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A 31-bp indel localised in the 5' untranslated region of *OsSUT3* affects the gene expression and rice (*Oryza sativa* L.) pollen development

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Abstract: *OsSUT* genes have been demonstrated to be relevant for diverse biological processes in rice. In this study, we identified the close relationship between a 31-bp insertion in a 5' untranslated region (5' UTR) of the *OsSUT3* gene and higher *OsSUT3* expression in rice panicles by qRT-PCR and transgenic research. Statistically significant results ($P < 0.01$) were found for this 31-bp insertions/deletions (indels) in the rice pollen development and other panicle traits, such as the pollen number, pollen fertility, seeding rate, and grain length. An evolution analysis showed that the proportion of the 31-bp insertion significantly increases in rice domestication. Therefore, the 31-bp Indel could be considered as a convenient molecular marker to screen more pollen and better panicle traits in rice breeding.

Keywords: cis-elements; genotype; panicle traits; polymorphism; regulation

Rice (*Oryza sativa* L.) is one of the most important cereal crops in the world. Panicles are a key component of the agronomic traits in rice, directly determining the rice yield. The formation of panicles is a systemic process and closely correlated with the pollen development (Gayathri & Stephen 2021). Thereby, it is of great value to screen excellent individuals with high viable rate of pollen in rice breeding.

Marker assisted selection (MAS) is an important selection means for the prediction of the breeding value of an individual (Lande & Thompson 1990). Through the development of MAS, it has been widely applied to screen key traits in most important food crops (Schuster 2011; Liu et al. 2020). Indels, as the common MAS, were crucial prognostic factors affecting the gene expression and plant phenotype in molecular-assisted breeding. In maize, indels derived from teosintes (a maize ancestor) were selected to be reserved or discarded in different types of *zein2* (*z2*) genes during domestication (Li et al. 2018). In the sunflower, a 999-bp upstream insertion in the promoter region of the *HaCYC2c* gene resulted in a garden variety with disc floret bilaterality (Chapman et al. 2012). Therefore, a small genetic change in the non-coding region plays an important role by affecting the morphology and physiology of organisms.

Sucrose transporter genes (*SUTs*/*STPs*) are key components that mediate the cross-membrane transport of sucrose in most plants. Several studies on pollen development have revealed that there is a strong correlation between pollen development

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and the expression of *SUTs*/*STPs*, as demonstrated in sorghum *SbSUT3* (Mizuno et al. 2016), cucumber *CsSUT1* (Sun et al. 2019), and apple *MdSTP13a* (Li et al. 2020). In rice, five *SUT* genes (*OsSUT1-5*) were isolated (Aoki et al. 2003). *OsSUT3*, a member of the *OsSUT* family, has been demonstrated to be specifically expressed at high levels. Its high expression is regulated by the 385-bp core pollen-specific regulatory sequence in the promoter region (Li et al. 2020), and may play a major role in rice pollen development (Hirose et al. 2010).

In this study, a 31-bp indel located in the 5' UTR of *OsSUT3* was first identified in rice. However, there is limited information about the effect of this indel variation on the mRNA expression of *OsSUT3* and its agronomic traits. Therefore, the aim of this study was to detect the associations of the 31-bp indel with the rice pollen and panicle traits, and to investigate the mRNA expression of the downstream gene in the panicles of different rice species. The results provide valuable information for rice breeding.

MATERIAL AND METHODS

Plant materials and growth conditions. The rice accessions used in this study were all provided by the Rice Research Institute of Yunnan Agricultural University (Kunming, Yunnan, China) and listed in Table S1 in the Electronic Supplementary Material (ESM). Nipponbare seeds were used for the rice transformants according to the methods as mentioned in previous report (Rachmawati et al. 2004).

Data collection and analysis of the panicle traits. All the rice plants growing under the same conditions in the Yuanyang breeding base (Honghe, Yunnan, China) were randomly selected in the mature period to detect the panicle traits. The data of the panicle traits were collected including the panicle length (PL), seeding rate (SR), grain length (GL), grain width (GW), grain thickness (GT), and fertile pollen number (FPN). The investigated rice accessions' spikelets were collected 1 or 2 days pre-anthesis and fixed in Carnoy's solution for 24 h. Then the samples were kept in 70% alcohol (Cat. No. A600108-0100; Fuyu Fine Chemical, Tianjin, China). Three spikelets were randomly selected from each plant, and six anthers from one spikelet were stained with a 1% (wt/vol) iodine potassium iodide (I_2- KI) (Cat. No. 7681-11-0; Shenbo Chemical, Shanghai, China) solution on a glass slide (Ghouri et al. 2019). The FPN of the five horizons were counted under a microscope (Motic BA200) and the average was taken. A significance analysis between the 31-bp indel and the rice panicle traits was performed using SPSS (Ver. 20.0).

Genomic DNA and total RNA extraction. The total genomic DNA was isolated from the rice leaf tissue at the four-leaf stage by the cetyltrimethylammonium bromide (CTAB) (Cat. No. A600108-0100; BBI Life Sciences, Cayman Islands) method for the polymerase chain reaction (PCR) amplification (Clarke 2009). The total RNA was isolated from the leaf and panicle (50–100 mg fresh weight) 1 or 2 days pre-anthesis using TRIzol (Cat. No. DP424; Tiangen, Beijing, China) following the manufacturer's instructions. Next, 1 μL of DNAse was added to the RNA solution (30–50 μL) for the DNA contamination removal. The quality of the RNA samples was by checked using a 1.2% denaturing agarose gel (Cat. No. 111860; XHLY, Beijing, China), and the quantity and concentration were estimated using a NanoDrop 1000 spectrophotometer (Thermo Scientific Inc., Waltham, USA). The first-strand cDNA was synthesised by addition of an equal quantity of RNA using a FastKing RT Kit (With gDNase) (Cat. No. FP205-02; Tiangen, Beijing, China) for the quantitative real-time PCR (qRT-PCR).

5' rapid amplification of cDNA ends (5' RACE) and PCR amplification. The 5' UTR of *OsSUT3* was obtained by the 5' RACE with a 5'/3' RACE Kit (Cat. No. RA101-1; Roche, Mannheim, Germany) according to the instructions. The primers used for the nested PCR and the polymorphism screening are listed in Table S2 in the ESM. The PCR cycles were 94 °C for 4 min, 35 cycles of 94 °C for 30 s, 55–59 °C for 1 min, 72 °C for 40 s, and 72 °C for 10 min.

Quantitative real-time PCR (qRT-PCR) analysis of gene expression. The qRT-PCR was performed using a CFX96 Real Time System (Bio-Rad, Hercules, CA, USA) and a SuperReal PreMix Plus (SYBR Green) (Cat. No. FP205-01; Tiangen, Beijing, China) according to the instructions. The *β-actin* gene was used as an endogenous control. Three independent biological replicates were analysed. The relative quantification values were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001).

Construction of the *GUS* expression vector. Two PCR fragments, 2029-bp Frag and 1998-bp Frag, were amplified with specific primers (Table S2 in the ESM) and fused with the *GUS* gene in the pBI 121 vector (KIB, CAS, Kunming, Yunnan, China) using *Eco*31I (*Bsa*I) restriction sites.

Histochemical staining and quantitative assaying of GUS activity. The leaves and panicles of the

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T1 plants were collected for a histochemical analysis 1 or 2 days pre-anthesis. All the explants were stained with a GUS blue Kit (Cat. No.GT0391/0392; Huayueyang, Beijing, China) according to the instructions. The GUS activities were assayed on a Tecan Infinite 200 PRO set (Tecan, Austria, Switzerland) using the fluorogenic substrate 4-methylumbellifery β-D-glucuronide (MUG), as described in Jefferson et al. (1987).

RESULTS AND DISCUSSION

Effect of the 31-bp indel on sequence and secondary structure of *OsSUT3* 5' UTR. An indel polymorphism was identified by the specific primer P1 (Table S2 in the ESM) (Figure 1A). Two different PCR products, 2029-bp Frag and 1998-bp Frag, were generated because of the 31-bp indel (CAACTTCGATCTCTTGGGATATAACTAGCTT) at the upstream region of the *OsSUT3* gene (Figure 1B). The amplified fragments were composed of three parts: the upstream region of *OsSUT3* (1628-bp and 1597-bp), the first exon of *OsSUT3* (156-bp), and a part of the first intron (245-bp) (Figure 1C). In addition, eight SNPs were found between the two fragments (Table 1). More clones and sequencing in the rice accessions with the 31-bp insertion and deletion

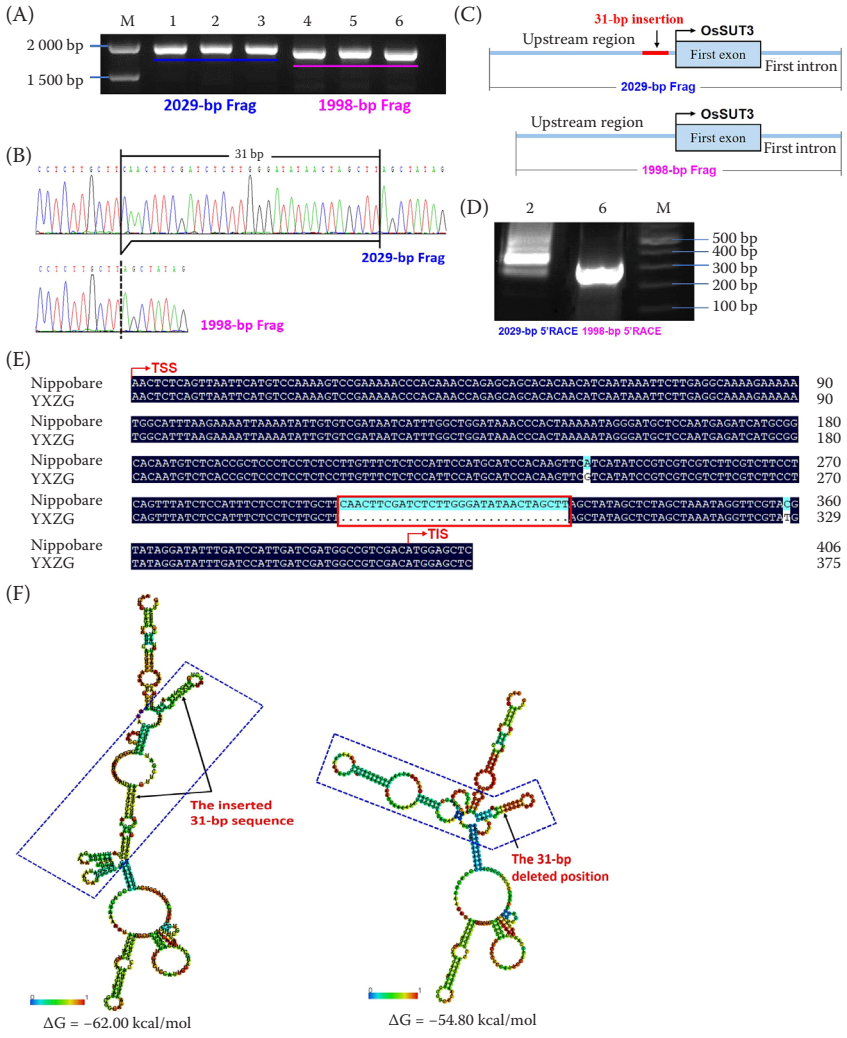

Figure 1. Polymorphism identification of the 31-bp indel in the 5' UTR of *OsSUT3*: electrophoresis pattern of the PCR amplification (A); sequence diagrams (B); location diagram of the 31-bp in the *OsSUT3* gene (C); electrophoresis pattern of the 5' RACE (D); sequence alignment of the 5' UTR (E); the secondary structures of the *OsSUT3* 5' UTRs (F)
1 – 93-11; 2 – Nipponbare; 3 – Pannong1hao; 4 – Daxianggu; 5 – Niubagu; 6 – Yuxizigu; red box shows the 31-bp sequence; TSS – transcription start site; TIS – translation initiation site; ΔG is the free energy predicted by RNAfold with default parameters; blue boxes show the structural changes affected by the 31-bp polymorphism

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Table 1. Information about the eight single-nucleotide polymorphisms (SNPs) loci in the amplified fragments

Nipponbare	YXZG	Distance (bp) to ATG	Location of the *OsSUT3*
A	T	–1 528	upstream region
A	G	–1 254	upstream region
A	G	–1 115	upstream region
G	C	–948	upstream region
C	A	–710	upstream region
A	G	–156	upstream region
C	T	–39	upstream region
T	A	289	the first intron

Nipponbare with the 31-bp insertion; YXZG without the 31-bp insertion; – and + denote the upstream and downstream region of the ATG of *OsSUT3*

were performed for a further differentiation analysis of eight SNPs. The results showed that these SNPs were just a nonsense mutation among the different varieties as they only appeared in one rice accessions (Figure S1 in the ESM).

To check whether the 31-bp indel affected the *OsSUT3* mRNA expression, 5' RACE was firstly carried out in Nipponbare (with 31-bp) and YXZG (without 31-bp) because the transcription start site (TSS) or the 5' UTR of *OsSUT3* was been determined in previous research. Two different PCR products, 5' UTR-385 and 5' UTR-354, were obtained (Figure 1D). Moreover, the sequencing results showed that the difference in the PCR products was caused by the 31-bp indel polymorphism (Figure 1E). The 5' UTR, as the binding site of a ribosome, contains various cis-regulators and affects the transcription and translation of the downstream gene (Warrier et al. 2018). Based on previous studies, the variations of 5' UTR may affect the gene function and phenotypic traits in many plants. For instance, the multi-indels in 5' UTRs of *Waxy* genes are strongly associated with the evolution of T*riticum* L. and *Aegilops* L. (Li et al. 2012).

Next, the regulatory elements in the 31-bp indel sequence were predicted by PlantCARE. The results revealed that four cis-acting factors (CAA-motif, OSE2ROOTNODULE, MYBST1, and GATA-box) were located in this region (Table 2). These cis-acting factors play important roles in the regulation of the gene expression (Torrent et al. 2010). Among them, the CAA-motif, the binding site of AtMYB1, achieves the specific gene expression in the promoter (Verma & Burma 2017). OSE2ROOTNODULE can enhance the gene expression by improving the promoter activity (Ramlov et al. 1993). MYBST1 was verified to upregulate the gene expression involving sugar/auxin pathways (Chen et al. 2017). The GATA-box is the core binding site of multiple transcription factors regulating promoter activity (Liu et al. 2019).

In addition, two secondary structures of 5' UTR-385 and 5' UTR-354 were predicted by RNAfold. The results showed that a long stem-loop structure formed in the 5' UTR-354 as the 31-bp deletion (Figure 1F). Moreover, the secondary structure of the 5' UTR-354 was more stable than that of the 5' UTR-385 for the 31-bp deletion. Many studies suggested the higher ΔG would down-regulate the gene expression by reducing the speed of the ribosome scan the mRNA (Shin et al. 2018). Therefore, the expression of *OsSUT3* gene may be upregulated in rice plants with the 31-bp fragment; e.g., as found for the *LAT59* gene and its suppressed expression for a stem loop structure of the 5' UTR in somatic cells and pollen (Curie & McCormick 1997).

Effect of the 31-bp indel on the expression of downstream gene. In rice, five *OsSUTs* genes were identified (Aoki et al. 2003). *OsSUT3* was an important member of the *OsSUT* family and was expressed specifically in the rice pollen (Li et al. 2020). To detect the effect of the 31-bp indel on the expression of *OsSUTs*, a qRT-PCR was performed (Figure 2). Interestingly, six tested rice cultivars were distinctly divided into two groups according to the expression of the *OsSUT3* gene. This is consistent with the results according to the genotypes. The *OsSUT3* expressions

Table 2. Cis-elements located in the 31-bp insertion

Cis-elements	Sequence motif	Function	References
CAA-motif	CAA	regulating the specific expression of the promoter in the tapetum	Verma and Burma (2017)
OSE2ROOTNODULE	CTCTT	enhancing the promoter activity	Ramlov et al. (1993)
MYBST1	GGATA	up-regulating the gene expression	Chen et al. (2017)
GATA-box	GATA	affecting the promoter activity	Liu et al. (2019)

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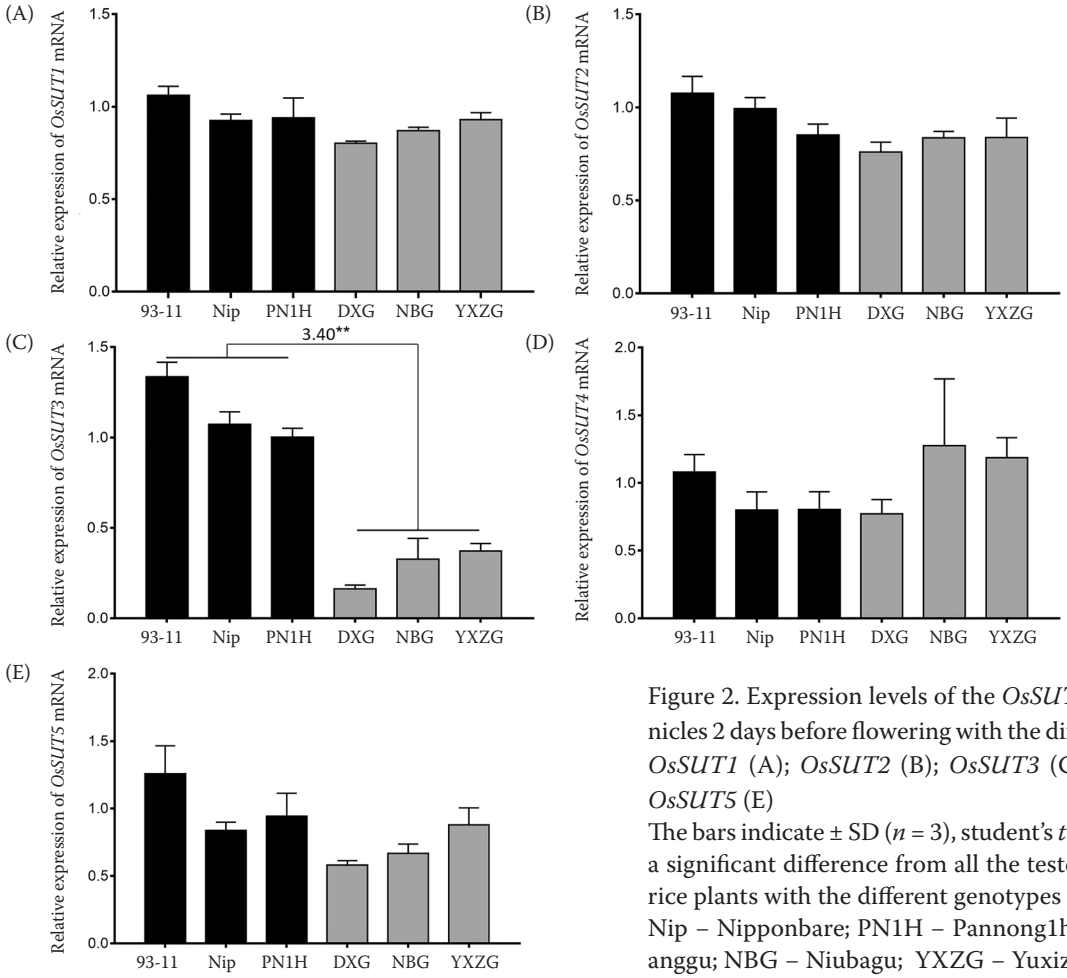

Figure 2. Expression levels of the *OsSUT* genes in the panicles 2 days before flowering with the different genotypes: *OsSUT1* (A); *OsSUT2* (B); *OsSUT3* (C); *OsSUT4* (D); *OsSUT5* (E)

The bars indicate ± SD (n = 3), student's t-test; **represents a significant difference from all the tested panicles of the rice plants with the different genotypes

Nip – Nipponbare; PN1H – Pannong1hao; DXG – Daxianggu; NBG – Niubagu; YXZG – Yuxizigu

were significantly higher, by about 3.40 times, in the panicles of the 31-bp insertion types compared with that of 31-bp deletion types ($P < 0.01$). However, there is no significant differences in the expressions of other *OsSUTs* in panicles between the 31-bp inserted and deleted genotypes ($P > 0.01$).

To further determine the effect of the 31-bp indel on the gene expression, two expression vectors, *2029-bp::GUS* and *1998-bp::GUS* (Figure 3A), were constructed and transfected into the Nipponbare callus. The histochemical staining of GUS showed the expression of *GUS* was obviously higher in the pollen than in the leaves (Figure 3B). Furthermore, much stronger GUS activities were observed both in the panicles and leaves with the 31-bp insertion (Figure 3C, D). In addition, the quantitative analyses of the *GUS* mRNA and protein were performed and showed similar results with the histochemical assay. The levels of the *GUS* transcript both in the pollen and leaves with the 31-bp insertion were all 2.92 times bigger of that without the 31-bp (Figure 3C). The protein activity also exhibited a significant increase. Compared to the 2.06-fold in the leaves, the GUS activity in the panicle of the 31-bp inserted plants was 5.97 times bigger as that in the panicle of the 31-bp deleted plants (Figure 3D).

Similarly, the absence of an 18-bp segment in the 5' UTR of *SOX9* gene showed a significant reduction in the RNA and protein levels in pigs (Brenig et al. 2015). Hence, the above results suggest that the 31-bp indel was sufficient to regulate the expression of the downstream gene.

The 31-bp insertion related to human-preferred traits was conserved during rice domestication. In organisms, the effect of the indel in the 5' UTR on the gene expression is universal. For example, the 573-bp deletion in the 5' UTR of the *YFT1* gene changed the colour of a tomato fruit from red to yellow (Zhao et al. 2020). A 4-bp indel in the upstream region of the *TaAFP* gene controlled the seed dormancy in wheat (Feng et al. 2019). In rice, the specific and higher expression of *OsSUT3* was detected in the pollen and

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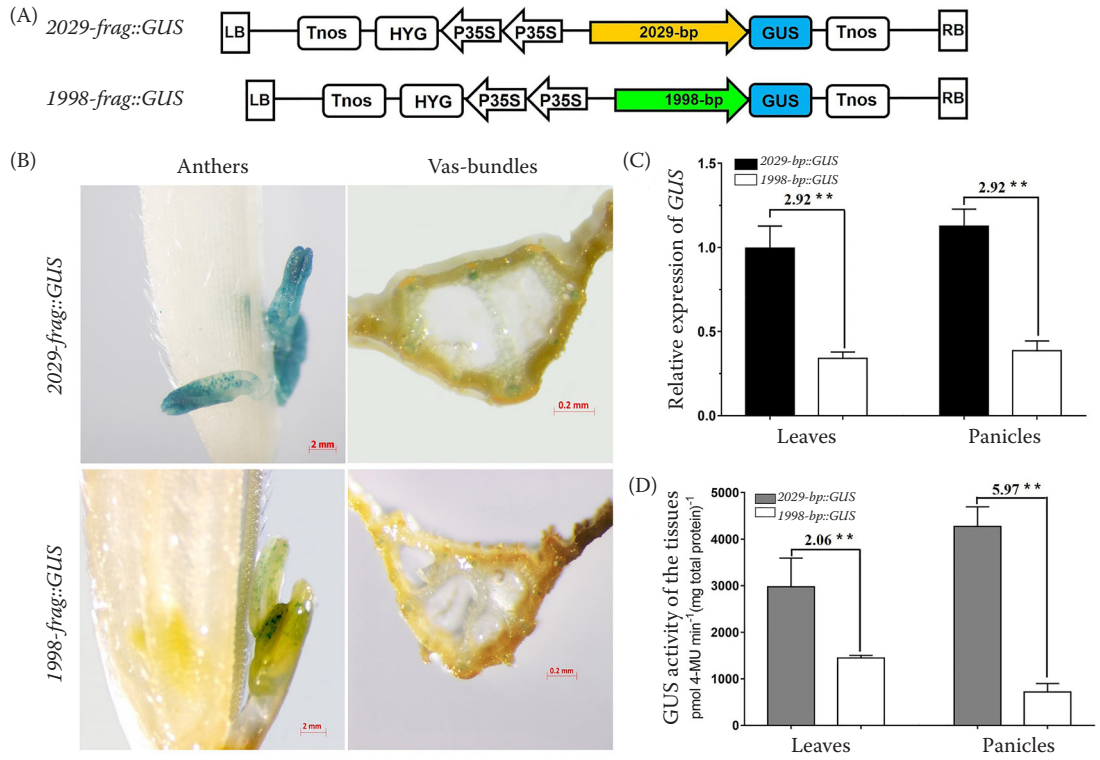

Figure 3. Regulation of the 31-bp indel on the expression of the downstream gene (*GUS*) by transgenic technology: linear maps of the *2029-bp::GUS* and *1998-bp::GUS* vectors (A); histochemical localisation of the GUS activity in the panicles and leaves of the transgenic plants (B); relative expression of the *GUS* mRNA in the panicles and leaves of the transgenic plants (C); GUS protein activity in the panicles and leaves of the transgenic plants (D)
The bars indicate ± SD (n = 3), student's t-test; **represents significant differences from all the tested tissues of each transgenic plant

affected the development of the rice grain (Adamski et al. 2009; Li et al. 2020). So, the five panicle traits, PL, SR, GL, GW, and GT, and pollen trait FPN were selectively examined in 85 collections of rice cultivars with a 31-bp indel (Table 3). The correlational analyses indicated that the 31-bp indel was particularly associated with the SR, GL, and FPN ($P < 0.01$).

In order to understand the distribution of the 31-bp indel, we randomly selected 560 rice accessions containing 58 wild rice, 154 landraces and 348 improved cultivars for PCR detection using the specific primer P1 (Table S2 in the ESM). The results showed that the proportions of the 31-bp insertion gradually increased with the rice domestication, from 79.31% in wild rice to

Table 3. Relationship between the 31-bp indel and the rice panicle and the pollen traits

Growth traits	Genotypes (No.)		*P* values
	insertion (mean ± SD)	deletion (mean ± SD)	
SR (%)	78.06 ± 5.91	68.62 ± 9.79	4.84E-07**
PL (cm)	19.31 ± 2.55	18.08 ± 2.29	0.027
GL (mm)	8.16 ± 0.48	7.50 ± 0.59	2.39E-07**
GW (mm)	3.47 ± 0.31	3.28 ± 0.44	0.023
GT (mm)	2.13 ± 0.21	2.23 ± 0.18	0.032
FPN	341.82 ± 63.79	188.43 ± 57.11	2.25E-18**

SR – seeding rate; PL – panicle length; GL – grain length; GW – grain width; GT – grain thickness; FPN – fertile pollen number; **highly significant difference ($P < 0.01$)

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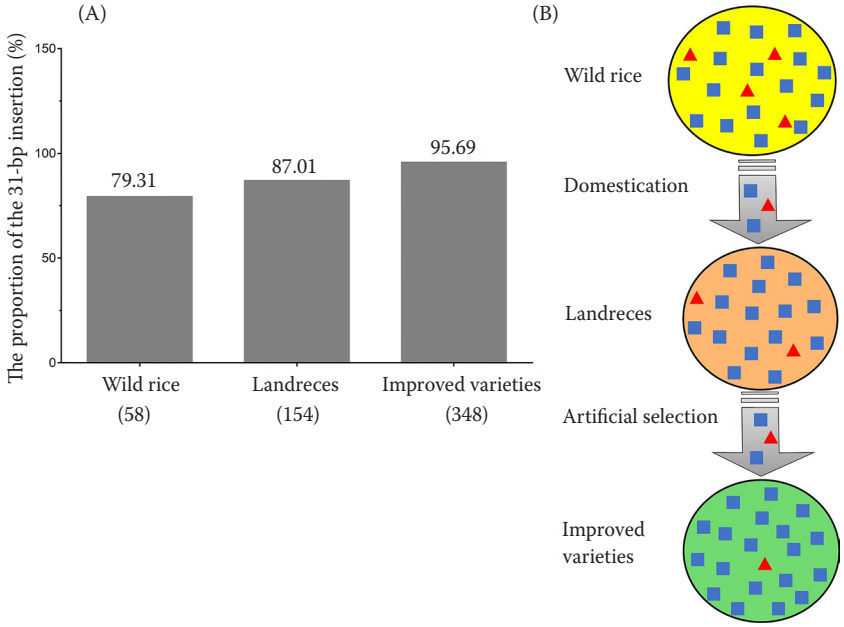

Figure 4. The proportion changes of the 31-bp insertion during rice domestication: the distribution of the 31-bp insertion in the different rice accessions (A); the schematic diagram of the 31-bp indel in rice domestication (B)
Wild rice – 58 varieties; landraces – 154 varieties; improved varieties – 348 varieties; blue square – the rice varieties with the 31-bp sequence; red triangle – the rice varieties without the 31-bp sequence

95.69% in improved cultivars (Figure 4A). Furthermore, the 31-bp insertion related with the higher FPN and bigger grain size was retained under the human-preferred selection (Figure 4B). It is similar to the preservation of the 3-bp insertion in the promoter of the Sl-*ALMT9* gene determined fruit malate contents and aluminium tolerance after tomato domestication (Ye et al. 2017). Therefore, a positive selection pressure in evolution or domestication process often results in the reduction of genetic diversity, so the selection of polymorphism locus would be significant (Li et al. 2018).

CONCLUSION

In conclusion, we identified a 31-bp indel in the 5' UTR of the *OsSUT3* gene, which was associated with the pollen and grain development in rice. In this short fragment, four cis-elements up-regulating the gene expression and affecting the gene function were predicted. The expression of the downstream gene decreased significantly when the 31-bp fragment was missing by qRT-PCR in the rice accessions and transgenic plant. Therefore, it is speculated that the 31-bp indel found in this study may affect the function of the *OsSUT3* gene, and this indel locus was most probably a potential target of artificial selection and modern breeding.

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