Mutation of *OsCAX1a* Results in Panicle Degeneration in Rice

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Abstract

Background: Rice is one of the most common cereal crops in China. Increasing the yield of rice has always been a primary purpose of rice breeding. However, panicle degeneration in rice, a complex characteristic regulated by many genes and commonly encountered in rice production, seriously reduces the yield.

Findings: In this study, we obtained a new apical panicle degeneration mutant named *ym48*, which exhibits a serious degeneration rate and reduced grain yield in rice. After fine mapping, the *OsCAX1a* gene responsible for Ca^{2+} selection and transportation was identified. In the *ym48* mutant of the *OsCAX1a* gene, a A to G substitution was noted at the 190^{th} nucleotide, and the corresponding 64^{th} amino acid was changed from threonine to alanine. Also, the tolerance from Ca^{2+} stress was damaged due to the mutation. Phylogenetics, protein sequence alignment and motif identification of CAX family members in *Arabidopsis* and rice indicated that this mutation site was highly conserved and might play an essential role in Ca^{2+} transportation. Moreover, the *OsCAX1a* expression pattern was analyzed in rice. qRT-PCR and GUS (β-glucuronidase) staining experiments showed that *OsCAX1a* was highly expressed in roots, stems and panicles and that its expression increased with panicle development.

Conclusions: These results demonstrated that *OsCAX1a* played an essential role in the regulation of panicle development for the first time and mutation of *OsCAX1a* would generate the panicle degeneration in rice. This study provided a new view point to explore the mechanism of panicle development and degeneration in rice.

Findings

Rice (*Oryza sativa*) is an important food crop that serves as a staple food for greater than half of the world's population (Tero et al. 2010). Increased yield represents the major purpose of rice genetics and breeding, which is of great significance for satisfying food requirements (Pushpendra et al. 2006). Grain yield in rice is a complex agronomic trait that is mainly determined by the number of differentiated spikelets per panicle, 1000-grain weight and the number of panicles, and the number of differentiated spikelets is a major contributor to grain yield (Xing and Zhang, 2010; Asif et al. 2019). The occurrence of panicle degeneration during floret development is widespread in rice breeding and production, which causes an approximately 20% reduction in yield. Moreover, the rate of panicle degeneration ranges from 50% to 60% under extreme weather conditions (Yamagishi et al. 2004; Zhang et al. 2017). Thus, understanding the molecular mechanisms and discovering the key genes involved in panicle development are important to breed high-yield rice varieties.

Panicle differentiation in rice is regulated by a complex network of genes and initiates with the change in shoot meristems to the progression of axillary meristems (AMs). Subsequently, AMs are derived by the genes that control meristem transition to floral meristems (FMs) (Huijser and Schmid, 2011). Transcriptome research has revealed that 357 genes play an essential role in panicle development, and

some of these genes are involved in the regulation of panicle degeneration (Wang et al. 2011). For example, LAX1/2 (LAX panicle 1/2), SPA (small panicle), MOC1 (MONOCULM 1) and OsH1 (*Oryza staiva* homeobox 1) are involved in AM development, and their mutants displayed fewer spikelets and reduced branching (Tabuchi et al. 2011; Komatsu et al. 2003; Li et al. 2003; Sinha et al. 1997). In FMs, APO1 regulate the expression of C-class homeotic genes, and their overexpression result in an increased panicle size (Ikeda et al. 2007). APO2 suppressed the transition from the inflorescence meristem to FMs through interaction with APO1 (Ikedakawakatsu et al. 2012).

Recently, research have found that the limitation of source transportation can also result in panicle degeneration in rice. SP1 (short panicle 1) regulates nitrate transportation to maintain panicle size by encoding PTR (peptide transporter), which is involved in the transportation of different assimilates to different parts of plants (Li et al. 2009). However, neither nitrate transporter activity nor any other compounds transported by known PTR proteins could be found, suggesting that SP1 may need other component(s) to be able to function as a transporter. Encoding a lipid transfer protein, OsC6 plays a crucial role in the development of lipidic orbicules and pollen exine during anther development in rice (Zhang et al. 2010). Tut-1 mutants exhibit defects in the arrangement of actin filaments in trichome, indicating that TUT1 is a functional SCAR/WAVE protein and plays an essential role in panicle development (Bai et al. 2015). OsALMT7 (aluminum-activated malate transporter 7) maintains sink size and grain yield in rice by transporting malate into the apical portion of the panicle, and its mutant exhibits apical panicle degeneration that was accompanied by cell death (Heng et al. 2018).

Inorganic cations are important components of plant nutrition and play an essential role in physiological and cellular processes. Their precise redistributions are regulated by vacuolar antiporters, which are important elements in mediating the intracellular sequestration of these cations (Manohar et al. 2011). In the *Arabidopsis* genome, 855 open reading frames code for transporters among the predicted 25498 genes (Shigaki and Hirschi, 2006). One class of transporter protein that mediates the vectorial transport of both Ca^{2+} and other metal ions is the Cation/H^+ exchanger (CAX), a secondary energised transporter that is dependent on a proton (H^+) gradient across a membrane (Pittman and Hirschi, 2016). The past several years has found that CAXs are involved in a number of important aspects of plants growth and development, especially in crops nutritional enhancement and mitigating pollutants in soils (Conn et al. 2011). However, there is no report of the relationship between Cation/H^+ exchanger and panicle development and degeneration yet.

In this study, to discover the key genes involved in panicle development, an *ym48* mutant was created from indica rice 93-11 through irradiation treatment. The *ym48* mutant was morphologically similar to wild-type 93-11 before the heading stage (Fig. 1A). However, serious degeneration appeared in the top panicle of the *ym48* mutant at the heading stage (Fig. 1B). Specifically, the withered spikelet in the *ym48* mutant was dried and malformed compared to the wild-type, and the flower appeared pale in color and smaller, especially in anthers and stigmas (Fig. 1C). Regarding the number of kernels, the wild-type had approximately 190 kernels in the main panicle. However, the *ym48* mutant had an average degeneration rate of only 72, and the degeneration rate was approximately 40% compared to 1% in wild-type (Fig. 1G

and H). With the exception of the bottom spikelet, which was not significantly different from the wild-type, the top and partial middle spikelets degenerated in the *ym48* mutant and largely led to a shorter panicle length (average 23.06 cm in 93-11, 16.72 cm in *ym48*) and plant height (average 116.99 cm in 93-11, 100.50 cm in *ym48*) (Fig. 1E and F). Regarding the tiller number and thousand seed weight, no obvious differences were noted between the wild-type and *ym48* mutant. In general, *ym48* is a typical panicle degeneration mutant that exhibits a significant reduction in grain yield.

To investigate when degeneration of the top panicle starts to occur during panicle development in the *ym48* mutant, developmental process analysis was performed to compare the wild-type 93-11 and the *ym48* mutant. As shown in Fig. 1D, the developmental process was divided into 8 stages according to panicle length. Here, a to h corresponded to 0.5 cm, 1 cm, 3 cm, 6 cm, 10 cm, 14 cm, 18 cm and 22 cm, respectively. During early-stage development (panicle length < 6 cm), no obvious difference was noted between 93-11 and the *ym48* mutant (Fig. 1D a-c). When the panicle developed to approximately 6 cm, the top panicle stared to degenerate in the *ym48* mutant, and the specific characteristic was a black and wizened spikelet (Fig. 1D d). From this stage, the degeneration in the *ym48* mutant became more serious with panicle development (Fig. 1D e-h).

To further determine the causal gene of the panicle degeneration phenotype in the *ym48* mutant, an F2 mapping population was constructed by crossing *ym48* with the indica cultivar “Yehesimiao”. In the F1 population, all individuals exhibited the normal phenotype. In the F2 population, approximately one-third of individuals (1520/4891) showed the panicle degeneration phenotype, indicating that the panicle degeneration phenotype was regulated by a single recessive gene. Next, these 1520 mutated individuals were used for mapping. The *ym48* locus was initially mapped to the long arm of chromosome 1 between the markers IL-20 and IR-19 (Fig. 2A). After fine mapping, the mutation was further narrowed down to a 70-kb genomic region between markers RM11162 and IR-4, in which eleven ORFs were annotated (Fig. 2A). After sequence comparison, a single nucleotide substitution of A to G in the 190th nucleotide in the first exon of LOC_Os01g37690 (*OsCAX1a*) was noted in the *ym48* mutant, and the corresponding 64th amino acids were substituted from threonine (T) to alanine (A) (Fig. 2B).

To verify whether the *OsCAX1a* mutation was responsible for panicle degeneration in the *ym48* mutant, transgenic knockout and overexpression were subsequently performed. In transgenic knockout analysis, target sequences of ACTGGTTGCGGTCGTAGGGCTGG were selected as sgRNA, and the CRISPR/Cas9 recombinant plasmid was introduced into wild-type 93-11. Two transgenic knockout lines (*cas9-1* and *cas9-2*) were obtained, and all individuals showed the panicle apical degeneration phenotype, which is similar to that noted in the *ym48* mutant (Fig. 2C left). Sequences comparing *cas9-1*, *cas9-2* and 93-11 indicated that dozens of nucleotides were deleted near the sgRNA (Fig. 2E). For transgenic overexpression analysis, the CDS region of the *OsCAX1a* plus UBI promotor was inserted into pCAMBIA1301, and the recombinant plasmid was introduced into the *ym48* mutant. Two transgenic overexpression lines (*oe-1* and *oe-2*) were obtained, and the panicle degeneration phenotype was significantly recovered (Fig. 2C right). qRT–PCR showed that the relative expression of *OsCAX1a* in transgenic overexpression lines was increased compared to that in the *ym48* mutant (P value < 0.01) (Fig.

2D). Collectively, these results demonstrated that the *OsCAX1a* mutation was responsible for panicle apical degeneration. To further identify whether the Ca^{2+} transportation was effect in *ym48* mutant, hydroponic solutions with 7 different concentrations of $CaCl_2$ (0, 0.2, 0.5, 1, 5, 20 and 100 mM) were used in hydroponic experiment. As shown in Supplementary Fig. 1, the shoots development of *ym48* mutant which cultivated in 0, 0.2, 0.5, 20 and 100 mM $CaCl_2$ was torpid compared to wild-type 93-11, especially in 0 and 100 mM $CaCl_2$ groups, and had no obviously different in roots and normal $CaCl_2$ groups. These results demonstrated that the A to G substitution at the 190^{th} nucleotide reduced the tolerance from extreme Ca^{2+} stress and the Ca^{2+} transportation was effect in *ym48* mutant.

OsCAX1a is a Cation/H^+ exchanger and plays an essential role in Ca^{2+} selection and transportation in rice (Kamiya and Maeshima, 2004). OsCAX1a is a member of the CAX family. Six CAXs have been identified in *Arabidopsis* and rice: AtCAX1, AtCAX2, AtCAX3, AtCAX4, AtCAX5, AtCAX6, AtCAX7, OsCAX1a, OsCAX1b, OsCAX1c, OsCAX2, OsCAX3, and OsCAX4 (Supplementary Table 3) (Pittman and Hirschi, 2016). To identify the relevance of these protein sequences, a phylogenetic tree of the CAX family in *Arabidopsis* and rice was constructed using the NJ method and divided into two groups (Supplementary Fig. 2A). OsCAX1a, OsCAX1b, OsCAX1c, AtCAX1, AtCAX3 and AtCAX4 (Group I) exhibited significant homology, especially between OsCAX1a and OsCAX1b. Moreover, in the analysis of protein sequence alignment in the CAX family, the amino acids mutated in *ym48* were all threonines in Group I and were highly conserved. In contrast, differentiation appeared in this amino acid in group II, and these sequences were deficient in OsCAX4 (Supplementary Fig. 2B). Previous research has suggested that gene structural diversity is an important resource for multigene family evolution (Liu et al. 2009). To elucidate the structural similarity and diversity of CAXs in *Arabidopsis* and rice, schematic diagrams of exons and introns were constructed. As shown in Supplementary Fig. 2C, slightly different numbers of exons were found in CAX genes, varying from 8 to 12. Except for OsCAX1c, the CAX family exhibited a similar construction of exons and introns, which would contribute to the explanation of functional conservation. Next, 20 conserved motifs were identified in CAX proteins using MEME tools. The composition and arrangement of these motifs were largely consistent with previous phylogenetic analysis (Supplementary Fig. 2D). Motif 9, which included the mutated amino acid in *ym48*, was present in all CAX family members except OsCAX4. These results implied that these sequences might play an essential role in Ca^{2+} selection and transportation. The specific sequence information is listed in Supplementary Table 2.

Next, the expression pattern of *OsCAX1a* was investigated by qRT–PCR. *OsCAX1a* expression was detected in all rice organs analyzed. Relatively higher expression was noted in roots, stems and panicles, and lower expression was noted in other organs, including leaves and sheaths (Fig. 3A). A detailed expression analysis focusing on the panicle implied that *OsCAX1a* expression increased continuously during panicle development (Fig. 3B). The *OsCAX1a* expression pattern was further evaluated in plants transformed with a GUS reporter gene driven by a 2500-bp promoter sequence of *OsCAX1a*. We observed GUS activity in various organs examined. The strongest staining was noted in the shoot, panicle and stem, and slight staining was noted in the leaf and sheath, which is consistent with the qRT–PCR analysis results (Fig. 3C). As a Ca^{2+} transporter, OsCAX1a is highly expressed in roots and stems and is

responsible for Ca^{2+} absorption and transportation. However, *OsCAX1a* was also highly expressed in panicles, and its expression increased with panicle development. The function of *OsCAX1a* in rice panicles remains unclear, and these results might indicate that *OsCAX1a* plays an important role in panicle development and differentiation.

Panicle degeneration is a widespread physiological problem that reduces grain yield in rice and other cereal crops (Yamagishi et al. 2004). However, the genetic and molecular mechanisms regulating panicle degeneration remain poorly understood. Various factors could lead to panicle degeneration in rice, including abnormal meristem development, phytohormone variation, source transport limitation, and abiotic stresses (Ali et al. 2019). Our research demonstrated that Ca^{2+} transportationmight be closely associated with panicle degeneration in rice. Ca^{2+} played an essential role in plants. First, Ca^{2+} is an essential nutrient element for plants. Ca^{2+} constitutes the main component of the plant cell wall and cell membrane, accounting for 10% of plant dry weight (Marschner H, 1995). Second, Ca^{2+} is one of the most important second messengers in plant cell signal transportation. A variety of external stimuli could cause changes in Ca^{2+} concentration ($[Ca^{2+}]_{CyT}$) in the plant cytoplasm, thus transforming external signals into internal signals that can be sensed by plants to induce a series of physiological and biochemical reactions, achieving plant perception and responding to environmental stimuli and developmental information (Sanders et al. 2002).

To maintain Ca^{2+} balance in the body, plants must complete Ca^{2+} transport through the Ca^{2+} transfer system. There were two types of Ca^{2+} channels: (1) Ca^{2+} inward transporters transport Ca^{2+} from the outside of the cell into the cytoplasm and (2) the outward transport system is responsible for transporting Ca^{2+} out of the cytoplasm or into organelles, such as vacuoles. These transport systems included Ca^{2+}-ATPase and Ca^{2+}/H^{+} reverse transporters. As a class of Ca^{2+} outward transporters, Ca^{2+}/H^{+} reverse transporters play an important role in plants. Firstly, after completing their messenger role, they restored Ca^{2+} concentration in the cytoplasm to the resting level in preparation for the initiation of the next signal. Second, Ca^{2+} was correctly allocated to each organelle to ensure the progress of various specific biochemical reactions (White et al. 2003). Third, Ca^{2+} is stored in intracellular and extracellular calcium banks and interacts with Ca^{2+} channels to ensure the generation and completion of cell signals (Sanders et al. 2002). Since the first Ca^{2+}/H^{+} reverse transporter (VCX1) was identified in the tonoplast *of Saccharomyces* Cerevissiae, an increasing number of Ca^{2+}/H^{+} reverse transporters have been found in plants, including maize, rice, *Arabidopsis* (Vicente et al. 1995; Shigaki et al. 2000; Kamiya et al. 2004).

In this study, fine mapping showed that A to G substitution at the 190^{th} nucleotide (64^{th} amino acid was changed from threonine to alanine) in OsCAX1a resulted in panicle degeneration in the *ym48* mutant. Actually, Ca^{2+} transportation was also affected by this mutation. In the hydroponic experiment, tolerance to extreme Ca^{2+} concentrations was altered in the *ym48* mutant compared to wild-type 93-11, which exhibited torpid development. Previous research has suggested that OsCAX1a has 11 predicted transmembrane domains (TMs) and is divided into three characteristic domains: the N-terminal

regulatory region, the calcium domain, and the C domain (Kamiya and Maeshima, 2004). The N-terminal regulatory region has been shown to suppress Ca^{2+} transport activity by interacting with its neighboring N-terminal sequence. The domain between TM1 and TM2 was thought to be involved in the selection of Ca^{2+}. In the *ym48* mutant, the 64^{th} amino acid was changed from threonine to alanine, and this amino acid is located in front of TM1. However, current studies have paid little attention to this domain, and its specific function remains unclear.

In conclusion, a highly conserved amino acid site mutation of Cation/H^+ exchanger OsCAX1a was identified in *ym48* mutant and generated the serious apical panicle degeneration in rice. Also, the tolerance from Ca^{2+} stress was damaged due to the mutation. *OsCAX1a* highly expressed in roots, stems and panicles and that its expression increased with panicle development. This novel relationship between Ca^{2+} transportation and panicle degeneration was not reported before and our research provided a new view point to explore the mechanism of panicle development and degeneration in rice.

Abbreviations

GUS: β-glucuronidase; AMs: axillary meristems; FMs: floral meristems; T: threonine; A: alanine; TMs: transmembrane domains

Declarations

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

All authors reviewed the manuscript and agreed to publish it.

Availability of Data and Material

The data sets supporting the results of this article are included within the article and its additional files.

Competing interests

The authors declare that they have no conflict of interest.

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Authors' contributions

QG, FS, CL and DN conceived and designed the experiments. QG implemented the experiments and prepared the manuscript. FS guided the molecular experiments. CL and DN collected the field data. QG analyzed the results. QG, FS, CL and DN revised the manuscript. All authors contributed to the article and approved the submitted version.

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Figures

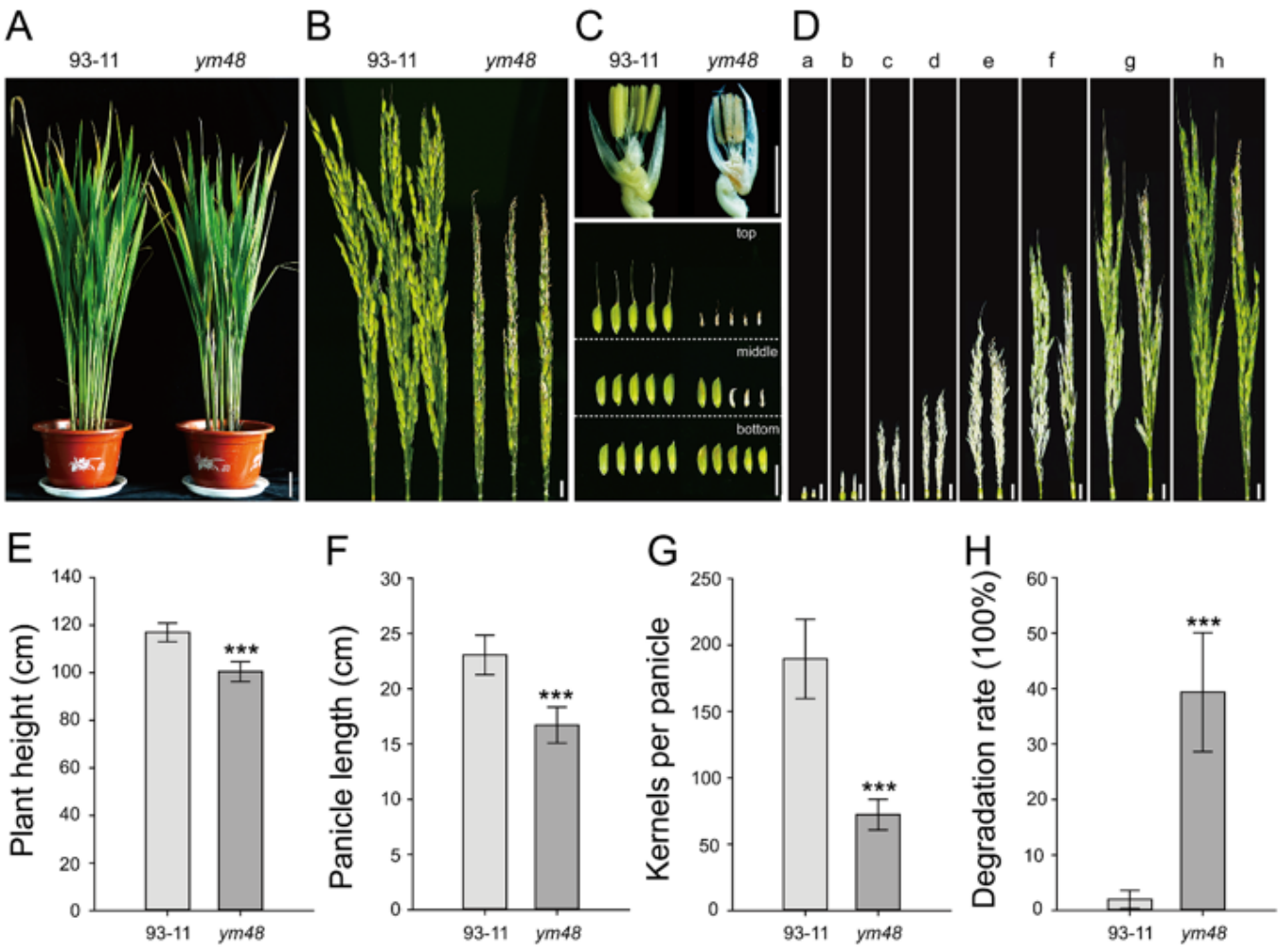

Figure 1

Phenotypic characterizations of wild-type 93-11 and ym48 mutant. A The plant type during the heading stage. Bars = 10 cm. B The panicle type before flowering. Bars = 1 cm. C Flowers and different vertical positions of grain type (top, middle and bottom). Bars in flowers indicate 0.5 cm, and bars in grain type indicate 1 cm. D Panicle type during the different development stages. a, 0.5 cm. b, 1 cm. c, 3 cm. d, 6 cm. e, 10 cm. f, 14 cm. g, 18 cm. h, 22 cm. Bars = 1 cm. E Plant height. F Panicle length. G Kernels per panicle. H Degeneration rate. All data shown are mean ± SE (n = 20). Asterisks represent statistically significant differences from wild-type 93-11, as determined by Student's t test. ***P < 0.001.

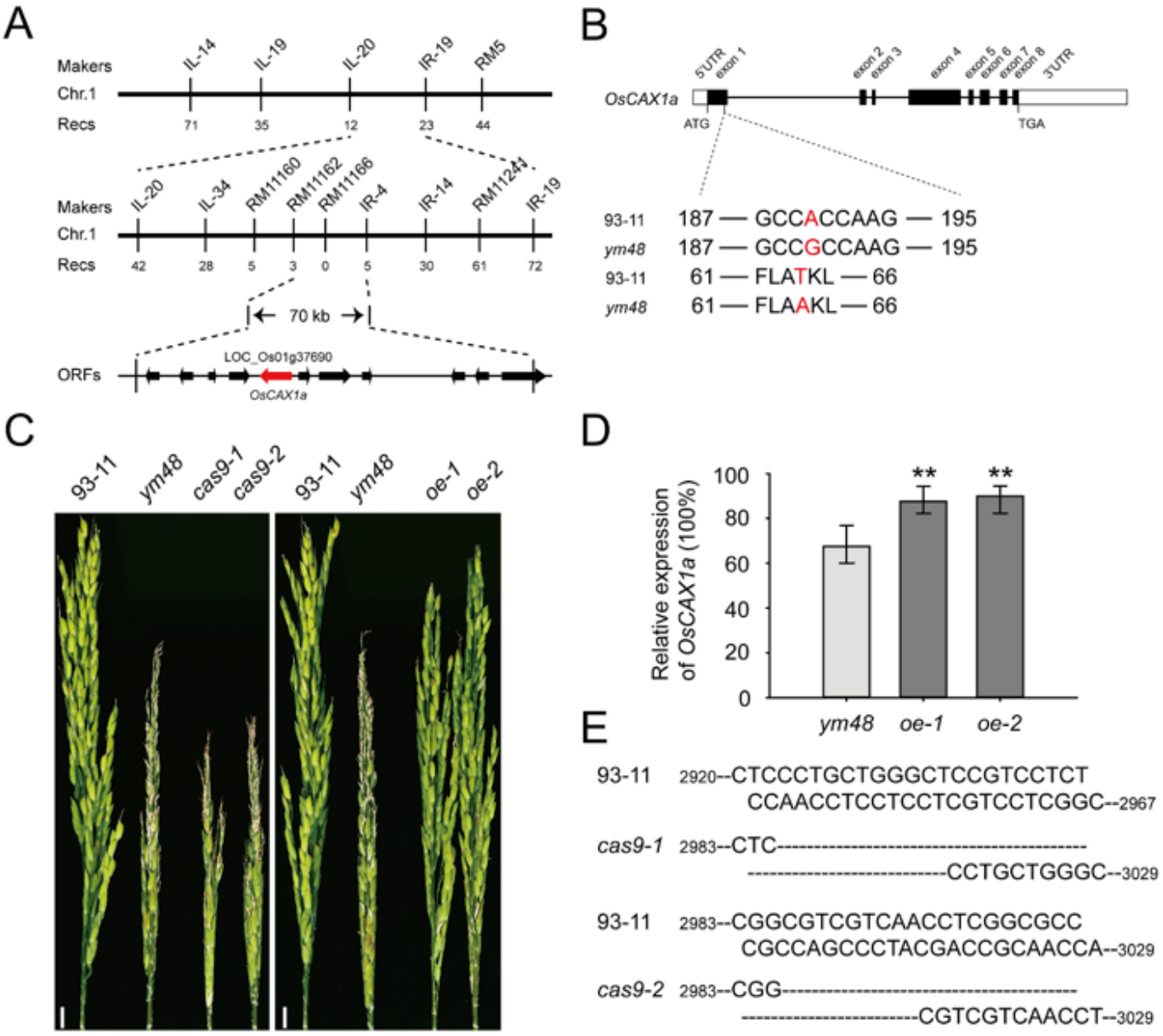

Figure 2

Map-based cloning of ym48. A Fine mapping of ym48. The molecular markers are indicated above the black line, which refers to chromosome 1, and the corresponding numbers of recombinants are indicated below the black line. The candidate ORF is highlighted in red. B Gene exon-intron structure of OsCAX1a. Black rectangles indicate different exons, black lines show introns, and white rectangles signify 5' and 3' UTRs. The mutation site in ym48 exists in exon 1, and specific sequences are listed below. C Genetic confirmation of OsCAX1a through transgenic knockout (cas9-1 & cas9-2) and overexpression (oe-1 & oe-2). Bars = 1 cm. D Relative expression of OsCAX1a in overexpression transgenic lines. Asterisks represent statistically significant differences from ym48, as determined by Student's t test. **P < 0.01. E Sequence information in two knockout transgenic lines. The specific position is marked at both ends.

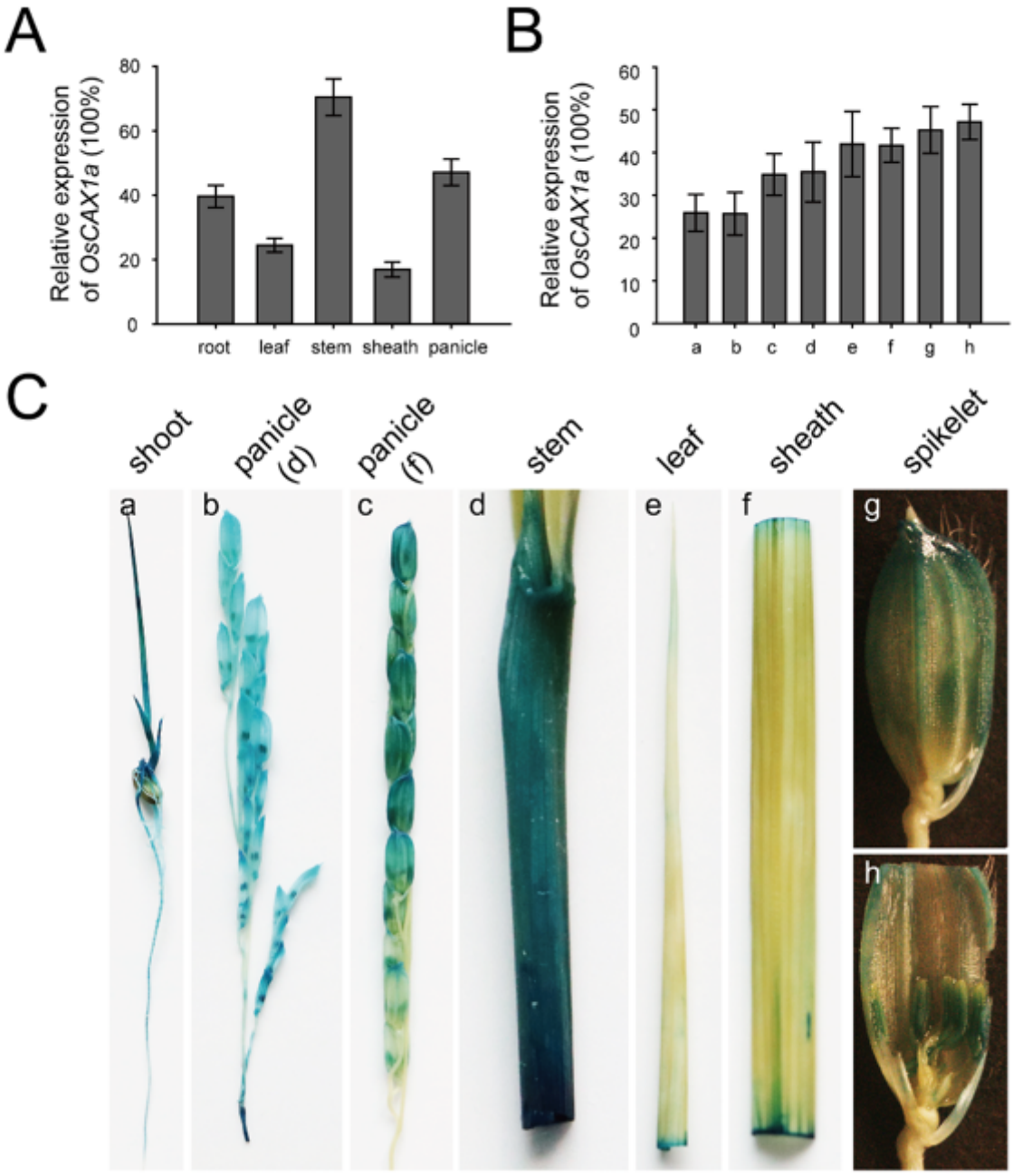

Figure 3

Expression pattern and subcellular localization of OsCAX1a. A Relative OsCAX1a expression in different tissues before flowering, including roots, leaves, stems, sheaths and panicles. B Relative OsCAX1a expression in different developmental stages, which is consistent with Fig. 1D. C Promoter activity of OsCAX1a as shown by GUS staining. a, shoot. b, panicle in VI stage. c, panicle in IV stage. d, stem. e, leaf. f, sheath. g and h, spikelet.

Supplementary Files

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