes (Jiang et al., 2007; Morita et al., 2009; Tang et al., 2011) were significantly lower in the fully expanded leaves of osckx11 than in those of the WT (Figure 5f). Several senescence-associated genes (SAGs) such as Osh36, OsS57 and OsS85 (Li et al., 2014) were expressed at lower levels in osckx11 than the WT (Figure S9). The results showed the expression levels of chlorophyll degradation and SAG genes were significantly reduced in the osckx11 mutants compared to that of WT.

The leaf senescence phenotype in osckx11 and WT was partially rescued by ABA and cytokinin administration

Leaf detachment extensively induces leaf senescence (Sakuraba et al., 2014). We examined the senescence-related phenotype of osckx11 via a detached leaf assay in darkness and under normal light and in the presence of ABA and 6-BAP. The aim was to verify
whether OsCKX11 participates in the crosstalk between the cytokinin and ABA pathways. The difference between WT and osckx11 in terms of total chlorophyll content and Fv/Fm increased with time in the dark and under normal light when the detached leaves were treated with water (Figure 6; Figure S9). This finding is consistent with the visible phenotype and suggests that the osckx11 mutation significantly delays detached leaf senescence. The ABA content was dramatically lower in the detached osckx11 leaves than the detached WT leaves (Figure S10a). As expected, OsNCED1 and SGR were down-regulated but OsABA8ox3 was up-regulated 3 d after the water treatment (Figure S10b). This observation aligned with those recorded for natural leaf senescence (Figure 5).

ABA treatment accelerated the decline in the chlorophyll and Fv/Fm values for the detached osckx11 leaves and partially rescued them to the WT level (middle in Figure 6a–c). Therefore, reduction in endogenous ABA accounted for the DLS phenotype in osckx11. On the other hand, 6-BAP treatment delayed senescence and chlorophyll reduction in the detached WT leaves such that their appearance was similar to that of the detached osckx11 leaves (right in Figure 6a–c). The expression levels of the senescence marker genes Osh36 and OsI57 were consistent with the chlorophyll content and Fv/Fm for the detached osckx11 and WT leaves under various treatments (Figure 6d). Thus, the osckx11 mutation significantly delayed ABA-induced leaf senescence compared to the WT.

**Figure 3** Biochemical properties of recombinant OsCKX11 protein in vitro. (a) HPLC profiles for reactions of recombinant OsCKX11 protein. (i) UV spectra of enzymatic reactions using boiled OsCKX11 recombinant enzyme and cZ as substrate. (ii) UV spectra of enzymatic reactions using OsCKX11 recombinant enzyme and tZ as substrate. (iii) UV spectra of enzymatic reactions using OsCKX11 recombinant enzyme and tZ as substrate. (iv) UV spectra of authentic tZ, cZ and adenine standards. (b) Substrate specificities of recombinant protein OsCKX11 towards various cytokinins. tZ, trans-zeatin; cZ, cis-zeatin; cZR, cis-zeatin riboside; tZR, trans-zeatin riboside; iP, isopentenyladenine; iPR, isopentenyladenine riboside; DHZ, dihydrozeatin. Kinetics of recombinant OsCKX11 protein towards tZ (c) and cZ (d). Data are means ± SD of three independent replicates. [Colour figure can be viewed at wileyonlinelibrary.com]
Figure 4 Characterization of osckx11 mutants in field rice plants. (a) Phenotypes of whole osckx11 mutant and WT plants at mature stage (35 days after heading, DAH). Bar = 20 cm. (b) Phenotypes of flag leaves of osckx11 mutant and WT from young to old stages. YL, NS, OS and LS: young, non-senescent, onset of senescence and late senescence stages, respectively. Bar = 10 cm. (c) DAB and NBT staining of osckx11 mutant and WT flag leaves at late senescence stage. Chlorophyll content (d) and fluorescence parameter (Fv/Fm) (e) of osckx11 mutant and WT flag leaves on different days after heading. Data are means ± SD of fifteen biological replicates (one leaf disc each). Statistical significance was determined by two-tailed Student’s t test (**P ≤ 0.01). (f) Net photosynthetic rate in osckx11 at grain-filling and grain-full maturity stages. Error bars: means ± SD of three biological replicates (two leaf discs each). Statistical significance was determined by two-tailed Student’s t test (**P ≤ 0.01). Quantification of endogenous cytokinin contents in flag leaves at YL (g) and onset of leaf senescence (h). tZ, trans-zeatin; cZ, cis-zeatin; cZR, cis-zeatin riboside; tZR, trans-zeatin riboside; iP, isopentenyladenine; iPR, isopentenyladenine riboside; DHZ, dihydrozeatin. Data are means ± SD of three biological replicates. Statistical significance (*P ≤ 0.05; **P ≤ 0.01) was established by one-way ANOVA. [Colour figure can be viewed at wileyonlinelibrary.com]
Figure 5 osckx11 mutation suppressed ABA-induced leaf senescence. (a) ABA quantification in non-senescent (NS) WT and osckx11 mutants flag leaves. FW, fresh weight. Data are means ± SD of three biological replicates. Statistical significance (*P ≤ 0.05; **P ≤ 0.01) was determined by one-way ANOVA. (b) RT-qPCR transcriptional analysis of ABA biosynthesis genes NCED1 and ABA degradation genes ABAox8-1 in non-senescent flag leaves. UBQ5 was the internal control. (c) ABA quantification in WT and osckx11 flag leaves at the onset of senescence. FW, fresh weight. Data in (a) and (c) are means ± SD of three biological replicates. Statistical significance (*P ≤ 0.05; **P ≤ 0.01) was established by one-way ANOVA. (d) RT-qPCR transcriptional analysis of ABA biosynthesis genes NCED1 and NCED3 in flag leaves at onset of senescence. (e) Expression of degradation genes ABAox8-1, ABAox8-2 and ABAox8-3 detected by RT-qPCR in flag leaves at onset of senescence. (f) Expression of chlorophyll-degrading genes RCCR1, SGR and NYC3 detected by RT-qPCR in flag leaves at onset of leaf senescence. (g) Expression analysis of senescence-associated genes (SAGs) (Osh36, OsI57 and OsI85) detected by RT-qPCR in flag leaves at onset of leaf senescence. UBQ5 was the internal control. Data in (b) and (d–g) are means ± SD of three technical replicates from one experiment. Three independent experiments were performed with similar results. Statistical significance (*P ≤ 0.05; **P ≤ 0.01) was analysed by one-way ANOVA. [Colour figure can be viewed at wileyonlinelibrary.com]
Figure 6 Dark-induced leaf senescence in WT and osckx11 mutants treated with ABA and cytokinin. (a) Dark-induced leaf senescence phenotypes of WT and osckx11 mutants treated with ABA and 6-benzylaminopurine (6-BAP). Detached WT and osckx11 mutant flag leaves at heading stage were incubated in water (left), 20 μM ABA (middle) or 5 μM 6-BAP (right) for 0, 3 or 6 days in darkness. Leaves were obtained from three biological samples. Quantification of chlorophyll content (b) and fluorescence parameter (Fv/Fm) (c) of treated leaves in (a). FW, fresh weight. Data are means ± SD of three biological replicates. Statistical significance (*P ≤ 0.05; **P ≤ 0.01) was established by one-way ANOVA. (d) Expression analysis of Osh36 and OsI57 in detached leaves incubated for 3 d under various treatments. UBQ5 was the internal control. Data are means ± SD of three technical replicates from a single experiment. Three independent experiments were performed with similar results. Statistical significance (*P ≤ 0.05; **P ≤ 0.01) was determined by one-way ANOVA. [Colour figure can be viewed at wileyonlinelibrary.com]
**OsCKX11 mutation increased grain number and crop yield compared to the WT**

As OsCKX11 was up-regulated in the panicle, we measured OsCKX11 expression from the branch primordium to the floret primordium stages via in situ hybridization (Figure 7a–d). OsCKX11 mRNA was highly expressed throughout panicle development, including the primary and secondary branches and the spikelet primordia. Therefore, OsCKX11 might regulate the SAM activity and grain number.

To confirm whether the OsCKX11 mutation affects the rice grain number, we measured several agronomic traits on a single-plant basis in the field (Figure 7e–l). Consistent with its expression pattern in the panicle (Figure 7a–d), osckx11 produced more primary branches by an increase ranging from 20.53% to 23.64% in three independent lines compared to the WT (Figure 7f,g). As expected, the independent osckx11 lines displayed higher tiller numbers ranging from 14.83% to 27.07% than the WT (Figure 7h). Accordingly, the grain number per panicle was dramatically increased by 15.11% to 27.96% and the total grain number per plant was increased by 21.62% to 27.29% in osckx11 mutants compared with the WT plants (Figure 7i,j). Thus, OsCKX11 regulates rice grain number. Compared to the WT, however, all three independent osckx11 lines had lower fertility by a decrease ranging from 7.33% to 9.96% (Figure 7k), lower seed weight (thousand grain weight) by a decrease ranging from 6.99% to 8.92% and smaller size (seed width) by a decrease ranging from 3.85% to 5.22% (Figure S1l). Nevertheless, a statistical analysis indicated that the grain yield per plant was significantly higher (11.61% to 16.97%) in the three independent osckx11 lines than the WT (Figure 7l). In addition, small-field tests showed that the grain yield of the osckx11 mutant was significantly higher (by 7.47% and 7.58%, respectively) than that of the WT based on two independent plots (Figure 7m). To confirm whether the mutation influences rice harvest timing, we assessed panicle senescence from the heading to full grain maturity stages. Although the leaf senescence was moderately delayed in osckx11 compared to the WT, there were no obvious differences in panicle ageing between osckx11 and the WT (Figure S12). Overall, the study reveals that OsCKX11 regulates photosynthesis activity, sink strength, grain number and grain yield, but does not delay grain harvest.

**Discussion**

Cytokinins are implicated in several aspects of plant growth and development such as leaf senescence and grain number. Cytokinin activity in vivo is mediated by a balance of biosynthesis, inactivation, reactivation and degradation (Galuszka et al., 2000; Sakakibara, 2006). However, cytokinin homeostasis in the aforementioned metabolic processes is complex. The members of CKX family have different enzymatic properties and various spatiotemporal distributions which enable them to maintain cytokinin homeostasis in different tissues and exhibit multiple functions (Jablonski et al., 2020; Werner et al., 2003). Here, we applied a reverse genetic strategy to characterize a gene family member, OsCKX11, that simultaneously controls rice leaf senescence and grain number.

A phylogenetic tree disclosed that OsCKX11 belongs to a CKX family, a few members of which have been well studied. Rice OsCKX2, OsCKX4 and OsCKX9 have been studied in detail and were found to participate in the reproductive meristem, crown root formation and tillering (Ashikari et al., 2005; Duan et al., 2019; Gao et al., 2014). Compared to OsCKX1 to OsCKX10, OsCKX11 is highly abundant in senescing leaves (Figure 2a,b). This finding corroborates its unique role in leaf senescence. Similarly, TaCKX11-7D, a homolog of OsCKX11 in wheat, is also highly expressed in the senescing leaves (Chen et al., 2020), suggesting it may play an important role in wheat leaf senescence. On the other hand, OsCKX11 is the most highly expressed member of the CKX family in early rice panicles (Yamburenko et al., 2017). This discovery aligned with the results of the in situ hybridization and supports the concept that OsCKX11 is another CKX which regulates rice grain number in addition to OsCKX2 (Ashikari et al., 2005). Regarding the enzyme subcellular localization, three CKXs including OsCKX11 (Figure 2f), OsCKX4 and OsCKX9 are all localized to the cytosol which is similar to maize ZmCKX10 (Duan et al., 2019; Gao et al., 2014; Smehilova et al., 2009). In contrast, Arabidopsis has only one cytokinin CKX, AtCKX7, which is highly homologous with OsCKX11 (Figure S1b) and plays a role in root growth and xylem differentiation (Kollmer et al., 2014). The localization of these CKXs in cytosol suggests that they affect the cytokotosolic cytokinin pool and signalling.

Cytokinins have evolved various derivatives with multiple functions and different binding affinities for various receptors. For these reasons, the substrate specificities of OsCKX11 may clarify the physiological functions of various cytokinins. The in vitro enzyme assays indicated that OsCKX11 catalyses multiple cytokinins but preferentially reacts with iZ and cZ (Figure 3). Its close homologs, ZmCKX10 and AtCKX7, exhibit similar enzymatic/metabolic behaviours (Kowalska et al., 2010; Smehilova et al., 2009). While the substrate specificities of rice OsCKX2 and OsCKX4 were not assayed in detail (Ashikari et al., 2005; Gao et al., 2014), the OsCKX9 has measurable activity towards iZ (Duan et al., 2019). Analysis of the biochemical properties of the other rice CKX members in the future may help identify their substrate preferences and their physiological functions in vivo. The iZ, cZ and iP levels were significantly higher in the young and senescing osckx11 rice leaves than in those of the WT (Figure 4), suggesting that these cytokinins are likely to maintain rice leaf longevity. The present study demonstrated that cZ-type cytokinins may be strongly associated with, and likely play important roles in rice leaf senescence. Our results corroborate those of a previous report which postulated that cZ and iZ have comparable activity levels in rice (Kudo et al., 2012). The discovery that OsCKX11 participates in leaf senescence may account for the fact that foliar cytokinin levels decrease during senescence.

ABA is an essential phytohormone that plays vital roles in leaf senescence (Mao et al., 2017; Zhang and Gan, 2012). We found that cytokinin and ABA homeostasis were reciprocally and antagonistically regulated during leaf senescence in rice. Compared with the WT, the endogenous ABA levels were markedly lower in the natural and detached leaves of osckx11 (Figure 5a,c; Figure S10a). In osckx11, the ABA biosynthetic genes were relatively down-regulated and the ABA-degrading genes were comparatively up-regulated (Figure 5b,d,e; Figure S10). Furthermore, exogenous ABA application to detached osckx11 leaves partially rescued their senescent phenotype to the WT (Figure 6). Hence, ABA accumulation in osckx11 was suppressed during leaf senescence. At the transcriptional level, OsCKX11 expression was significantly induced by ABA which, in turn, created a feedforward loop in which cytokinins were degraded and ABA accumulation increased in vivo. Therefore, OsCKX11 might mediate leaf
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Figure 7  osckx11 mutation increased grain number and crop yield. In situ hybridization assay of OsCKX11 in young WT panicles in primary branch primordia (a), secondary branch primordia (b), and floret primordia (c), respectively; PBM, primary branch primordia; SBM, secondary branch primordia; FM, floret primordia. (d) is negative control preparation made with sense OsCKX11 probe. Bar = 50 μm. (e) Panicle morphologies of WT and three mutant alleles of osckx11. Bar = 4 cm. (f) Stretched panicle morphologies of WT and three mutant alleles of osckx11. Bar = 4 cm. (g)–(m) Agronomic traits of WT and osckx11 mutant plants. (g) Primary branch number; (h) tiller number; (i) grain number per panicle; (j) total grain number; (k) seed setting rate (%); (l) yield per plant (g). Data are means ± SE of ≥20 biological replicates (n = 24 for NIP; n = 21 for osckx11-1 and osckx11-2; n = 20 for osckx11-3) in (g)–(l). Statistical significance was analysed by two-tailed Student’s t test (*P ≤ 0.05; **P ≤ 0.01). (m) Grain yield per plot (kg). Data are means ± SD of duplicate field tests. Control represents WT plants isolated from heterozygous osckx11 mutants. Statistical significance analysed by two-tailed Student’s t test (*P ≤ 0.05; **P ≤ 0.01). [Colour figure can be viewed at wileyonlinelibrary.com]
senescence in a feedforward mode via the ABA metabolic pathway which may play a partial role in cytokinin-inhibited leaf senescence (Figure 8). In a recent report, strigolactones were found to specifically and significantly up-regulate OsCKX9. This led to a decrease in cytokinin content, revealing crosstalk between strigolactones and cytokinins (Duan et al., 2019). The findings that OsCKX11 and OsCKX9 are involved in the crosstalk between CK and ABA/SLs respectively suggest that CKXs may mediate crosstalk between cytokinins and other hormones.

Leaf senescence is an internally programmed degradation process that overlaps with the reproductive stages and grain filling (Gregersen et al., 2013). Foliar photosynthesis furnishes carbohydrate for panicle development. Thus, premature senescence seriously hinders photosynthetic assimilation, rational grain filling and canopy expansion (Woo et al., 2013). Premature leaf senescence may limit hybrid rice yield potential and quality. However, rice yield could be enhanced by delaying leaf senescence appropriately (Liang et al., 2014; Mao et al., 2017). In practice, unfortunately, delayed leaf senescence has not always increased crop yield (Gregersen et al., 2013; Uauy et al., 2006). Staying ‘green’ for too long will retard stored carbohydrate remobilization and nitrogen recycling efficiency and can lead to undesirable crop productivity (Yang and Zhang, 2010; Yang et al., 2003). The sink size may limit the availability of the total photosynthetic activity realized by delayed leaf senescence. In our study, the osckx11 knockout mutants grown in the field presented with moderately delayed leaf senescence and high photosynthetic performance during leaf development (Figure 4; Figure S7); meanwhile, the osckx11 mutants exhibited enhanced sink strength by increasing the tiller and grain number, thereby resulting in higher yield (Figure 7e–m). The gain in yield achieved with osckx11 could be explained by the enhanced photosynthetic capacity of this mutant before and during leaf senescence and an enhanced sink strength through grain number increase.

Previous reports proposed that modulation of the cytokinin level by selectively targeting the biosynthesis or degradation pathways of this phytohormone may enlarge the plant source or sink (Chen et al., 2020; Jameson and Song, 2016). Our findings indicate that OsCKX11 affects rice leaf senescence and grain number and simultaneously regulates both the source and the sink. Besides the morphological improvement of the agricultural traits, the elevated cytokinins may affect the shift in sink and source identities of organs (Roitsch and Ehne, 2000). During the process, the key enzymes such as trehalose 6-phosphate (TEP) synthase (Wingerl et al., 2012) and extracellular invertase (Balibrea Lara et al., 2004) which are associated with source–sink balance could be altered in the osckx11 mutants. Unfortunately, the fertility and thousand grain weight of osckx11 were lower than that of WT. The low fertility could refer to induction of strong cytokinin homeostatic mechanisms in its panicles or nutrient flow process. The reduction of thousand grain weight may be caused by the elevated tiller number or grain number in osckx11 plants which could form competing sinks resulting in lower grain-filling efficiency. Similarly, the elevated IZ in spikes of TaCKX1 RNAi plants also led to lower thousand grain weight, but the plants still had an overall higher grain number and yield (Jablonski et al., 2020).

Taken together, we propose that OsCKX11 has a dual role in cytokinin-mediated leaf senescence and grain number in rice (Figure 8). The future research objectives might include the correction of low osckx11 fertility and seed size with weak gene alleles for generation of a rice variety that is highly useful for breeding purposes. Moreover, the crosstalk between cytokinins and ABA at the molecular level will be investigated in order to understand cytokinin-inhibited leaf senescence.

**Experimental procedures**

**Plant materials and growth conditions**

*Oryza sativa* L. ssp. japonica (cv. Nipponbare) was used as wild type (WT) in this study. For hormone-induced assays at seedling stage, the seeds were germinated in wetted filter paper at 37°C for two days in darkness and then grown in a growth chamber under a 12-h-light (28°C)/12-h-dark (22°C) photoperiod at 500–600 μmol m⁻² s⁻¹ light intensity and 50% humidity. For expression profiling analysis and detached leaf assays, the tissue samples were collected from the WT and knockout (KO) mutants osckx1-11. In the field experiments, the WT and mutants were grown under conventional cultivation environment in a paddy field with a distance of 20 × 20 cm and the independent lines were arranged in each plot (2 m × 2 m) in the Botany Garden of Zhejiang Normal University from June to October in 2016-2018 in Jinhua (119°63'E, 29°13'N), China.

**Treatments**

To determine the transcriptional response of OsCKX11 to phytohormones, rice plants were grown for 7 days and then immersed in nutrient solution containing different phytohormones, including 20 μM 6-BAP, 20 μM trans-zeatin, 50 μM indole-3-acetic acid (IAA), 50 μM abscisic acid (ABA), 50 μM jasmonic acid (JA), 50 μM salicylic acid (SA) and 50 μM 1-aminoacyclopropane-1-carboxylic acid (ACC) at 30°C for the indicated time. The seedlings were collected and frozen.
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Phylogenetic analysis
The full-length CKX protein sequence alignment of Arabidopsis (AtCKXs), Oryza sativa L. (OsCKXs) and Zea mays (ZmCKXs) was performed using ClustalW (2.0). The full-length sequences were searched from the TAIR (https://www.arabidopsis.org), the RAPDB rice database (https://rapdb.dna.affrc.go.jp) and the MaizeGDB database (https://chinese.maizegdb.org/gene_center/gene/40675) according to a method previously described (Gu et al., 2010). Phylogenetic analyses were conducted using MEGA6 as described previously (Tamura et al., 2013). A neighbour-joining method was conducted to build the phylogenetic tree. Then, the Poisson correction distance was used to compute the proportions of amino acid differences in order to estimate the evolutionary distance. By using the bootstrap method with 1000 replicates, different phylogenetic clusters were evaluated. The FAD-binding and cytokinin-binding domains in the OsCKXs proteins were predicted using ARAMEMNON-plant membrane protein database (http://aramemnon.botanik.uni-koeln.de).

Generation of mutants by CRISPR/Cas9 gene editing
The loss-of-function mutants of OsCKX1-OsCKX11 genes were generated by the CRISPR-Cas9 technique following a method described previously (Wang et al., 2015). The target gRNA sequences of OsCKX11 were designed by CRISPR Primer Designer (http://www.genome.arizona.edu/crispr/CRISPRsearch.html). The sgRNAs were digested with Kpn I/Bgl II and then inserted into pc1300-Cas9 binary vector digested with Kpn II/BamHI I, respectively (Wang et al., 2015). To identify the mutants, the gene fragments were amplified and sequenced. All the primers used in this study are provided in Table S1.

Subcellular location of OsCKX11
To examine the subcellular location of OsCKX11, the gene was amplified from cDNA using primers OsCKX11-Of and OsCKX11-OR. The PCR fragment was cloned into plasmid pENTR/D-TOPO using Gateway technology (Invitrogen, Carlsbad, CA) and then to the destination vector pGWB405 to generate OsCKX11-eGFP. Rice protoplasts were prepared from the sheaths of 15-days-old rice seedlings following the method previously described (Zhang et al., 2011). 35S:eGFP and 35S:OsCKX11-eGFP plasmids were transformed into rice protoplasts using a polyethylene glycol-calcium-mediated transformation method and were detected by a confocal laser scanning microscope (Leica TCS SPS) using an excitation 488-nm laser with an emission wavelength of 505–530 nm for GFP.

Expression and purification of recombinant protein OsCKX11
The open reading frame of OsCKX11 was PCR-amplified using a pair of primers pMAL-F and pMAL-R. The fragment was ligated into pMAL-c2X vector as an N-terminal fusion to the maltose-binding protein (MBP) to generate construct pMAL-OsCKX11 using Clonexpress II One Step Cloning Kit (Vazyme, Nanjing, China). The plasmid was then transformed into E. coli BL21 (DE3, pLYS3; Invitrogen), and the recombinant protein was purified following the method described previously (Zhang and Gan, 2012). The electrophoretic separation of proteins was performed on 12% (w/v) polyacrylamide gels and quantified according to the method of Bradford with BSA as a standard.

Enzymatic assays of recombinant protein OsCKX11
The assay of OsCKX11 activity was performed according to a modified method (Frebort et al., 2002). The reaction mixtures containing 0.1 ml of appropriate concentrations of the enzyme, 0.2 ml different substrates (0.1 mM Cz, tz, ip, CzR, tzR, ipR and DHZ) and electron acceptor 2,6-dichlorophenolindophenol (DCIP, 0.5 mM) in a 75 mM Tris-HCL buffer (with pH ranging from 3.5 to 10.5) was incubated at different temperatures (different Tm from 5–50°C) for 10 min (2 min for kinetics assay). Then, 0.3 ml 40% trichloroacetic acid was added to stop the reaction and followed by centrifuging at 15 000 g for 20 min. The substrate in the supernantant solution was quantified by HPLC with ultraviolet absorption at 254, 280 and 310 nm as described previously (Miao and Liu, 2010). OsCKX11 activity (nmol/mg protein min) was defined as the amount of substrates (nmol) degraded by one mg protein per min under the optimal reaction conditions. The enzyme kinetics profiling analysis was conducted using various concentrations of substrate under the optimal conditions.

RNA extraction and real-time RT-qPCR analysis
Total RNA was isolated from tissues using the TRizol Reagent (Invitrogen, Carlsbad, CA). Total RNA (2 μg) was treated with HiScript QRTsupermix for RT-qPCR (Vazyme, Nanjing, China) for reverse transcription and first-strand complementary DNA synthesis. For quantitative reverse transcription-PCR analysis, 4.6 μl of diluted first-strand complementary DNA (corresponding to 10 ng RNA) was used as a template in 10 μl reaction with the gene-specific primers. RT-qPCR was performed with the SYBR Green Mix (Takara) using the ABI PRISM 7700 system (Applied Biosystems). Rice UBQ5 gene was used as an internal control to normalize different samples.

Chlorophyll content measurements in detached leaves
For determination of total chlorophyll content of detached leaves, about 0.2 g materials were weighed and extracted with 2 ml 80% acetone (v/v). The mixture solution was shaken overnight in darkness and centrifuged at 12 000 g at 4°C for 10 min, and the supernatant was separated and used for the chlorophyll assay according to a described method (Arnon, 1949). The absorbance at 663 and 645 nm was measured and the results expressed as mg per gram fresh weight (mg/g FW).

Chlorophyll content index and chlorophyll fluorescence assays
The leaf chlorophyll content index (CCI) and chlorophyll fluorescence parameter (Fv/Fm) of leaves were measured from 6 days after flowering for every 6 days using the fully expanded flag leaves. The tip, middle and base of each leaf were measured and the average value was calculated. The chlorophyll content was measured with a portable chlorophyll content meter (CCM 200 plus, Opti-Sciences, Hudson, NH) and SPAD meter (SPAD-502 plus, Japan). Fv/Fm was measured with a portable fluorescence system (model OS1p, Opti-Sciences, Hudson, NH). The CCI and Fv/Fm measurements were done between 8:00-9:00 AM and...
4:00-5:00 PM on sunny days, respectively. Leaves were kept in darkness for 30 mins to acclimate to the ambient conditions in the laboratory or in the field. The net photosynthetic rate was measured with portable photosynthesis system Li-6800 (LI-COR).

Histochemical staining

The detection of H2O2 and O2− accumulation was performed using leaf blades following the described method (Mao et al., 2017). Briefly, the leaves were soaked in 1.25 mg/mL DAB and 2 mg/mL NBT using vacuum infiltration for 15 min and then were left at room temperature overnight in darkness. The green pigments were removed by using de-staining solution (ethanol : acetic acid : glycerinum = 3 : 1 : 1) for several times before photography.

Quantification of endogenous cytokinins and ABA

The extraction and quantification of endogenous cytokinins and ABA were following a method previously described (Zhao et al., 2019). Approximately 0.1 g fresh leaves were frozen in liquid N2 and powdered by the tissue lyser. After the addition of 1.5 ml 80% methanol with internal standards including [2H5]tZ, [2H5]JZR, [2H6]JP, [2H6]PR (each 45 pg) and [2H6]ABA (100 pg), the mixture solution was agitated for at least 2 h at 4°C. Followed by centrifugation, the supernatant was concentrated under N2 and re-solubilized in 300 μL 30% methanol; the filtered extracts were quantified by UPLC/MS/MS system (AB SCiEX/QTRAP 5500) following a method previously described (Zhao et al., 2019).

RNA in situ hybridization

RNA in situ hybridization was performed using the method previously described (Shao et al., 2019). A 452-bp fragment (979-1430bp) of OsCKX71T DNA was subcloned into pGEM-T Easy vector (Promega) and used to generate sense and antisense RNA probes.

Agronomic traits analysis

All lines were grown in a standard paddy field and grown under conventional conditions as described previously. All the agronomic traits were measured using 24 plants for WT, 21 plants for osckx11-1 and osckx11-2 and 20 plants for osckx11-3, which were harvested from the plot randomly. The agronomic traits including primary branch number, tiller number, grain number per panicle, total grain number, seed setting rate and yield per plant were measured on a single-plant basis. For the small-field test, the grain yield per plot (kg) was estimated with two replicates. Each plot was approximating 4 m2 in paddies.

Accession numbers

Genes and their accession numbers used in this study from GenBank/EMBL, Phytozone and Rice Annotation Project are shown in Table S2.

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Conflict of interest

The authors declare no conflict of interests.

Author contributions

K.Z., Z.C., D.Z. and Y.W. conceived and designed the experiments. W.Z., K.P., F.C., D.W., J.Z., Y.Z. and N.Y. performed the experiments; K.Z., W.Z., K.P., F.C., D.W., D.Z., Z.C. and Y.W. analysed the data. W.Z. and K.Z. wrote and revised the manuscript. All the authors discussed the results and collectively edited the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 OsCKX1-11 distribution on rice chromosomes and phylogenetic CKX trees for rice, Arabidopsis, and maize.
Figure S2 Targeted OsCKX1-11 mutagenesis by CRISPR/Cas9.
Figure S3 Detailed information of three loss-of-function mutant alleles of OsCKX11 generated by CRISPR/Cas9 gene editing.

Figure S4 Chlorophyll content (A) and fluorescence parameter (Fv/Fm) (B) in detached osckxs mutant and WT leaves incubated in water and under darkness.
Figure S5 Chlorophyll content (A) and fluorescence parameter (Fv/Fm) (B) of detached osckxs mutant and WT leaves incubated in water and under normal light.
Figure S6 Recombinant OsCKX11 protein purification and reaction optimization in vitro.
Figure S7 Phenotypes of fully expanded osckx11 mutant flag leaves.
Figure S8 RT-qPCR measurement of relative expression levels of rice type A response regulator (OsRR) genes in the flag leaves of osckx11 mutants and WT.
Figure S9 Light-induced leaf senescence in WT and osckx11 mutants treated with cytokinin and ABA.
Figure S10 Crosstalk of cytokinin and ABA in dark-induced senescence of detached osckx11 and WT leaves.
Figure S11 Morphological phenotypes of WT and osckx11 flag leaves and panicles.
Figure S12 osckx11 mutation decreased seed weight and seed size.

Table S1 Primers used in this study.
Table S2 Gene accession numbers in this study.