https://doi.org/10.17221/103/2020-CJGPB

TPR domain coding gene *ST2* may be involved in regulating tillering and fertility in rice

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Citation: Wei G., Yang H., Xiong Z., Wu J., Chen D., Liu Y., Ban Y., Liu W., Shang L., Wang N. (2021): TPR domain coding gene *ST2* may be involved in regulating tillering and fertility in rice. Czech J. Genet. Plant Breed., 57: 83–90.

Abstract: A decrease in the tiller number and male sterility will lead to a decline in the rice yield. Therefore, it is significant to study the molecular mechanism of controlling the tiller number and regulating the male reproductive development. The mutant *st2* (*single tiller 2*) was induced by ethyl methane sulfonate (EMS) in the indica maintainer line Xinong 1B and showed single tillering and male sterility. I_2-KI staining showed that the *st2* pollen was aborted. The scanning electron microscope (SEM) observation underlined that the anther of *st2* became smaller, the wax of the epidermis reduced, the inner wall shrank and the Ubisch body decreased, the pollen collapsed, and the germination pore developed abnormally. The genetic analysis discovered that the trait was controlled by a single recessive nuclear gene located on chromosome 3. *LOC_Os03g05540* encoding a tetratricopeptide repeat (TPR) domain was identified as the candidate gene by sequencing. The quantitative real-time polymerase chain reaction (qRT-PCR) analysis indicated that *ST2* was highly expressed in the stem apical meristem (SAM) and the initial stage of meiosis during the anther development. The subcellular localisation indicated that ST2 is a nuclear and plasmic localisation protein. The homology analysis demonstrated that *ST2* was evolutionarily conserved. These results laid a foundation for further study of the *ST2* function.

Keywords: gene mapping; male sterility; *Oryza sativa* L.; tiller; tetratricopeptide repeat (TPR)

Improving the grain yield and quality is a vital goal of basic plant science and applied science research (Ren et al. 2019). The tillering ability is a key factor in determining the plant structure, which develops independently of the main culm and contributes significantly to the grain yield (Spielmeyer & Richards 2004). Rice tillering is mediated by the interplay of the environment and endogenous signals (such as plant hormones) (Li et al. 2016).

There are a large number of proteins containing tetratricopeptide repeat (TPR) in nature, and it is gradually becoming an important determinant for signal transduction pathways mediated by most plant hormones (Schapire et al. 2006). SPYDLY (SPY), a TPR motif contained protein, plays an active role in the signal transduction of cytokinin that plays a role in the early stage of bud initiation to control the activity of the meristem (Schmülling 2002). It is reported that the TPR domain is related to fertility. The wheat gene *FKBP73* containing TPR and CaMbd (CaM-binding domain), are essential for the correct calmodulin binding and dimer formation as well as for the male fertility in transgenic rice (Kurek et al. 2002). In addition, a close relationship is observed

https://doi.org/10.17221/103/2020-CJGPB

between rice male sterility and endogenous hormones (Risheng et al. 1996), gibberellins (GAs) and cytokinin promote male development in *Arabidopsis thaliana* and tobacco (*Nicotiana tabacum*) (Huang et al. 2003). Understanding the complex biological process of male reproductive development can help breeders understand the characteristics of increasing the yield and ensuring the reproductive capacity of rice (Peipei et al. 2017).

The mutant *st2*, showed the phenotype of a single tiller and male sterility, reported in this study was induced by ethyl methane sulfonate (EMS) (concentration: 0.05–0.5 mol/L) from the indica maintainer line Xinong 1B. A mapping interval of 1.23 Mb was determined by a map-based cloning technique, and a candidate gene *LOC_Os03g05540* was identified by sequencing and named *ST2* according to the phenotype. The TPR domain coding gene *ST2* has a base substitution that occurs in its second exon, which contributed to glycine, an important acid in the TPR domain, change into valine. The transcriptional level analysis underlined that *ST2* is highly expressed in the stem apical meristem (SAM), and it is speculated that it might play a role in the regulation of the tiller number. In addition, *ST2* is specifically expressed at the initial stage of meiosis during the anther development. The TPR function was lost after the mutation, resulting in a single tiller phenotype and a male sterility phenotype. The study of *ST2* is of great significance for further understanding the molecular mechanism of proteins containing a TPR domain in regulating the rice tiller number and rice fertility, as well as for the construction of an ideal plant type of rice.

MATERIAL AND METHODS

Material. The material in this experiment was derived from the indica maintainer line Xinong 1B (1B) by an EMS induction mutation. After the stable inheritance of the self-crossing traits for successive generations, the F_1 generation was obtained by crossing with the restorer line Jinhui 10 (J10), and the F_2 generation was obtained by the self-crossing of F. F_2 was planted together with the parents for the genetic analysis and gene mapping.

Genetic analysis. The separation of the characteristics in the F_2 generation population was investigated, and the separation ratio between the normal plants and the mutant plants was calculated and tested by the chi-squared method (Gai 2000).

Observation with asana mirror and identification of pollen fertility. The fresh samples were observed and photographed at different magnification rates. The anthers 1B and *st2* were pounded in an I_2-KI solution, dyed in the dark and photographed (Xu et al. 2017).

Observation and analysis by SEM. The fresh samples were placed in a S-3500 NiI scanning electron microscope (Hitachi, Japan) with a –20 ℃ cooled stage (Ren et al. 2019).

Genomic DNA extraction and PCR amplification. The DNA was extracted according to the improved cetyltrimethylammonium bromide (CTAB) method (Rogers & Bendich 1985) for the gene mapping. The polymerase chain reaction (PCR) refers to Ren et al. (2016).

Molecular marker analysis and construction of the genetic map. The genetic mapping was performed using the bulked segregant analysis (BSA) method (Michelmore et al. 1991). The http://gramene.org/ database and Vector NTI Advance 11.5 software (Invitrogen, USA; http://www.invitrogen.com/) were used to develop new polymorphic simple sequence repeats (SSR) and insertion-deletion (InDel) molecular markers. The molecular marker primers were synthesised by Chengdu Tsingke Biotechnology Co., Ltd.

Candidate gene analysis. The http://gramene.org/ website was used to find the genes in the interval, and the http://www.ricedata.cn/index.htm website was used to annotate and analyse the genes.

Extraction of the total RNA and synthesis of the cDNA. The RNA was extracted according to the instructions of the total RNA extraction and purification kit provided by Tiangen Biochemical Technology (Beijing) Co., Ltd. The reverse transcription kit PrimeScript 1st Strand cDNA Synthesis Kit was produced by TaKaRa Biology. The qRT-PCR analysis was performed using the BIO-Rad CFX Connect Real-Time PCR System and a TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus) kit (Takara Bio Inc., China). The $2^{-\Delta\Delta CT}$ method was used to analyse the relative changes in the gene expression (Liu et al. 2016).

Construction of evolutionary tree. The amino acid sequence of ST2 was downloaded from the http://gramene.org/ website, and the ST2 homologous sequences were downloaded from the https://www.ncbi.nlm.nih.gov/ website. The phylogenetic analysis was carried out with MEGA7.0 (Kumar et al. 2016).

Subcellular localisation. The full-length coding sequence of *ST2* was amplified from the 1B cDNA. The amplified fragment and vector were digested with *Xba*I and *BamH*I to construct CaMV *35S*-ST2–GFP. Operation steps refer to Xing et al. (2021). The speci-

https://doi.org/10.17221/103/2020-CJGPB

mens were imaged under an inverted confocal laser scanning microscope (LSM710, Carl Zeiss Microscopy GmbH, Cologne, Germany).

RESULTS AND DISCUSSION

Phenotypic observation and analysis. The tillering has a great effect on the yield of monocotyledon crops such as rice (Na et al. 2014). In order to understand the molecular mechanism of the tiller formation, some mutants, such as *moc1* and *moc2*, were isolated and identified (Komatsu et al. 2003; Koumoto et al. 2013). In the aspect of tillering, compared with the wild type (WT), *st2* showed a single tillering from the tillering stage (Figure 1A, B) to the mature stage (Figure 1D, E). At the mature stage, WT can blossom normally, so the panicle is bent downward, and the glume of *st2* is shrivelled and without contents, so it cannot blossom normally, showing that the panicle is upright (Figure 1C). The cause of the tiller degeneration is mainly due to the competition of hormones or the change in the hormone balance (Li-Na et al. 2010). Plant hormones play an important role in regulating the maintenance and differentiation of meristem cells (Lu et al. 2015). The genetic interpretation of the tiller number has become a hot topic in rice genetics and breeding (Liu et al. 2010). Therefore, the study of *st2* will help to reveal the molecular mechanism of tillering in rice.

Identification of the development of the male organ and pollen fertility of *st2*. Male sterility in plants is very significant for the commercial application of heterosis and hybrid seed production, and it also provides a means to explore the molecular mechanism of a plant's reproductive development (Gustafson et al. 2007). The spikelet (Figure 2A) and anther (Figure 2E) of *st2* were significantly shorter than that of WT, and the colour of the anther has no obvious change compared with the WT (Figure 2B, E). Using an I_2-KI staining solution to detect the filling of the pollen starch, only a few *st2* pollens could be stained compared with the WT, and the pollen grains showed an abnormal state (Figure 2C, D). It is suggested that *st2* is a male sterile mutant, and the shortening of the anther indicates that the anther development of *st2* is blocked, which may be

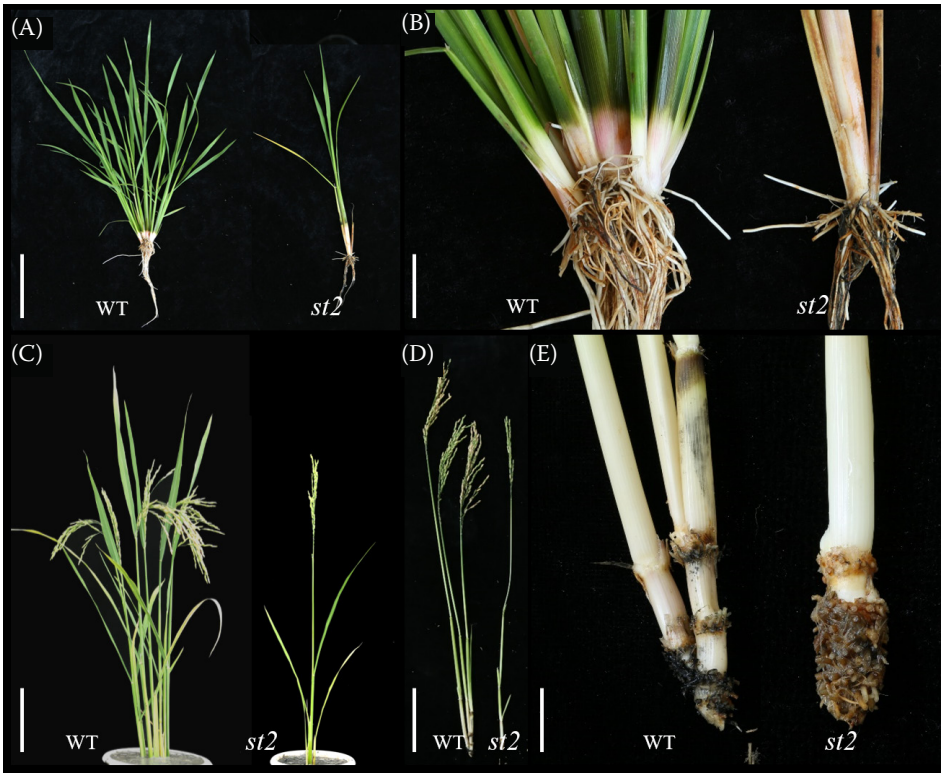

Figure 1. Phenotypic analysis of wild type (WT) and *st2*: WT and *st2* at the tillering stage (A), the base of WT and *st2* at the tillering stage (B), WT and *st2* at the mature stage (C), the tillering of WT and *st2* at the mature stage (D), the tillering nodes of WT and *st2* at the mature stage (E); bar = 9.2 cm (A) or 1.1 cm (B) or 15 cm (C) or 15.92 cm (D) or 1.7 cm (E)

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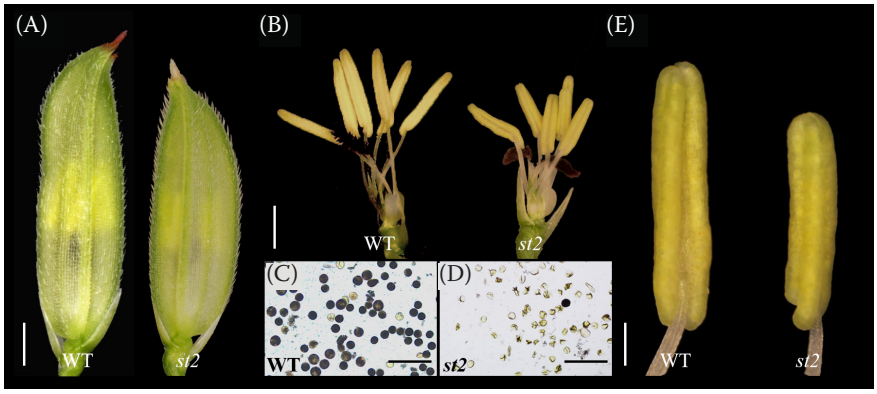

Figure 2. Asana microscope observation of wild type (WT) and *st2*: spikelets of WT and *st2* (A), anthers of WT and *st2* (B), pollen of WT and *st2* (C-D), single anther of WT and *st2* (E); bar = 1.75 mm (A) or 1.5 mm (B) or 136 μm (C, D) or 0.5 mm (E)

caused by male sterility. The anther of *st2* was observed by scanning electron microscope (SEM), and the results indicated that it is smaller and showed a state of shrinkage compared with the WT (Figure 3A, B), and the structure of the waxy layer of the anther epidermis was significantly reduced (Figure 3C, D), thus, it is speculated that the protective effect on the pollen is reduced, which may be one of the causes of male sterility. The Ubisch body of *st2* obviously decreased, and the inner wall of the anther abnormally accumulated (Figure 3E, F). The Ubisch body plays an important role in the material transport from the tapetum to the pollen (Jing 2009). Therefore, a decrease in the Ubisch body may lead to a decrease in all kinds of substances transported to the pollen, resulting in abnormal pollen development and eventually leading to male sterility. The pollen grains of *st2* were smaller and showed a state of shrinkage and collapse, and there were defects in the development of the germination pores which was characterised by the collapse of the germination pore cover (Figure 3G, H), which may be caused by male sterility. Male sterility will inevitably lead to a significant decrease in the rice yield, so it is very important to study the related mutants and genes, therefore, the study of *ST2* will contribute to a deeper

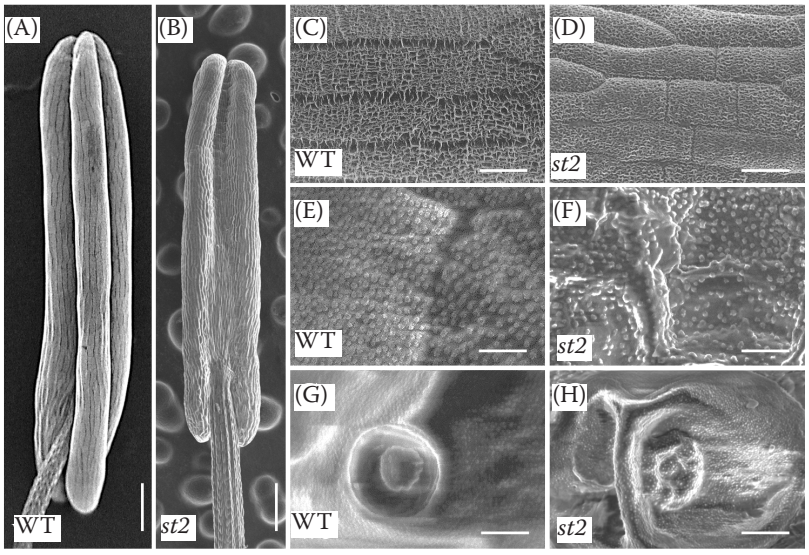

Figure 3. Scanning electron microscope observation of wild type (WT) and *st2*: anthers of WT and *st2* (A–B); the structure of the anther epidermis of WT and *st2* (C–D); the structure of the inner wall of the anther of WT and *st2* (E–F); pollen germination pores of WT and *st2* (G–H); bar = 310 μm (A, B) or 22 μm (C, D) or 4.4 μm (E, F) or 3.3 μm (G, H)

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understanding of the regulatory mechanism of male sterility in rice.

Gene mapping and determination of candidate genes. The F_1 plants of the hybrid between J10 and *st2* showed normal tillering and fertility, indicating that the mutation character of *st2* was controlled by a recessive gene. The F_2 population was obtained by selfing the F_1 seeds. It was found that there were two types of single tiller plus a sterile phenotype and normal phenotype in the F_2 population. Through field statistical characteristics, there were 387 normal phenotypic plants and 122 mutant phenotypic plants in the F_2 population. The chi-squared test indicated that the segregation ratio conformed to the Mendelian genetic segregation ratio at 3 : 1 ($\chi^2 = 1.607 < \chi^2_{0.05, 1} = 3.84$) (Table S1 in the Electronic Supplementary Material (ESM)). The results suggest that the mutation character of the mutant *st2* was controlled by a single recessive nuclear gene.

The polymorphic molecular marker primers that were provided by the Rice Research Institute, Southwest University were used to analyse the gene pool of J10 and *st2*; there was a linkage relationship between the target gene and the molecular marker ZTQ27 on chromosome 3. A further linkage analysis was carried out on the adjacent marker primers on both sides of the marker ZTQ27, and the target gene was preliminarily mapped between ZTQ27 and W25-28. The SSR markers and InDel markers (Table S2 in ESM) were further developed by us within the initial positioning interval, and finally *ST2* was mapped between the markers FL-1 and W25-38, and the physical distance was 1.23 Mb. There was a base substitution (Figure 4, Figure S2A, B in ESM) in the second exon of the TPR domain coding gene *LOC_Os03g05540*, which contributed to the glycine, an important amino acid in the TPR domain, change into Valine (Figure S1A and Figure S2C in ESM), which may affect the function of the gene. Therefore, it was identified as a candidate gene and named *ST2*, and its amino acid evolution is conservative (Figure S1B in ESM). The protein containing TPR is an important element in plant hormone signal transduction, indicating that the formation of a protein complex mediated by TPR may be the common mechanism of plant hormone regulation (Rosado et al. 2006). Plant endogenous hormones are indispensable to the normal development of stamens. It has been noted that there is a certain relationship between plant male sterility and hormones (Zhong-Ming et al. 2015). Similarly, TPR is also related to fertility (Zhang & Tang 2005). Therefore, the TPR domain encoding gene *ST2* may jointly affect the tillering and fertility of the rice through hormonal regulation.

Transcriptional level analysis of candidate genes. qRT-PCR was used to analyse the transcriptional level of *ST2*, the primer (Table S3 in ESM) developed by us was located on the coding sequence (CDS) of *ST2*. It was expressed in all the parts, but highly expressed in the SAM compared with the others (Figure 5A), which suggested that *ST2* may be involved in the formation of rice tillers. Due to the male sterility phenotype of *st2*, we analysed the expression of *ST2* at the different anther development stages in the rice. The classification method referred to Zhang et al. (2011), and the results indicated that *ST2* was specifically expressed at stage 7, which is the beginning of meiosis, which suggests that ST2 may

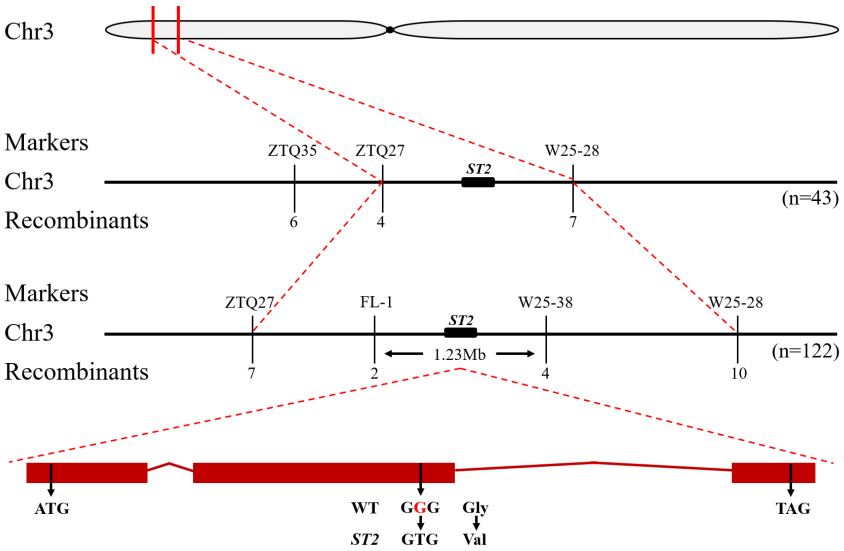

Figure 4. Gene mapping of *ST2*

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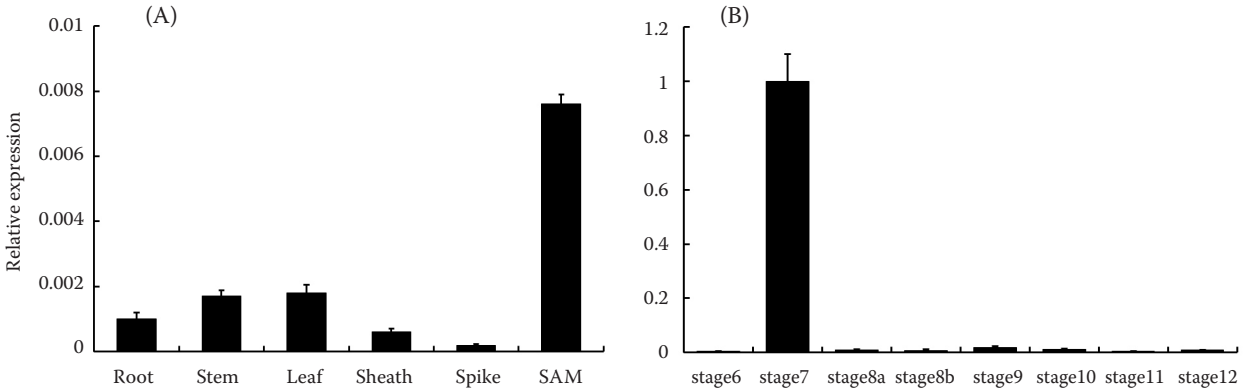

Figure 5. Transcriptional level analysis of *ST2*: expression of *ST2* in various parts of the wild type (WT) (A), expression of *ST2* in the anthers of the WT at different stages (B)

regulate the male fertility of rice by affecting the meiosis process (Figure 5B).

Subcellular localisation analysis. In order to explore the function of ST2, we constructed a Green Fluorescent Protein (GFP) fusion expression vector for the subcellular localisation analysis, the primers used were developed by us and are listed in Table S4 in the ESM. The results indicated that ST2 was a nuclear and plasmic localisation protein (Figure 6), which indicated that ST2 might play a role in the nucleus. A steroid receptor has a special preference for the TPR protein, and its localisation is controlled by the TPR protein recruitment (Banerjee et al. 2008). The TPR domain exists in the components of the animal steroid receptor complex. The mutant *bri1* is dwarfed, and the brassinosteroids bind to the extracellular domain of the receptor kinase BRI1 to activate its kinase activity and activate the signal transduction cascade that regulates the nuclear gene expression and plant development (Tang et al. 2008). The ST2 protein containing a TPR domain is located in the cytoplasm and the nucleus, which may transmit hormone signals into the nucleus by recruiting hormone receptors, thus regulating the plant growth.

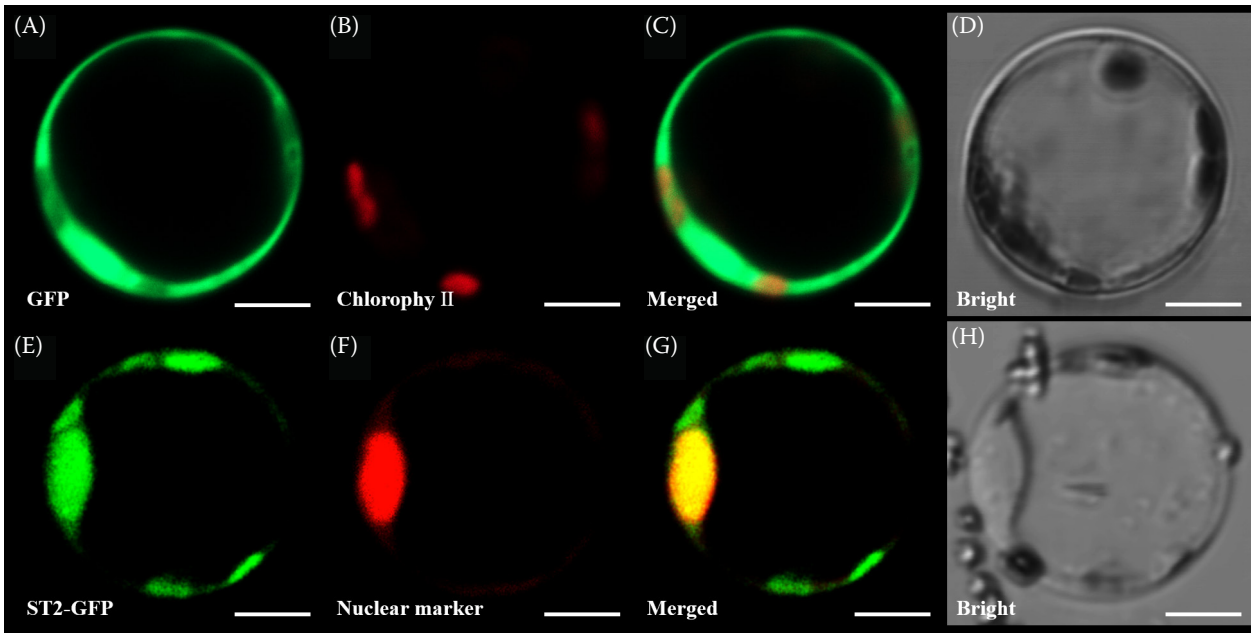

Figure 6. Subcellular localisation of ST2: expression of *35S*-GFP in the rice protoplast (A–D), expression of *35S*-ST2-GFP in the rice protoplast (E–H); bar = 5 μm (A–H)

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CONCLUSION

In this study, we identified a single tiller mutant *st2*, which could not produce any tillers, and its male organs were abnormal, such as having a shorter anther, a reduced wax layer of the anther epidermis, a fold in the anther inner wall and a significantly reduced urn structure, and finally having pollen collapse and the absence of a germination pore cover, showing male sterility. The inheritance analysis indicated that the mutation was controlled by a single recessive nuclear gene. By gene mapping, *ST2* was mapped between the molecular markers FL-1 and W25-38 on chromosome 3, and the physical distance was 1.23 Mb, there were 16 cloned genes and 3 uncloned ones, but reported genes in the interval. Among the corresponding mutants of these reported genes, we did not find the phenotype of the single tiller and male sterility at the same time. Therefore, we infer that *st2* is a new mutant with both a single tiller and male sterility phenotype. A TPR domain coding gene *LOC_Os03g05540* was identified by sequencing and identified as a candidate gene. The transcriptional level analysis suggested that *ST2* was highly expressed in the SAM. It is speculated that *ST2* is a gene related to the regulation of the number of tillers. In addition, *ST2* is specifically expressed at the initial stage of meiosis during the anther development, suggesting that *ST2* may control the rice fertility by affecting meiosis. The functional deletion of this gene affects the production of the tillers and the development of the male organs in *st2*, resulting in a single tiller and male sterility phenotype. The subcellular localisation indicated that ST2 is a nuclear and plasmic localisation protein. At present, there are few studies on the role of TPR inclusion proteins in the regulation of the tiller production and reproductive organ development in rice. The study of ST2 may be of great significance for the deeper understanding of the molecular mechanism of TPR inclusion proteins regulating the production of tillers and the development of reproductive organs in rice and is also helpful to breed the ideal plant type and produce the genetic improvement of the rice.

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Received: December 16, 2020
Accepted: June 10, 2021
Published online: July 1, 2021