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ORIGINAL ARTICLE

The wall-associated kinase GWN1 controls grain weight and grain number in rice

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Abstract

Grain size is a crucial agronomic trait that determines grain weight and final yield. Although several genes have been reported to regulate grain size in rice (*Oryza sativa*), the function of Wall-Associated Kinase family genes affecting grain size is still largely unknown. In this study, we identified *GRAIN WEIGHT AND NUMBER 1* (*GWN1*) using map-based cloning. *GWN1* encodes the OsWAK74 protein kinase, which is conserved in plants. *GWN1* negatively regulates grain length and weight by regulating cell proliferation in spikelet hulls. We also found that *GWN1* negatively influenced grain number by influencing secondary branch numbers and finally increased plant grain yield. The *GWN1* gene was highly expressed in inflorescences and its encoded protein is located at the cell membrane and cell wall. Moreover, we identified three haplotypes of *GWN1* in the germplasm. $GWN1^{hap1}$ showing longer grain, has not been widely utilized in modern rice varieties. In summary, *GWN1* played a very important role in regulating grain length, weight and number, thereby exhibiting application potential in molecular breeding for longer grain and higher yield.

Introduction

Rice (*Oryza sativa* L.) is one of the staple crops, feeding more than half of the global population. Rice yield is mainly determined by panicle number per plant, grain number per panicle and grain weight (Xing et al. 2010). Grain size is determined by grain length, width and thickness, which is the main trait that determines grain weight and final yield (Zhu et al. 2021). Therefore, grain weight is one of the main targets of cereal crop domestication and improvement breeding. Many genes affecting grain size and grain weight have been identified in rice (Ren et al. 2023). According to their genetic regulatory mechanisms, these genes could be divided into several categories: ubiquitination-protease pathway, G-protein signaling pathway, mitogen-activated protein kinase (MAPK) signaling pathway, plant hormone perception and homeostasis pathway, transcriptional regulator pathway, microtubule regulatory pathway, secretory peptide regulation pathway and other functional proteins (Ren et al. 2023; Li et al. 2019). For example, *GRAIN WIDTH 2* (*GW2*) is the first QTL cloned in rice, which encodes a RING-type E3 ubiquitin ligase (Song et al. 2007). Loss of function of *GW2* result in increased grain width and yield, by regulating ubiquitination-mediated degradation of *expansin-like 1* (*EXPLA1*) and *WIDE GRAIN 1* (*WG1*) (Hao et al. 2021; Choi et al. 2018). *GS3* is a major QTL for grain size, encoding a noncanonical G-γ and negatively regulating grain length (Fan et al. 2006). *GS3* acts antagonistically with *DENSE AND ERECT PANICLE 1* (*DEP1*) and *GGC2*, which encodes another two noncanonical Gγ and positively

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regulates grain length (Sun et al. 2018). The OsER1-OsMKKK10-OsMKK4-OsMAPK6 module positively regulates grain size in rice by enhancing cell proliferation in spikelet hulls (Xu et al. 2018; Guo et al. 2020). *OsWRKY53* works downstream of this module, but it regulates grain size by promoting cell expansion (Tian et al. 2021). *GRAIN SIZE AND NUMBER 1* (*GSN1*) negatively regulates grain size by dephosphorylating OsMPK6 (Guo et al. 2018). *GRAIN SIZE 5* (*GS5*) controls grain size in rice by regulating the brassinosteroid (BR) signaling pathway (Li et al. 2011). GS5 enhances BR signaling and enlarges grain width by preventing the endocytosis of OsBAK1-7 (Xu et al. 2015). *GRAIN LENGTH AND WEIGHT ON CHROMOSOME 7* (*GLW7*) is a major QTL for rice grain size, encodes the *SQUAMOSA promoter binding protein-like* (SPL) family transcription factor OsSPL13, and positively regulates grain size by promoting spikelet hull cell expansion (Si et al. 2016).

However, there is a trade-off between grain weight and grain number. Only a few genes have been reported to increase grain size, weight and grain number at the same time. *GRAIN NUMBER AND SIZE ON CHROMOSOME 4* (*GNS4)* positively regulates grain size, grain weight and grain number in rice. Overexpressing *GNS4* enhanced grain yields, with possible application in high-yielding rice breeding (Zhou et al. 2017). *CONTROL OF GRAIN YIELD 1* (*COG1*) works with *OsMADS1* and *OsAP2-39* simultaneously increasing both grain number and grain size. The favorable variants of these three genes can also increase grain yield (Li et al. 2023a, b). More genes that simultaneously regulate grain size, weight and number urgently need to be discovered.

Wall-Associated Kinase (WAK) represents a unique receptor-like kinase (RLK) subfamily in plants (Shiu et al. 2001). It has a typical eukaryotic Ser/Thr kinase domain and an extra cytoplasmic domain (ectodomain) with several EGF-like repeats (Wanger et al. 2001; Kohorn et al. 2006). The *WAK* genes play important roles in cell expansion, pathogen resistance and stress tolerance in *Arabidopsis* (Lally et al. 2001; He et al. 1998; Sivaguru et al. 2003). There are 125 OsWAKs in rice, and the biological roles of most of them are little known (Zhang et al. 2005). *OsWAK1* plays an important role in the rice disease resistance response. The expression of *OsWAK1* was induced by mechanical injury, salicylic acid (SA) and methyl jasmonate (MeJA). OsWAK1 could interact with and phosphorylate the transcriptional regulator OsRFP1 (Li et al. 2009). *OsWAK10* was recently found to positively control rice stem height by regulating cell expansion in the stem (Cai et al. 2023). *OsWAK11* was recently found to negatively regulate stature, leaf angle, and grain length in rice by suppressing cell elongation. It also negatively regulates BR signaling by phosphorylating BRASSINOSTEROID INSENSITIVE 1 (OsBRI1) and inhibiting its kinase activity (Hu et al. 2014; Yue et al. 2022). However, the function of most WAK family genes remains unclear in rice and the relationship between WAKs and rice grains needs to be further explored.

In this study, we cloned a novel quantitative trait locus (QTL), *GRAIN WEIGHT AND NUMBER 1 (GWN1)*, by map-based cloning. *GWN1* encodes the WALL-ASSOCIATED KINASE 74 (OsWAK74), which negatively regulates grain length and weight by regulating cell proliferation in spikelet hulls, and simultaneously negatively influenced grain number per panicle by influencing secondary branch numbers.

Materials and methods

Plant materials and growth conditions

SLG-1 (*ssp. japonica*, SLG) was crossed with Nipponbare (*ssp. japonica,* Nip) to produce the F_1 generation, and subsequently backcrossed with Nip to generate BC_4F_2 and BC_4F_3 populations were used for primary QTL mapping (Yu et al. 2018).

The plants were grown under natural paddy conditions in Beijing or Sanya, in Hainan province. The planting density was 23.3 cm between rows and 13.3 cm between plants. The T_3 or later generations of transgenic plants were used for phenotypic evaluation.

QTL fine mapping

A total of 146 BC_4F_4 plants and 4537 BC_4F_5 plants were used for fine mapping. Polymorphic molecular markers were screened by the BSA mixed pool, and genotypes were identified by the SDS-PAGE method. QTL IciMapping3.1 was used for QTL analysis (Li et al. 2007).

Plasmids construction and plant transformation

To generate the CRISPR/Cas9 vector, a 20 bp PAM sequence from the *GWN1* coding sequence (CDS) was selected for specific recognition and cloned into the vector sgRNA-Cas9 as previously described (Mao et al. 2013). To produce the complementation construct *ProGWN1*:*GWN1*^{Nip}, the 2982 bp promoter and full-length genomic region of *GWN1* was amplified from Nip and cloned into the *Pme*I and *Sac*I sites of the plant binary vector pMDC163. For the *ProGWN1*:*GUS* vector, the 2982 bp promoter region of the *GWN1* was amplified and cloned into the *Pme*I and *Sac*I sites of the vector pMDC162. For subcellular localization, the *GWN1* coding region without stop codon from Nip or SLG was amplified and cloned into the *Kpn*I and *Hin*dIII sites of the binary plant expression vector pSuper1300-GFP, respectively, to generate *Pro35S*:*GWN1*^{Nip}- green fluorescent

protein (GFP) and *Pro35S*:*GWN1*^{SLG}-GFP vector. The DNA constructs in this study were utilized Tks Gflex™ DNA Polymerase (TAKARA). The correct vectors were introduced into *Agrobacterium tumefaciens* strain EHA105 and then transformed into rice calli via *Agrobacterium*-mediated transformation (Hiei et al. 1994; Toki et al. 2006).

RNA extraction and expression analysis

For expression analysis, fresh tissues were flash-frozen in liquid nitrogen and total RNA was extracted using the RNApure Total RNA Kit (Aidlab, Beijing, China) according to the manufacturer's instructions. 1 μg of the DNase-treated RNA was reverse transcribed using HiScript II Reverse Transcriptase (Vazyme, Nanjing, China). The resulting cDNA samples were diluted three times and used as templates for quantitative RT-PCR (qRT-PCR). qRT-PCR analysis was performed on the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR® Premix Ex Taq II (TaKaRa, Kyoto, Japan) (Duan et al. 2012; Xiong et al. 2014). The rice *ubiqutin1* gene (*LOC_Os03g13170*) was used as an internal control. Data normalization was conducted using the $2^{-\Delta\Delta t}$ method (Livak et al. 2001).

GUS staining and subcellular localization of GWN1

For GUS-staining assays, different fresh tissues from *ProGWN1*:*GUS* transgenic plants were stained in GUS solution for 12 h at 37°C in the dark, and then dehydrated in a graded series of ethanol to remove the chlorophyll as previously reported (Zhang et al. 2018).

The *Pro35S*:*GWN1*^{Nip}-GFP plasmids were transformed into Nipponbare via *Agrobacterium*-mediated transformation. The roots of 2-week-old transgenic seedlings were used for fluorescence observation. For plasmolysis, the roots was treated with 1 M sorbitol for 15 min. Moreover, the *Pro35S*:*GWN1*^{Nip}-GFP and *Pro35S*:*GWN1*^{SLG}-GFP plasmids were introduced into *A. tumefaciens* strain EHA105 and infiltrated into the leaves of *Nicotiana benthamiana* plants. After 2 days of incubation at 25°C, the leaves were sampled for confocal microscopy observation using an LSM 880 confocal laser scanning microscope (Zeiss, Oberkochen, Germany). Membrane protein, PIP2;2 fused with mCherry, was used as a plasma membrane marker (Bai et al. 2021).

Scanning electron microscopy (SEM)

Spikelets of NIL-*qGL8a*^{SLG} and NIL-*qGL8a*^{Nip} were taken one week before pollination and fixed with 2.5% glutaraldehyde. The tissue material was immersed in the fixing solution under vacuum, placed at 4℃ for 12 h, and then dehydrated in a graded series of ethanol as previously reported (Zhang et al. 2018). After gold spray treatment, samples were observed and photographed using an S-2460 Scanning electron microscope (Hitachi, Tokyo, Japan).

Haplotype analysis

The single nucleotide polymorphisms (SNPs) and Indel datas of cultivated accessions were downloaded from the Rice 3 K project (Wang et al. 2018). Among them, 1,383 accessions with a publicly available grain length data, clear subgroup information and high quality genotypes were selected for haplotype analysis, and the relative information were listed in Table S1. We further choose 1,067 accessions for combined haplotype analysis of *GWN1* and *OsLG3b*, and the relative information were listed in Table S2.

Phylogenetic and haplotype network analysis

SNPs data of 2952 cultivated accessions and 242 wild rice accessions were obtained from the Rice 3 K project and a previous publication (Wang et al. 2018; Jing et al. 2023). MEGA 11.0 was used to conduct the neighbor-joining phylogenetic tree (Tamura et al. 2021) and then visualized and annotated using iTOL (Letunic et al. 2007). For evolutionary analysis, the *GWN1* gene region and upstream and downstream 20 kb flanking regions were chosen to calculate nucleotide diversity value (π) and Tajima's *D* value using VCF tools. Information on cultivated rice accessions for phylogenetic and haplotype network analysis is given in Table S3.

Primers

The primers used in the study are listed in Table S4.

Results

Phenotypic analysis of NIL-*GWN1*^{SLG} and NIL-*GWN1*^{Nip}

To identify genes affecting grain size, the improved *temperate japonica* variety SLG showing large grain size as a donor was crossed with a typical *temperate japonica* Nip to construct the segregating population (Fig. S1A–D). The BC_4F_2 and BC_4F_3 populations were used for QTL analyses. A novel QTL conferring grain length, *qGL8a*, was identified on the long arm of chromosome 8, explaining 14.7% (LOD score = 4.6) of the phenotypic variation in grain length (Fig. S1E; Table S5) (Yu et al. 2018).

Next, a pair of near-isogenic lines (NILs) were developed in the Nip genetic background for the *qGL8a* locus from the BC_4F_5 population, namely NIL-*qGL8a*^{SLG} containing

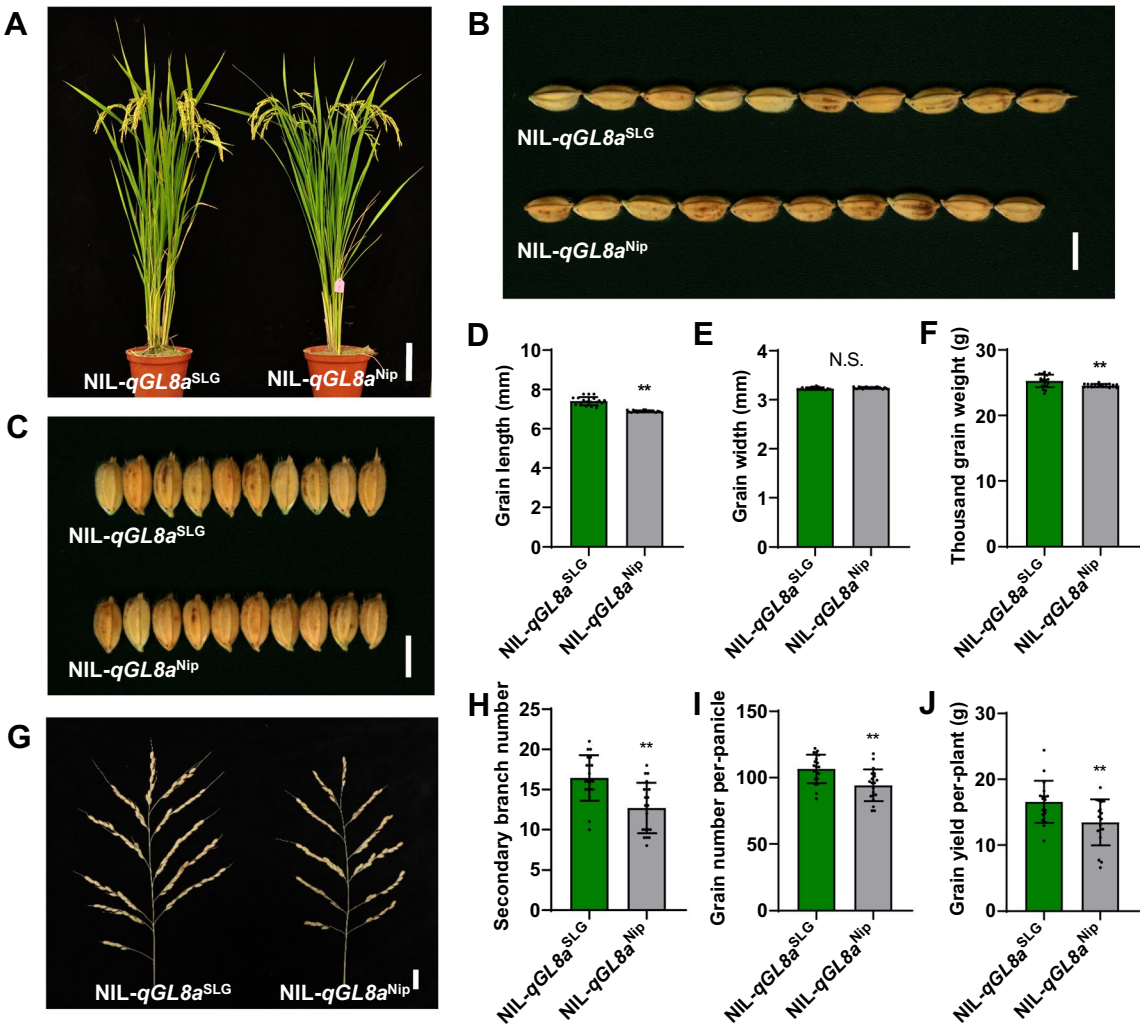


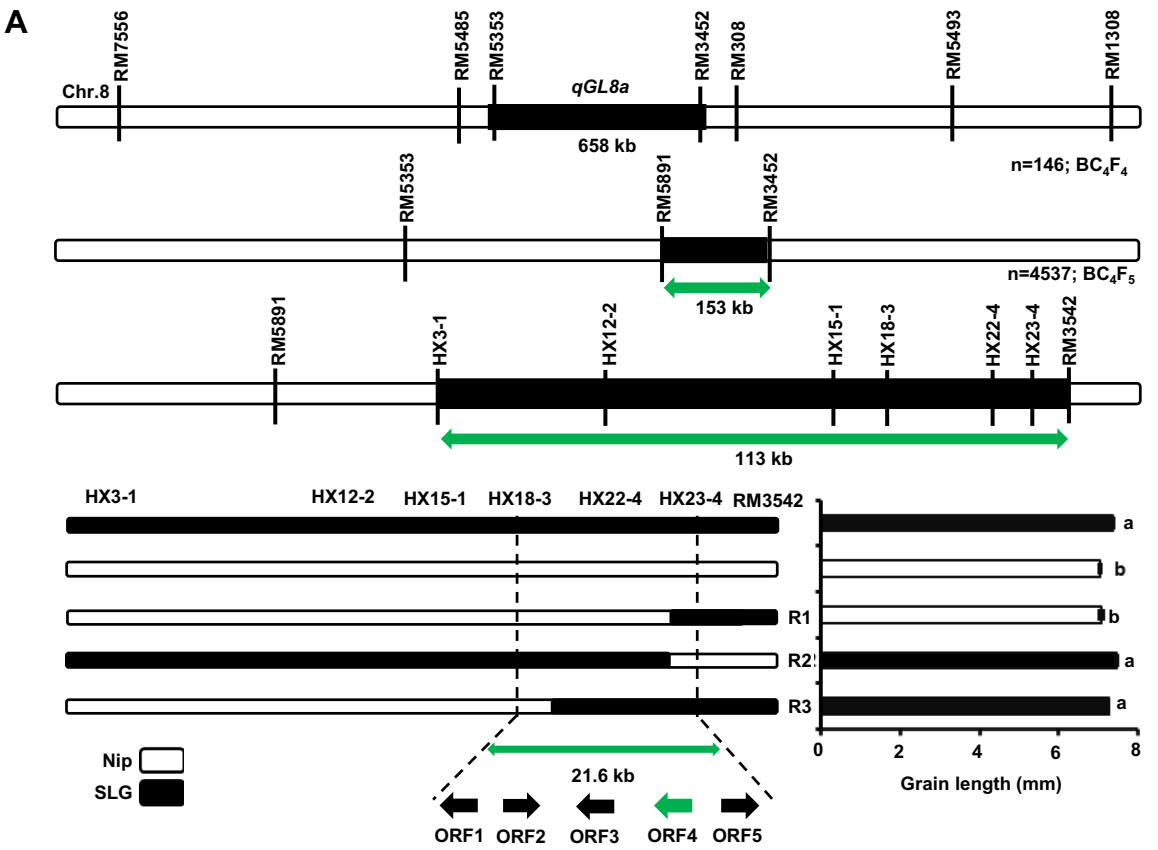
Fig. 1 Phenotype of NIL lines. **A** Representative phenotypes of NIL-*qGL8a*^{SLG} and NIL-*qGL8a*^{Nip}. Scale Bar, 20 cm. **B**–**F** Phenotype of grain length (**B**, **D**), grain width (**C**, **E**), thousand grain weight (**F**) of NIL-*qGL8a*^{SLG} and NIL-*qGL8a*^{Nip} (n = 20). Scale bar, 5 mm. **G** Representative panicles of NIL-*qGL8a*^{SLG} and NIL-*qGL8a*^{Nip}. Scale Bar, 2 cm. **H**–**J** Secondary branch number (**H**), grain number per panicle (**I**), per-plant grain yield (**J**) of NIL-*qGL8a*^{SLG} and NIL-*qGL8a*^{Nip} (n = 20). Data are presented as mean ± SD. *P*-values were determined using two-tailed Student's *t*-tests. *$P < 0.05$, **$P < 0.01$

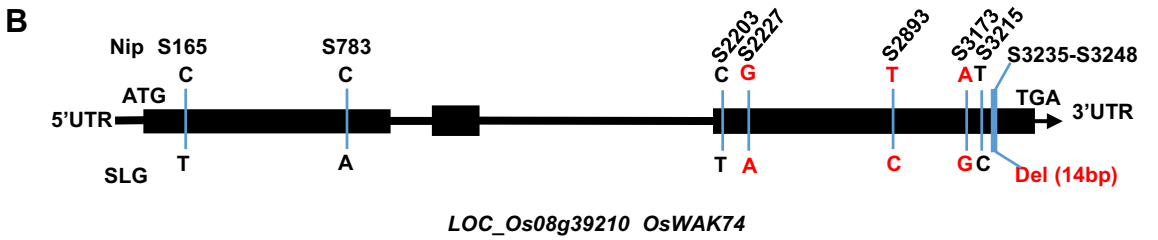
qGL8a fragment from SLG and NIL-*qGL8a*^{Nip} containing *qGL8a* fragment from Nip (Fig. 1A). Compared with NIL-*qGL8a*^{Nip}, the grain length and thousand grain weight of NIL-*qGL8a*^{SLG} were significantly increased, but there were no significant differences in grain width (Fig. 1B–F). Meanwhile, the secondary branch number and grain number per panicle were also significantly increased in NIL-*qGL8a*^{SLG} than in NIL-*qGL8a*^{Nip} without influencing panicle length or primary branch number (Fig. 1G–I; Fig. S2A, B). These traits contribute to a higher plant grain yield of NIL-*qGL8a*^{SLG} (Fig. 1J). Other important agronomic traits of NIL-*qGL8a*^{SLG} and NIL-*qGL8a*^{Nip} were also investigated, and similar plant height, panicle number, flag leaf length and width were found between them (Fig. S2C–F). Moreover, no significant differences were found in chalky grain ratio or chalkiness degree of grain between NIL-*qGL8a*^{SLG} and NIL-*qGL8a*^{Nip} (Fig. S3). These findings demonstrated that the *qGL8a* allele from SLG increases grain length, weight and number without loss of grain quality.

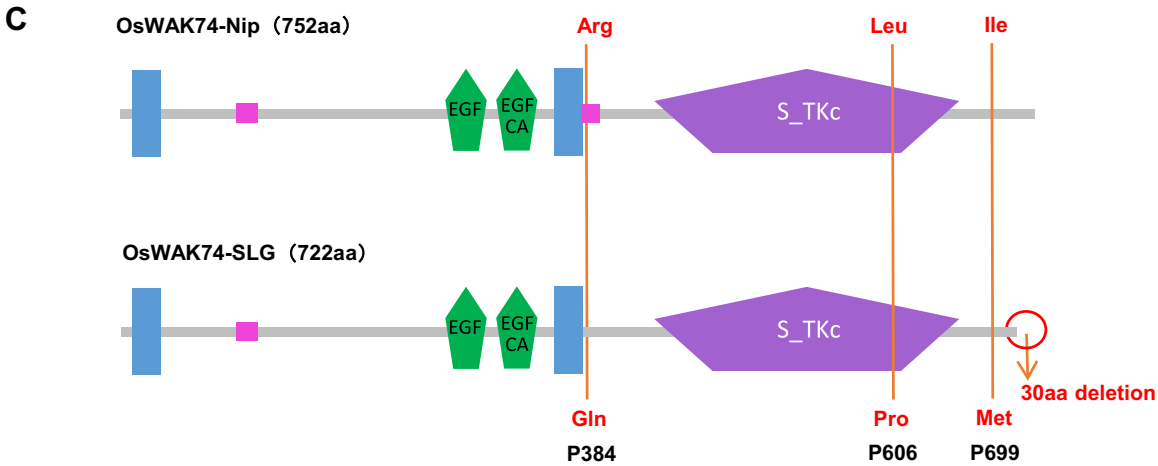
Fig. 2 Identification of *GWN1*. **A** Fine-mapping of *GWN1*. **B** *GWN1* CDS variation sites between two parents. The red letters indicate non-synonymous SNP. **C** *GWN1* protein variation sites between two parents

Fine mapping and candidate gene analysis of *qGL8a*

146 BC_4F_4 individual plants were used for preliminary mapping and localized *qGL8a* into a 658 kb region between RM5353 and RM3452. Then a total of 4,537 BC_4F_5 individual plants were used for fine mapping, and finally three


A
Chr.8
RM7556
RM5485
RM5353
qGL8a
RM3452
RM308
RM5493
RM1308
658 kb
n=146; BC4F4
RM5353
RM5891
RM3452
n=4537; BC4F5
153 kb
RM5891
HX3-1
HX12-2
HX15-1
HX18-3
HX22-4
HX23-4
RM3542
113 kb
HX3-1
HX12-2
HX15-1
HX18-3
HX22-4
HX23-4
RM3542
R1
R2
R3
a
b
b
a
a
Nip
SLG
21.6 kb
ORF1
ORF2
ORF3
ORF4
ORF5
0
2
4
6
8
Grain length (mm)


B
Nip S165
C
S783
C
S2203
S2227
C G
S2893
T
S3173
S3215
AT
S3235-S3248
ATG
TGA
5'UTR
3'UTR
SLG
T
A
T A
C
G C
Del (14bp)
LOC_Os08g39210 OsWAK74


C
OsWAK74-Nip（752aa）
Arg
Leu
Ile
EGF
EGF CA
S_TKc
OsWAK74-SLG（722aa）
EGF
EGF CA
S_TKc
30aa deletion
Gln
P384
Pro
P606
Met
P699

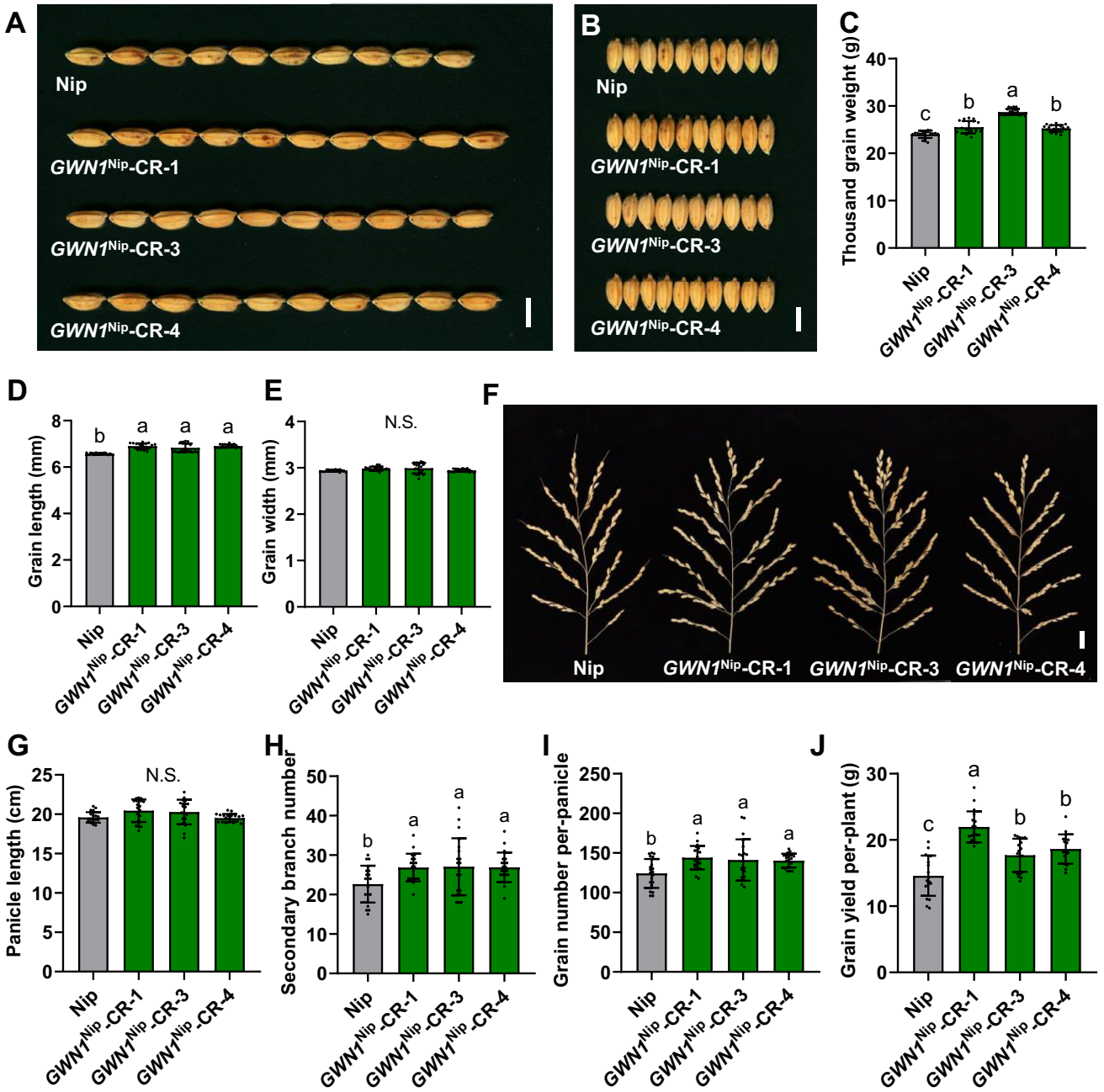


Fig. 3 Phenotype of *GWN1* knockout lines. **A**–**E** The phenotype of grain length (**A**, **D**), grain width (**B**, **E**) and thousand grain weight (**C**) of WT and *GWN1*-CR lines (n = 20). Scale bar, 5 mm. **F** Representative panicles of WT and *GWN1*-CR lines. Scale Bar, 2 cm. **G**–**J** Panicle length (**G**), secondary branch number (**H**), grain number per panicle (**I**), per-plant grain yield (**J**) of WT and *GWN1*-CR lines. (n = 20). Data are means ± SD. Different lowercase letters denote a significant difference (one-way ANOVA: $P < 0.05$)

recombinant plants, namely R1, R2 and R3, were identified between STS markers HX18-3 and HX23-4. R1 plants had short grains similar to Nip and R2 plants had significantly longer grains, indicating that the gene conferring *qGL8a* was located at the left of HX23-4. R3 plants showing long grains indicated that the gene conferring *qGL8a* was located at the right of HX18-3. These results showed that *qGL8a* was located in this 21.6 kb interval region containing five predicated open reading frames (ORF) (Fig. 2A). The DNA sequencing result revealed no difference in the coding regions of *ORF2* and *ORF3* between SLG and Nip. Thereafter, the transcriptome data of *ORF1* (*LOC_Os08g39180*), *ORF4* (*LOC_Os08g39210*) and *ORF5* (*LOC_Os08g39220*) from the Rice Bio-Analytic Resource for Plant Biology website (https://bar.utoronto.ca/eplant_rice/) were explored, which revealed that *ORF1* was not expressed at any growth

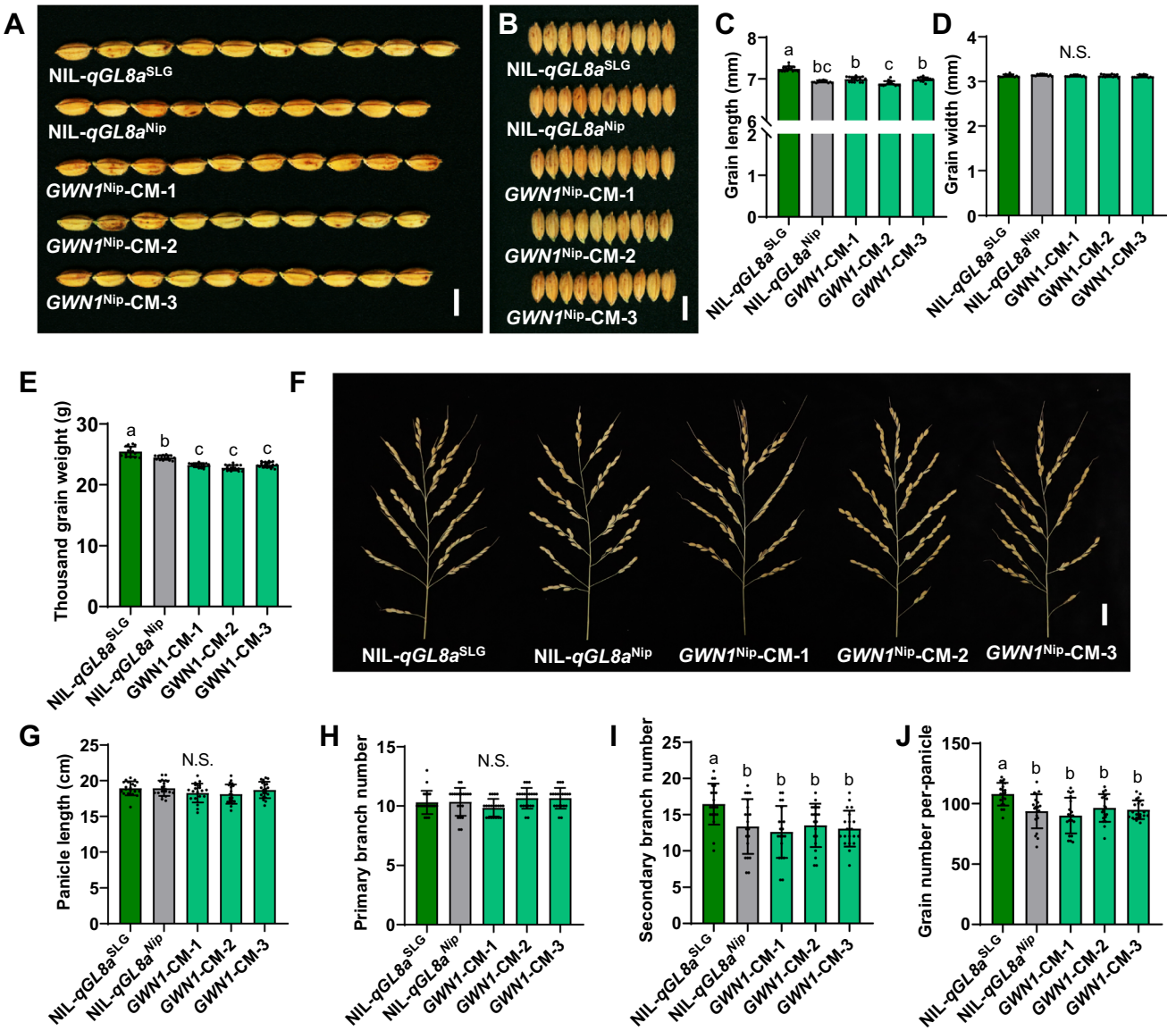


Fig. 4 The phenotype of *GWN1* complementary lines. **A**–**E** The phenotype of grain length (**A**, **C**), grain width (**B**, **D**) and thousand grain weight (**E**) of NIL-*qGL8a*^{SLG}, NIL-*qGL8a*^{Nip} and *GWN1*-CM lines (n = 20). Scale bar, 5 mm. **F** Representative panicles of NIL-*qGL8a*^{SLG}, NIL-*qGL8a*^{Nip} and *GWN1*-CM lines. Scale Bar, 2 cm. **G**-**J** Panicle length (**G**), primary branch number (**H**), secondary branch number (**I**), grain number per panicle (**J**) of NIL-*qGL8a*^{SLG} and NIL-*qGL8a*^{Nip} (n = 20). Data are presented as mean ± SD. Different lowercase letters denote a significant difference (one-way ANOVA: $P < 0.05$)

stage. *ORF4* was highly expressed in inflorescences at different stages compared to *ORF5* (Fig. S4A–C). To confirm this result, the expression level of these three genes were checked in different length inflorescences of NIL-*qGL8a*^{SLG} and NIL-*qGL8a*^{Nip}. It was found that *ORF4* showed significantly higher expression in NIL- *qGL8a*^{SLG} compared to NIL- *qGL8a*^{Nip}, while *ORF1* and *ORF5* showed no significant difference in expression level between the two NILs (Fig. S5A). Haplotype analysis was also conducted using nonsynonymous SNPs and Indel in the coding region of these three genes severally. The result showed that the accessions containing *ORF1*$^{hap2(SLG)}$ or *ORF1*$^{hap3(Nip)}$ and *ORF5*$^{hap1(SLG)}$ or *ORF5*$^{hap2(Nip)}$ exhibited similar grain length, but the accessions containing *ORF4*$^{hap1(SLG)}$ exhibited longer grain than *ORF4*$^{hap3(Nip)}$ (Fig. S5B–G). These results indicated that *ORF4*, namely *LOC_Os08g39210*, might be the candidate gene of *qGL8a*. According to the Rice Genome Annotation Project (RGAP, http://rice.plantbiology.msu.

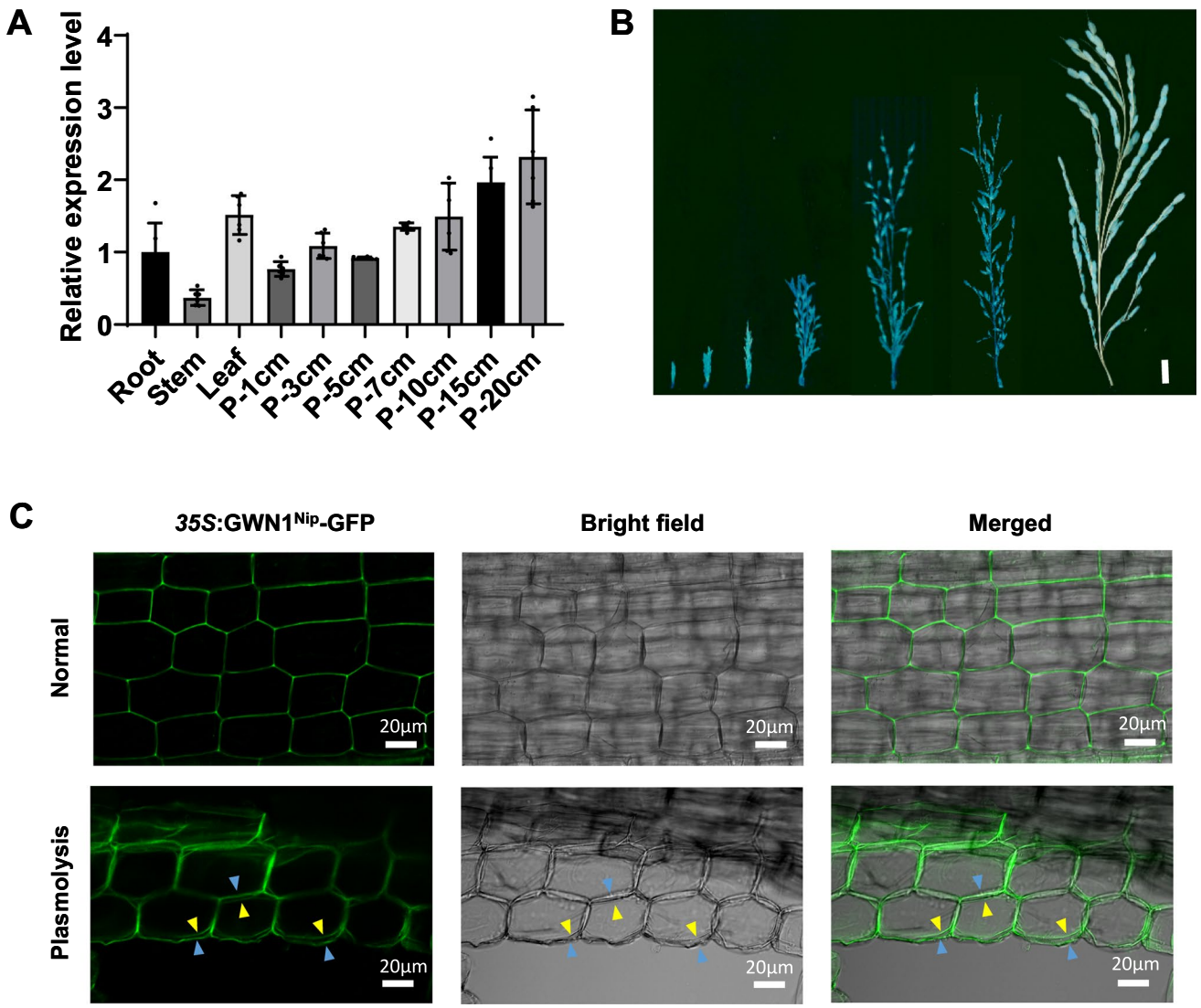


Fig. 5 Expression pattern and subcellular localization of GWN1. **A** Relative expression level of *GWN1* in different tissues of WT (n = 3). **B** GUS staining of different length panicles in *ProGWN1*:*GUS*-transgenic plants. Scale bar, 1 cm. **C** Subcellular localization of GWN1. The roots of *Pro35S*:*GWN1*^{Nip}-GFP seedlings was observed by confocal laser-scanning microscopy. Scale bar, 20 μm. The blue triangle indicates the position of cell wall, the yellow triangle indicates the position of plasma membrane. Data are presented as mean ± SD

edu/), *LOC_Os08g39210* encodes the OsWAK74, which is a receptor-like protein kinase. DNA sequencing results showed that there were 4 synonymous SNPs (S165, S783, S2203 and S3215) and 3 non-synonymous SNPs (S2227 (24768682), G-to-A, Arg-to-Gln; S2893 (24768016), T-to-C Leu-to-Pro; S3173 (24767736) A-to-G Ile-to-Met) and a 14 bp deletion (S3235-3248, 24767657–24767670) in the coding region of *LOC_Os08g39210* between SLG and Nip. (Figs. 2B, C; S6A). Among them, the 14 bp deletion results in the loss of 30 amino acids (aa) at the end of the C terminal of OsWAK74, which is near the S_TKc domain (Fig. 2C). This domain is highly conserved in plants (Fig. S6B). These results indicated that *LOC_Os08g39210* was the most likely candidate gene for *qGL8a*, and was renamed as *GWN1*.

GWN1 regulates grain weight and grain number in rice

To confirm the function of *GWN1*, mutants in *GWN1* were generated via clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9)-mediated gene editing in the Nip background, and three types of knockout lines were obtained: *GWN1*^{Nip}-CR-1 contains a 1 bp insertion, *GWN1*^{Nip}-CR-3 contains a 7 bp

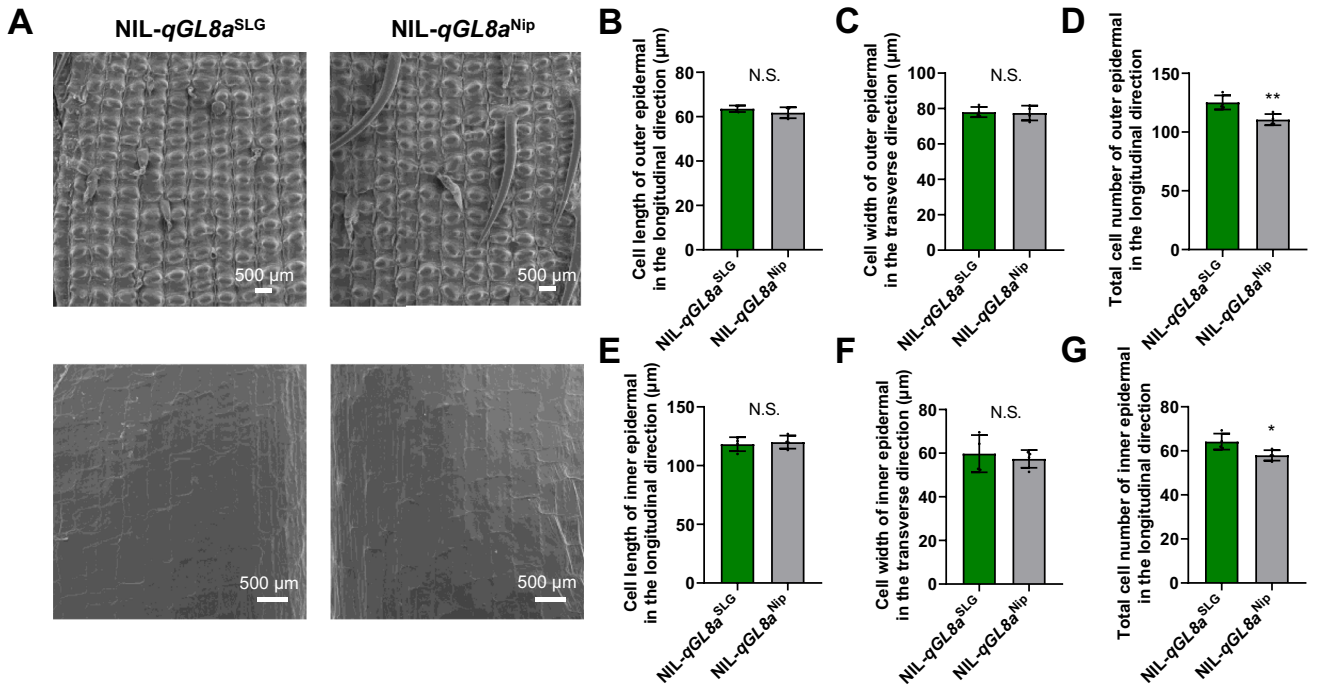


Fig. 6 SEM of the lemma of two *NILs* **A** SEM of outer and inner epidermal cells of two NILs lemmas in the longitudinal direction. Scale bar, 500 μm. **B**–**D** Cell length (**B**), cell width (**C**) and cell number (**D**) of outer epidermal (n = 4). **E**–**G** Cell length (**E**), cell width (**F**) and cell number (**G**) of inner epidermal (n = 4). Data are presented as mean ± SD. *P*-values were determined using two-tailed Student's *t*-tests. $^{*}P < 0.05$, $^{**}P < 0.01$

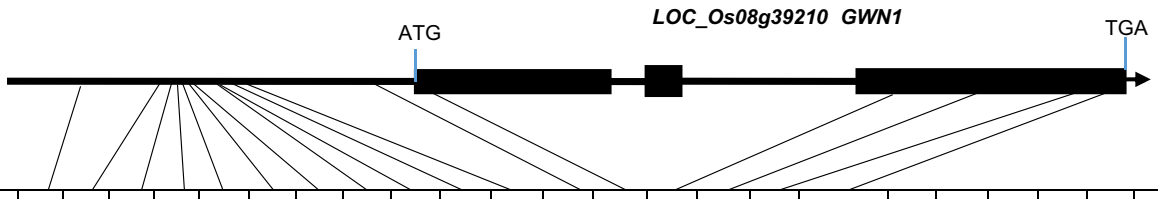
deletion, and *GWN1*^{Nip}-CR-4 contains a 2 bp deletion in the first exon of the gene (Fig. S7A, B). Compared with Nip, all three CRISPR lines showed significantly increased grain length and thousand grain weight, while the grain width showed no obvious difference (Fig. 3A–E). It was also found that the panicle length, primary branch number and panicle number showed no obvious difference with WT, whereas the secondary branch number and grain number per panicle significantly increased, resulting in increased plant grain yield. (Fig. 3F–J; Fig. S7C, D). Meanwhile, the grain length and grain number of *GWN1*^{Nip}-A, *GWN1*^{Nip}-B, and *GWN1*^{Nip}-H plants isolated from *GWN1*^{Nip}-CR-1 heterozygotes plants were also evaluated (Fig. S7A), and it was found that the grain length and grain number of *GWN-1*^{Nip}-H were between those of *GWN1*^{Nip}-A and *GWN1*^{Nip}-B (Fig. S8A–D). These results showed that the genetic effect of *GWN1* was semidominant.

In addition, a complementary construct of the *GWN1* allele from Nip was constructed and transformed into NIL-*qGL8a*^{SLG}. Three complementary lines, namely CM1, CM2 and CM3, were selected for phenotypic analysis (Fig. S9A). The results showed that the grain length and thousand grain weight of CM1, CM2 and CM3 were fewer than those of NIL-*qGL8a*^{SLG}, but were similar to NIL-*qGL8a*^{Nip} (Fig. 4A–E). The grain width of CM1, CM2 and CM3 did not change. Moreover, the secondary branch number and grain number of CM1, CM2 and CM3 also significantly decreased compared with NIL-*qGL8a*^{SLG}, while the panicle length, primary branch number and panicle number showed no obviously changed (Fig. 4F–J; Fig. S9B). These results indicated that *GWN1* negatively regulates grain length, weight and grain number in rice, and its allele from SLG result in a larger grain size and a higher grain number.

Expression pattern and subcellular localization of GWN1

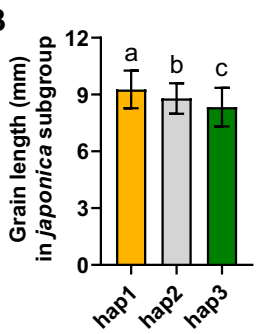
To characterize the spatiotemporal expression pattern of *GWN1*, qRT-PCR analysis was performed. It showed that *GWN1* was expressed in different tissues, but the expression level was relatively high in panicles (Fig. 5A). Similarly, β-glucuronidase [GUS] activity with the *GWN1* promoter driving the GUS reporter gene was also detected in different length panicles (Fig. 5B). We also searched *GWN1* expression data on the eFP browser of rice microarray data (https://bar.utoronto.ca/eplant_rice/). Expression levels of *GWN1* in the inflorescence, seeds and shoot apical meristems (SAM) were higher than in other tissues and organs (Fig. S4B).

A

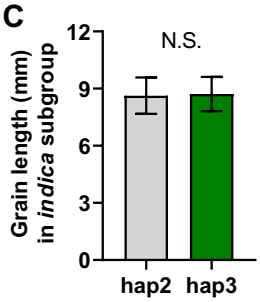


hap	24772418	24772096	24772047	24772029	24772010	24771997	24771989	24771842	24771838	24771783	24771742	24771082	24771009	24768682	24768016	24767736	24767657 - 24767670	tej	trj	ind	aus	bas	Grain Length (mm)
hap1	T	C	G	A	C	A	T	C	T	C	GCGGA	C	T	A	C	G	-	1	85				9.27 [a]
hap2	C	T	A	C	T	G	C	T	C	T	-	T	A	G	T	G	ATGTGATTATTTGT	1	21	664	3	1	8.64 [b]
hap3	C	T	A	C	T	G	C	T	C	T	-	T	A	G	T	A	ATGTGATTATTTGT	117	156	119	164	51	8.35 [c]

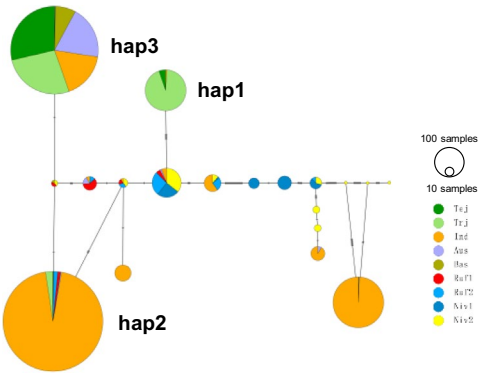
B



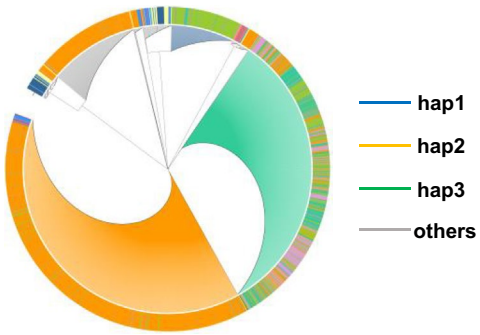
C



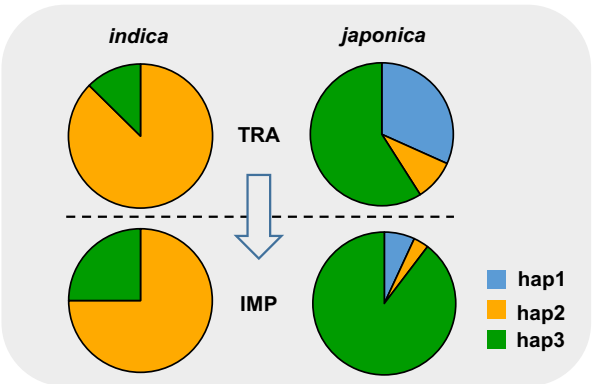
D



E



F



G

Sub	hap	OsLG3b 6053302	6053303	6053305	6053306	GWN1 24772418	24772096	24772047	24772029	24772010	24771997	24771989	24771842	24771838	24771783	24771742	24771082	24771009	24768682	24768016	24767736	24767657-24767670	No. of Accession	Grain Length (mm)
jap	H3	T	A	A	T	T	C	G	A	C	A	T	C	T	C	GCGGA	C	T	A	C	G	-	65	9.36 [a]
	H4	T	A	A	T	C	T	A	C	T	G	C	T	C	T	-	T	A	G	T	G	ATGTGATTATTTGT	19	8.92 [b]
	H5	T	A	A	T	C	T	A	C	T	G	C	T	C	T	-	T	A	G	T	A	ATGTGATTATTTGT	93	8.54 [c]
	H2	C	C	T	C	C	T	A	C	T	G	C	T	C	T	-	T	A	G	T	A	ATGTGATTATTTGT	114	7.73 [d]
ind	H1	C	C	T	C	C	T	A	C	T	G	C	T	C	T	-	T	A	G	T	G	ATGTGATTATTTGT	658	8.63
	H2	C	C	T	C	C	T	A	C	T	G	C	T	C	T	-	T	A	G	T	A	ATGTGATTATTTGT	118	8.69

◂**Fig. 7** Haplotypes, original and evolutionary analysis of *GWN1*. **A** Haplotype analysis of *GWN1*. **B–C** Grain length of different haplotypes of *GWN1* in *japonica* subgroup (**B**) or *indica* subgroup (**C**). **D–E** Haplotypes minimum spanning tree (**D**) and phylogenetic analysis (**E**) of *GWN1* in a natural rice population. The color of the outer circle in (**E**) refers to eight ecological groups as in (**D**): *Ruf1*, *Ruf2*, *Niv1*, *Niv2*, *indica* (*ind*), *temperate japonica* (*tej*), *tropical japonica* (*trj*), *aus*, and *bas*. The color lines of the inner circle in (**E**) refer to different haplotypes. **F** Frequency of alleles in traditional landraces (TRA) and improved varieties (IMP). **G** Combined haplotype analysis of *GWN1* and *OsLG3b*. Red number indicated three nonsynonymous SNPs and one Indel in CDS region of *GWN1*. Data are presented as mean ± SD. Different lowercase letters denote a significant difference (one-way ANOVA: $P < 0.05$)

These results are consistent with the function of *GWN1* in regulating grain size and grain number.

To examine the subcellular localization of GWN1, the roots of *Pro35S*:*GWN1*^{Nip}-GFP transgenic plant were used for confocal microscopy investigation. The green fluorescence was detected at both the cell membrane and cell wall (Fig. 5C). The *Pro35S*:*GWN1*^{SLG}-GFP vector was also generated and transformed it into *N. benthamiana*. The result shows there is no difference in subcellular localization between GWN1^{Nip}-GFP and GWN1^{SLG}-GFP (Fig. S10).

GWN1 regulates grain length by affecting cell proliferation

Grain size is usually determined by cell division or cell expansion on spikelet hulls (Li et al. 2018). Therefore, the cell size and cell number of the lemma of NIL-*qGL8a*^{SLG} and NIL-*qGL8a*^{Nip} were surveyed in this study (Fig. 6A). The longitudinal direction cell length and transverse direction cell width of both the outer and inner epidermal of the lemma show no significant difference between NIL-*qGL8a*-^{SLG} and NIL-*qGL8a*^{Nip}, but the cell number of NIL-*qGL8a*-^{SLG} was significantly higher than that of NIL-*qGL8a*^{Nip} (Fig. 6B–G). Besides, the cell size of the inner parenchyma cells and the cell width of the outer and middle thick-walled cells in the outer glume have no obvious difference (Fig. S11A–E). These results showed that *GWN1* regulates grain length by promoting longitudinal cell proliferation.

Natural variation of *GWN1*

To further explore the variation of *GWN1* in germplasm, a haplotype analysis was carried out using a panel of 1,383 accessions from the 3 K project, including 119 *temperate japonica* (*tej*), 262 *tropical japonica* (*trj*), 783 *indica* (*ind*), 167 *aus* and 52 *basmati* (*bas)* accessions (Table S1). Based on 15 nonsynonymous SNPs and 2 Indels in the promoter and CDS region of two parents, *GWN1* could be divided into three haplotypes, namely hap1, hap2 and hap3 (Fig. 7A). The *japonica* subgroup contains all three haplotypes, but the *indica* subgroup only includes hap2 and hap3. SLG belongs to hap1, and Nip belongs to hap3. In the *japonica* subgroup, accessions with hap1 had a longer grain length than accessions with hap2 and even longer than accessions with hap3. In *indica* subgroup, however, accessions with hap2 and hap3 have no obvious difference in grain length (Fig. 7B, C). This suggests that *GWN1*^{hap1} was the elite allele for grain size in the *japonica* subgroup.

Haplotype network analysis and phylogenetic analysis based on 46 common SNPs between 2786 cultivars and 242 wild rice accessions revealed that *GWN1*^{hap2} and *GWN-1*^{hap3} were present in both cultivated and wild rice accessions, whereas *GWN1*^{hap1} only present in cultivated rice (Fig. 7D; Table S3). While *GWN1*^{hap2} mainly spread into *indica*, *GWN1*^{hap1} and *GWN1*^{hap3} were mainly restricted to *japonica*, suggesting that the differentiation of *GWN1* into its three haplotypes might originate from different wild rice and may contribute to the differentiation of *indica* and *japonica* (Fig. 7D, E). Because only 86 accessions belonging to hap1 showed the largest grain length, and the majority of *GWN1*^{hap1} allele was present in traditional landraces (TRA), we speculate that the elite allele *GWN1*^{hap1} might be a rare mutation that plays an important role in regulating grain length in rice. It has significant potential for utilization in improved (IMP) rice varieties (Fig. 7F).

In our previous study, a QTL *OsLG3b* regulating grain size was identified using the population also constructed by crossing SLG and Nip. The *OsLG3b*^{SLG} allele also shows longer grains (Yu et al. 2018). To ascertain the utilization rate of *GWN1*^{SLG} and *OsLG3b*^{SLG} in cultivars, the joint haplotype analysis of *GWN1* and *OsLG3b* was conducted. A panel of 1,067 accessions was divided into five haplotypes (Table S2). H3 carries the *OsLG3b*^{SLG} allele and the *GWN1*^{SLG} allele only consists of the *japonica* subgroup and exhibits the longest grains. At the same time, we conducted genetic sequence testing on 43 elite rice cultivars. Among them, only Wuyoudao 4 (also known as Dao Hua Xiang 2) was discovered to possess both elite alleles of *GWN1* and *OsLG3b* from SLG (Table S6), which is a famous and popular rice variety in the Chinese market (Jiang et al. 2023). These results indicated that the advantage of the *GWN1* and *OsLG3b* genes in controlling grain length has been preserved and utilized by breeders in the *japonica* subgroup, but it has not been widely adopted.

The nucleotide diversity and Tajima's *D* value of the *GWN1* gene region and its upstream and downstream 20 kb flanking region in different subgroups were then analyzed (Fig. S12), and observed lower nucleotide diversity as well as a significant difference in Tajima's *D* value in the *GWN1* gene region of *tej* (Table S7). When *tej* was further divided into *tej*-hap1 and *tej*-hap3, *tej*-hap1 showed a significant Tajima's *D* value (Table S8). These results indicated that

there has been selective divergence of *GWN1* in *tej*, mainly caused by the selection of *tej*-hap1.

Discussion

The S2893 (24768016) variation is localized in the Ser/Thr kinase domain (Fig. 2B, C), which probably affects the phosphorylation or autophosphorylation of GWN1. We speculate the variation in S3173 (24767736) might be important in regulating grain length (Fig. 7A, B). According to the SMART website (http://smart.embl.de/), the S3173 (24767736) variation, led to a deficiency of the Kinase-associated protein B (KapB) domain in the $GWN1^{SLG}$. KapB, a major histidine kinase, provides phosphate input in the phosphorelay. The absence of KapB might affect the activity or phosphorylate ability of GWN1 in SLG (Dartois et al. 1997). The 14 bp deletion resulted in a loss of 30aa at the end of the C terminal of GWN1 leading to a deficiency of the RAS domain in the $GWN1^{SLG}$. RAS encodes small GTPases, which might influence protein folding or conformation, thus altering its function. We could directionally edit these sites to confirm which site determines the function of *GWN1* in controlling grain length and number.

Knockout of *GWN1* results in longer and heavier grains, accompanied by a higher grain number and higher plant grain yield (Fig. 3), indicating *GWN1* has great potential in breeding for a higher yield. There were mainly three haplotypes in the investigated germplasm (Fig. 7A). $GWN1^{hap1}$ was a rare allele, and the accessions containing it showed the longest grains in *japonica*. In the future, knocking out *GWN1* or editing its natural variation in the promoter or coding region is a good way to improve rice yield.

Rice WAKs play important roles in cell expansion (Cai et al. 2023; Hu et al. 2014; Yue et al. 2022). However, this study found that *GWN1* as a WAK regulates grain length by influencing cell proliferation rather than cell expansion. According to the STRING website (https://cn.string-db.org/), ten predicated interacting proteins were identified, most of them are related to the ubiquitin–proteasome pathway. It has been reported that ubiquitin–proteasome pathway-related factors participate in regulating grain size in rice (Hao et al. 2021; Huang et al. 2017, 2021; Li et al. 2023a, b; Zhu et al. 2021; Du et al. 2016). More research is required to determine whether GWN1 interacts with and phosphorylates these factors and then affects the ubiquitination of their substrates, which might be the regulators of cell proliferation.

In addition to the roles of WAK members in regulating plant growth and development, WAKs also play a vital role in plant responses to various biotic stress (Malukani et al. 2020; Zhang et al. 2022; Delteil et al. 2016; Harkenrider et al. 2016; Li et al. 2009). *OsWAK1* has been reported to positively regulate rice blast disease resistance, and its expression could be induced by MeJA (Li et al. 2009). According to the data in the Rice Expression Profile (RiceXPro) database (https://ricexpro.dna.affrc.go.jp), the expression of *GWN1* could be highly induced by JA in both roots and shoots, and slightly induced by auxin in roots and by abscisic acid in shoots (Fig. S13). These findings implied that *GWN1* tended to have pleiotropism and might perform important roles in the growth and development of rice and its response to the external environment, which are worthy of being confirmed in the future.

In this work, we uncovered a novel gene *GWN1* by map-based cloning. As a negative regulator of grain length, weight and number, *GWN1* influences grain length by regulating cell proliferation in spikelet hulls and regulates grain number by influencing secondary branch numbers. $GWN1^{hap1}$, as a rare natural allele, shows longer grains and has not been extensively utilized in modern rice varieties. *GWN1* might have huge application potential in molecular breeding for longer grains and a higher yield.

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Author contribution statement Zhiqi Ma: Conceptualization, Investigation, Formal analysis, Software, Writing-original draft-review & editing. Jinli Miao: Formal analysis, Investigation. Jianping Yu: Data curation. Yinghua Pan: Funding acquisition. Danting Li: Resources. Peng Xu: Funding acquisition. Xingming Sun: Methodology. Jinjie Li: Supervision. Hongliang Zhang: Methodology. Zichao Li: Conceptualization, Supervision, Project administration. Zhanying Zhang: Conceptualization, Funding acquisition, Supervision, Writing-review & editing.

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Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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