Letter to the Editor

Generation of new glutinous rice by CRISPR/Cas9-targeted mutagenesis of the

***Waxy* gene in elite rice varieties**

Running Title: CRISPR/Cas9 create new glutinous rice varieties

Jinshan Zhang[1, 2†], Hui Zhang[1, 3†]*, José Ramón Botella[4], and Jian-Kang Zhu[1, 3]*

[1]. Shanghai Center for Plant Stress Biology, and Center for Excellence in Molecular Plant Sciences, Chinese Academy of Sciences, Shanghai 201602, China

[2.] University of Chinese Academy of Sciences, Shanghai 201602, China

[3]. Department of Horticulture and Landscape Architecture, Purdue University,

West Lafayette, IN 47906, USA

[4]. Plant Genetic Engineering Laboratory, School of Agriculture and Food Sciences, University of Queensland, Brisbane QLD 4072, Australia.

[†]These authors contributed equally to this work.

***Correspondences:** Hui Zhang (huizhang@sibs.ac.cn); Jian-Kang Zhu

(jkzhu@sibs.ac.cn, Dr. Zhu is fully responsible for the distribution of all materials

associated with this article)

Edited by: Li-Jia Qu, Peking University, China

Summary   In rice, amylose content (AC) is controlled by a single dominant *Waxy* gene. We used CRISPR/Cas9 to introduce a loss-of-function mutation into the *Waxy* gene in two widely-cultivated elite *japonica* varieties. Our results show that mutations in the *Waxy* gene reduce AC and convert the rice into glutinous ones without affecting other desirable agronomic traits, offering an effective and easy strategy to improve glutinosity in elite varieties. Importantly, we successfully removed the transgenes from the progeny. Our study provides an example of generating improved crops with potential for commercialization, by editing a gene of interest directly in elite crop varieties.

Yield and quality traits are two of the most important targets for rice breeders. Recent technical advances in high-throughput sequencing have accelerated the mapping and identification of many Quantitative Trait Loci (QTLs) making it possible to use tools such as CRISPR/Cas9 to perform precise gene editing for plant improvement. In the last few years, a number of reports have described the use of gene editing to study yield-related QTLs, such as *Gn1a*, *IPA1*, *GS3* and *DEP1*, and explore their function in different varieties (Li et al. 2016). Food security considerations make yield an obvious target for rice improvement. However, economic growth and improved living standards in many rice-majority consuming countries are shifting public attention towards quality characteristics such as flavor, cooking, appearance and nutrition, which are strongly linked to starch physical properties. Starch is the major component in rice grain endosperm and it is a mix of two glucans: amylose and amylopectin. Amylose is primarily a linear polysaccharide while amylopectin is highly branched. The amylose content (AC) refers to the percentage of starch instead of the percentage of total weight in the grain, and is arguably the most important quality indicator in rice, especially for cooking and eating quality (Juliano 1998). Based on AC values, rice is commercially classified into five groups: waxy (0–5%), very low (5%–12%), low (12%–20%), intermediate (20%–25%), and high (25%–33%) AC (Juliano 1992). In general, cooking of high AC varieties results in dry, firm and well separated rice grains, becoming hard after cooling; Intermediate AC varieties is soft but not sticky after cooking; Low and very low AC (5%–20%) varieties, have a soft and sticky texture after cooking; Finally, waxy rice, also called glutinous rice, is especially sticky when cooked (Juliano 1998). The quality of some of the hybrid rice grown in China, especially the *indica* hybrids, is considered poor owing to their high AC that makes them hard and dry when cooked. Therefore, a major objective for breeders is to improve grain quality by decreasing AC in rice, mostly in the *indica* hybrids.

The *Waxy* (*Wx*) gene (LOC_Os06g04200) of rice which encodes a granule-bound starch synthase (GBSS), also known as Waxy protein, is responsible for the synthesis of amylose in endosperm (Wang et al. 1995). There are two major *Wx* alleles in cultivated rice, *Wx^a* and *Wx^b*, with *indica* cultivars mostly containing the *Wx^a* allele while most *japonica* cultivars contain the *Wx^b* allele (Wang et al. 1995). The main difference between the alleles is a G/T polymorphism that results in differential splicing of the gene affecting mRNA stability (Wang et al. 1995; Larkin and Park 2003). As a result, the *Wx^a* allele produces 10-fold higher mRNA and protein levels than *Wx^b* (Isshiki et al. 1998). *Waxy* expression levels are positively correlated with AC, making it feasible to alter the AC by manipulating the *Waxy* gene and a number of approaches have been attempted including conventional breeding and genetic manipulation. Ma et al (2015) showed that CRISPR/Cas9-generated mutations in the *Waxy* gene led to reduced AC in a *japonica* cultivar of rice, Taichung 65 (Ma et al. 2015). Transgenic rice lines containing *Waxy* antisense constructs also produced seeds with reduced amylose levels and improved quality; and the decreased AC was also observed in hybrids obtained with the transgenic lines (Terada et al. 2000; Liu et al. 2003; Liu et al. 2005). In contrast, introduction of the *Wx^a* cDNA into *Waxy* null-mutant *Japonica* rice lines increased the AC by 6%–11% (Itoh et al. 2003). Traditional breeding methods are effective but time-consuming and laborious, therefore we used CRISPR/Cas9-mediated gene editing to introduce a loss-of-function mutation into the *Waxy* gene to reduce AC in two widely-cultivated elite *japonica* varieties, Xiushui134 (XS134) and Wuyunjing 7 (9522). Our results show that mutations in the *Waxy* gene produce decreased AC in rice offering an effective strategy of generating improved crops in elite varieties without affecting other desirable agronomic traits.

We sequenced the waxy gene XS134 and 9522 and confirmed that both varieties contain the typical Wx^b allele (Figure S1). We designed a CRISPR/Cas9 construct targeting the first exon of the *Waxy* gene with the expectation to produce a null mutation (Figure 1A). The 20-nt target sequence for the sgRNA was carefully chosen to avoid off-target effects using the web-based tool CRISPR-P (http://cbi.hzau.edu.cn/cgi-bin/CRISPR). Embryogenic calli from the elite rice varieties XS134 and9522 were co-cultivated with *Agrobacterium tumefaciens* carrying binary vectors with the CRISPR/Cas9 cassette and transgenic T0 plants analyzed to detect the presence of mutations in the target regions using Sanger sequencing. A very high mutagenesis efficiency was observed with 82.76% of T0 transformants carrying mutations in XS134 plants and 86.96% in 9522 (TableS1). It has been reported that CRISPR/Cas9-induced mutations mainly take place in transformed calli cells and homozygous mutations in the target genes can be readily found in T0 plants (Zhang et al. 2014). In our case we found one homozygous T0 plant in XS134 (4.17%) and 3 in 9522 (15%) (Table S1). Most of the mutation types detected in the target sequence produced frameshifts in the coding region giving rise to non-functional proteins (Figure 1B). To test for possible off-target effects, we identified the locus in the rice genome with the highest probability for such effects based on the sequence of the sgRNA used in our study. No mutations were found in any of the 46 T0 plants analyzed for both varieties by sanger sequencing (Table S2) suggesting that off-target effects can be avoided or reduced by careful selection of the target site.

A number of T1 plants containing CRISPR/Cas9-induced homozygous frameshift mutations in the *Waxy* gene (hereupon referred to as "CRISPR-*wax*y" mutants) were

selected for phenotypic analysis. All plants were grown under natural field conditions in the Shanghai region, China (30°N, 121°E) during the normal rice-growing season from mid-May to mid-October. For both varieties, the mutated *waxy* plants were indistinguishable from WT controls in plant height (Figures 1C, S2A), grain number per panicle (Figure S2B), panicle number per plant (Figure S2C), yield per plot (Figure S2D), seed width (Figure S3A, C, F and H), seed length (Figure S3B, D, G and I) and 1,000 grains weight (Figure S3E, J). These results confirm our expectation that mutation of the *Waxy* gene does not affect important agronomic traits in rice, an essential pre-requisite to achieve our aim of crop improvement without compromising agronomic characteristics in elite varieties.

Grains of CRISPR/Cas9 mutated plants had a 'waxy' appearance being white and fully opaque in contrast with the typical 'non-waxy' translucent appearance of 9522 and XS134 seeds (Figure 2A). Cross-sections of seeds also revealed a milky white and opaque appearance in the CRISPR-*waxy* mutants, compared to translucent endosperm of WT seeds (Figure 2A). The two starch components in rice, amylose and amylopectin, have different iodine binding capacity. Amylose has a high binding capacity and turns deep blue when stained with iodine, while amylopectin has much lower iodine-binding capacity, turning red-brown upon staining. As expected, endosperms in cross-sections of 9522 and XS134 WT seeds turned dark blue when stained with an iodine solution while CRISPR-*waxy* endosperms turned red-brown (Figure 2A), revealing that CRISPR-*waxy* endosperms had a lower amylose/amylopectin ratio than WT. Quantification of the amylose content confirmed the staining results with CRISPR-*waxy* seeds showing a significant reduction in AC compared to XS134 and 9522 WT controls (Figure 2B; Table S3). Total starch content was unchanged in CRISPR-*waxy* and control plants (Figure 2B; Table S3). The low

AC in the CRISPR-*waxy* seeds qualify then as waxy or glutinous rice, implying that we successfully transformed two non-glutinous varieties, XS134 and 9522 into new glutinous varieties by using CRISPR/Cas9 to edit the *Waxy* gene. The *Waxy* gene has been reported to affect the gel consistency (GC) and gelatinization temperature (GT) of rice (Liu et al. 2003; Tian et al. 2009). Our analysis showed a 2-fold increase in GC and a marked reduction in GT for CRISPR-*waxy* seeds compared to their respective WT (Table S3). Scanning electron microscopy revealed clear differences in the starch structure of CRISPR-*waxy* and WT seeds with WT showing sharp edges while CRISPR-*waxy* seeds were more irregular (Figure 2C). Finally we addressed the issue of social acceptance of genetically modified foods by removing the transgenes from CRISPR/Cas9-edited *waxy* rice lines by self-pollination in the T2 generation (Table S4; Figure S4). To identify transgene-free lines we analyzed individuals by PCR using specific primers for the U6-sgRNA, 35S promoter and Nos terminator sequences. Two out of 48 *waxy*-XS134 and 4 out of 20 *waxy*-9522 T2 plants were transgene-free (Table S4).

Glutinous rice (waxy rice) is popular in many Asian countries. Compared with non-glutinous rice, waxy rice provides unique characteristics for numerous food and non-food applications, such as brewing. In this study, we used CRISPR/Cas9 to mutate the *Waxy* gene and converted two elite non-glutinous rice varieties into glutinous varieties without changing any of the important agronomic traits that had been laboriously introduced over long and costly breeding programs. Therefore, the newly created variety is already elite and can be immediately used without the process of trait introgression by repeated backcrossing to parental lines. In addition, the ability to remove the transgene cassettes in the T2 generation allowed us to produce non-GM lines containing the desired mutation with surgical precision, thus helping to

circumvent GM-related regulations and public controversy.

Aside from conventional elite varieties, CRISPR/Cas9 editing of the *Waxy* gene can also be used in the production of hybrid rice. The *Waxy* gene has a dosage effect in the triploid rice endosperm (Kumar and Khush 1986), making it possible to manipulate AC in hybrid rice by editing the *Waxy* gene in sterile or restorer lines. *Waxy* mutations in sterile lines would give rise to a *Wx/wx/wx* endosperm genotype; while mutations in restorer lines, would result in a *Wx/Wx/wx* genotype allowing the production of hybrid rice with different AC.

In summary, we have used CRISPR/Cas9 to mutate the *Waxy* gene in two elite cultivated rice lines, XS134 and 9522 and developed new rice lines with lower amylose content while maintaining all the desired agronomic traits and successfully removed the transgenes from the progeny to produce transgene-free lines. Our study provides an example of generating improved crops with potential for commercialization, by editing genes of interests directly in elite crop varieties.

MATERIALS AND METHODS

Plant materials and growth conditions

XS134 is an elite *japonica* rice variety with high yield and good quality, which is mainly planted in Jiangsu Province, Zhejiang Province and Shanghai area, China (http://ricedata.cn). 9522 is also an elite *japonica* rice variety with high yield, high disease resistance and good quality, which is cultivated widely in the south of Jiangsu Province, China (http://ricedata.cn). The CRISPR/Cas9 vector targeting the *Waxy* gene was constructed as previously described (Zhang et al. 2014). The CRISPR/Cas9 cassette was transferred into 9522 and Xiushui 134 (XS134) callus by *Agrobacterium*

tumefaciens-mediated transformation using the strain EHA105 (Hiei et al. 1997). Transgenic rice lines were grown in paddy fields in Shanghai, China, during normal rice-growing seasons. Mature seeds were harvested and dried for germination. T1 and T2 seeds were collected following the same procedure. Sequences of the primers used for vector construct and detection are listed in Table S5.

Phenotype and genotype assays

Plant height, grain number per panicle, and panicle number per plot weremeasured in the paddy fields. For plot experiment, the planting density was 20 plants in 65.5cm×101.5cm area and he plot yields determined when the seeds were harvested and dry. Seeds were collected for each plant and weighted after drying at 37℃ for two weeks. 1,000 grains weight, grain length and width were measured using an SC-A grain analysis system (Wseen company, China). PCR amplification was carried out using primer pairs flanking the sgRNA target site and putative off-target designated locus (Table S5). The PCR products were sequenced by sanger method.

Grain phenotype and iodine-staining of endosperm

The hull of rice seeds was removed to observe the external appearance of the grain in both 9522 and XS134 varieties. Grains were cut through the center to expose the endosperm. 0.2% iodine reagent was dropped on the endosperm surface and photographs taken after 3-5 minutes.

Determination of total starch and amylose content

Total starch content was determined according to the Megazyme Total Starch assay procedure (Megazyme Intenational Ireland, www. megazyme.com). D-Glucose stains with GOPOD (Glucose Oxidase Plus Peroxidase and 4-aminoantipyrine) reagent were determined at 510nm. Amylose content was measured following the procedure described in GB/T 15683-2008/ ISO 6647-1: 2007. The amylase-iodine blue color

was determined at 720 nm.

Evaluation of grain GC and GT

Gel consistence (GC) was evaluated according to Cagampang et al. (Cagampang et al. 1973). Quartic measurements were performed for each sample. Gelatinization temperature (GT) was indirectly estimated via the alkali digestion test (Little et al. 1958). Six whole-grain and same size, milled rice samples were placed in small plastic boxes containing 2 mL 1.7% potassium hydroxide (KOH) and incubated at 30 ℃ in an oven. After 23 hours, grain appearance and disintegration were visually rated based on a standard numerical scale.

Scanning electron microscopy of starch granules

Rice grains were dried in an oven at 42℃ for 2 days and cooled in a desiccator. Cross-sections of the samples were manually snapped and sputter-coated with gold palladium on copper studs. Magnifications of 50× and 2000× were used to observe endosperm and starch granule morphology.

AUTHORS CONTRIBUTIONS

J.-K.Z. and H.Z. designed research and supervised the study; J.Z. and H.Z. performed research; H.Z., J.Z., J.R.B., and J.-K.Z. analyzed data; and H.Z., J.R.B., J.-K.Z. and J.Z. wrote the paper.

REFERENCES

Cagampang GB, Perez CM, Juliano BO (1973) A gel consistency test for eating quality of rice. **J Sci Food Agric** 24 (12):1589–1594

Hiei Y, Komari T, Kubo T (1997) Transformation of rice mediated by Agrobacterium tumefaciens. **Plant Mol Biol** 35 (1-2):205–218

Isshiki M, Morino K, Nakajima M, Okagaki RJ, Wessler SR, Izawa T, Shimamoto K (1998) A naturally occurring functional allele of the rice waxy locus has a GT to TT mutation at the 5' splice site of the first intron. **Plant J** 15 (1):133–138

Itoh K, Ozaki H, Okada K, Hori H, Takeda Y, Mitsui T (2003) Introduction of *Wx* transgene into rice *wx* mutants leads to both high-and low-amylose rice. **Plant Cell Physio** 44 (5):473–480

Juliano B (1992) Structure, chemistry, and function of the rice grain and its fractions. **Cereal Foods World** 37:772–772

Juliano B (1998) Varietal impact on rice quality. **Cereal Foods World** 43:207–222

Kumar I, Khush G (1986) Gene dosage effects of amylose content in rice endosperm. **J Genet** 61 (6):559–568

Larkin PD, Park WD (2003) Association of *waxy* gene single nucleotide polymorphisms with starch characteristics in rice (*Oryza sativa* L.). **Mol Breeding** 12 (4):335–339

Li M, Li X, Zhou Z, Wu P, Fang M, Pan X, Lin Q, Luo W, Wu G, Li H (2016) Reassessment

of the four yield-related genes *Gn1a*, *DEP1*, *GS3*, and *IPA1* in rice using a CRISPR/Cas9 System. **Front Plant Sci** 7 (12217):377

Little RR, HILDER GB, Dawson EH (1958) Differential effect of dilute alkali on 25 varieties of milled white rice. **Cereal Chem** 35 (2):111–126

Liu Q, Wang Z, Chen X, Cai X, Tang S, Yu H, Zhang J, Hong M, Gu M (2003) Stable inheritance of the antisense *Waxy* gene in transgenic rice with reduced amylose level and improved quality. **Transgenic Research** 12 (1):71–82

Liu Q, Yu H, Chen X, Cai X, Tang S, Wang Z, Gu M (2005) Field performance of transgenic indica hybrid rice with improved cooking and eating quality by down-regulation of *Wx* gene expression. **Mol Breed** 16 (3):199–208

Ma X, Zhang Q, Zhu Q, Liu W, Chen Y, Qiu R, Wang B, Yang Z, Li H, Lin Y (2015) A robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. **Mol Plant** 8 (8):1274–1284

Terada R, Nakajima M, Isshiki M, Okagaki RJ, Wessler SR, Shimamoto K (2000) Antisense *Waxy* genes with highly active promoters effectively suppress Waxy gene expression in transgenic rice. **Plant Cell Physiol** 41 (7):881–888

Tian Z, Qian Q, Liu Q, Yan M, Liu X, Yan C, Liu G, Gao Z, Tang S, Zeng D (2009) Allelic diversities in rice starch biosynthesis lead to a diverse array of rice eating and cooking qualities. **Proc Natl Acad Sci USA** 106 (51):21760–21765

Wang ZY, Zheng FQ, Shen GZ, Gao JP, Snustad DP, Li MG, Zhang JL, Hong MM (1995) The amylose content in rice endosperm is related to the post-transcriptional regulation of the *waxy* gene. **Plant J** 7 (4):613–622

Zhang H, Zhang J, Wei P, Zhang B, Gou F, Feng Z, Mao Y, Yang L, Zhang H, Xu N (2014) The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. **Plant Biotechnol J** 12 (6):797–807

SUPPORTING INFORMATION

Figure S1. Sequence alignment of the *Waxy* genomic sequence in *Nipponbare*, 9522 and XS134

The sequence of *Waxy* gene in 9522 is the same with that in XS134, which contain the typical Wx^b allele. Compared with *Nipponbare*, 9522 and XS134 contains 18 CT repeats in the CT-microsatellite regions, while *Nipponbare* contains 17 CT repeats. Two SNPs between 9522/XS134 and *Nipponbare* are indicated in red box. Start code and stop code are indicated in pink box.

Figure S2. Plant height, grain number per panicle, panicle number per plant and yield per plot in CRISPR-*waxy* mutants and their corresponding WT plants

(**A**) Plant height of *waxy*-9522, *waxy*-XS134 mutant and corresponding 9522, XS134 WT. (**B**) Grain number per panicle of *waxy*-9522, *waxy*-XS134 mutant and corresponding 9522, XS134 WT. (**C**) Panicle number per plant of *waxy*-9522, *waxy*-XS134 mutant and corresponding 9522, XS134 WT. (**D**) Yield per plot of *waxy*-9522, *waxy*-XS134 mutant and corresponding 9522, XS134 WT. Data are presented as means ±sd. n=20 in A-C and n=3 in D; two-tailed, two-sample Student t-test. NS, no significant

Figure S3. Grain width, length and 1,000 grains weight of CRISPR-*waxy* mutants and their corresponding WTs

(**A**) and (**C**) Grain width of *waxy*-9522 mutant and corresponding 9522 WT. (**B**) and (**D**) Grain length of *waxy*-9522 mutant and corresponding 9522 WT. (**E**) 1,000 grains weight of *waxy*-9522 mutant and corresponding 9522 WT. (**F**) and (**H**) Grain width of *waxy*-XS134 mutant and corresponding XS134 WT. (**G**) and (**I**) Grain length of *waxy*-XS134 mutant and corresponding XS134 WT. (**J**) 1,000 grains weight of *waxy*-XS134 mutant and corresponding XS134 WT. Data are presented as means ±sd. n= 50 in C, D, H and I; n=5 in E and J; two-tailed, two-sample Student t-test. NS: no significant

Figure S4. Detection of transgene DNA in CRISPR-*waxy* lines in 9522 and XS134 backgrounds

Red arrows indicate the transgene-free lines.

Table S1. Percentage of T0 plants with mutations in the target locus
*, the T0 homozygous mutations were further confirmed in the T1 generation.

Table S2. Mutation analysis of putative sgRNA off-target sites

Mismatching bases are shown in red color; the PAM motif (NGG) is shown in blue color.

Table S3. Amylose content (AC), gel consistency (GC) and gelatinization temperature

(GT) in mature seeds of *waxy* mutants and corresponding WT lines

mm, millimetres; ASV, alkali spreading value. Data are presented as means $\pm$ sd. **P < 0.01.

Table S4. Production of transgene-free homozygous lines in CRISPR-*waxy* mutants

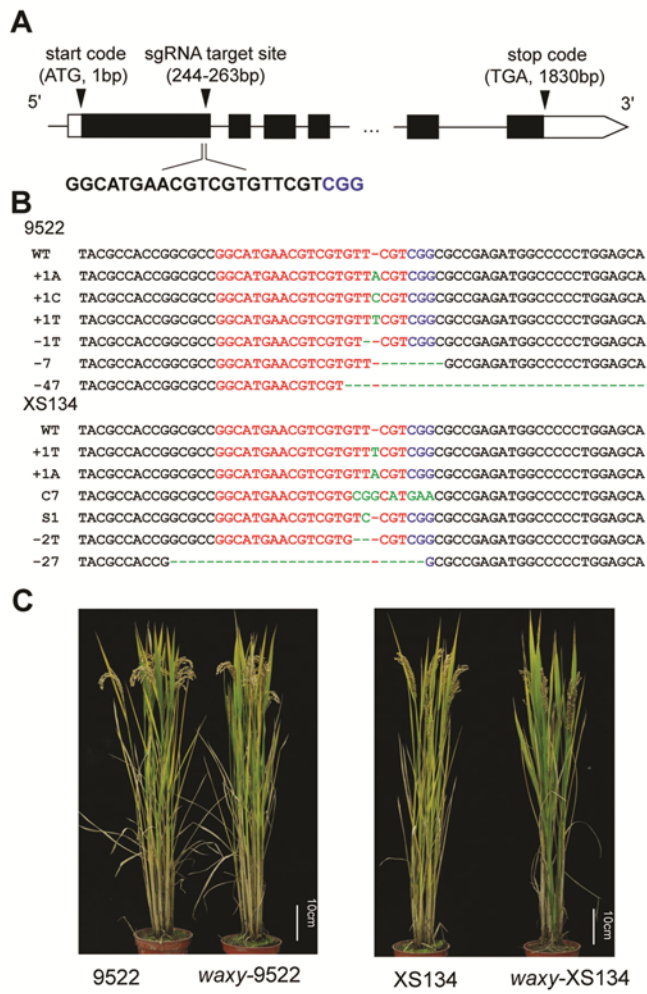**Table S5.** Primers used in this study

Figure legends

Figure 1. Generation of new glutinous rice by CRISPR/Cas9-targeted mutagenesis of the *Waxy* gene in elite rice varieties

(**A**) Schematic diagram of the targeted site in the *Waxy* gene. Black arrows indicate the start codon, targeted site and stop codon. The numbers in brackets indicate the distance to the start codon (ATG). The sequence of the targeted site is shown with the protospacer adjacent motif (PAM) sequences labeled in blue color. (**B**) Examples of mutations at the *Waxy* locus in CRISPR-*waxy* T0 generation plants of two rice varieties, 9522 and XS134. The targeted sequence is highlighted in red and the PAM sequences are in blue. Mutations are marked in green color. (**C**) Phenotypes of CRISPR-*waxy* mutants and their corresponding WTs.
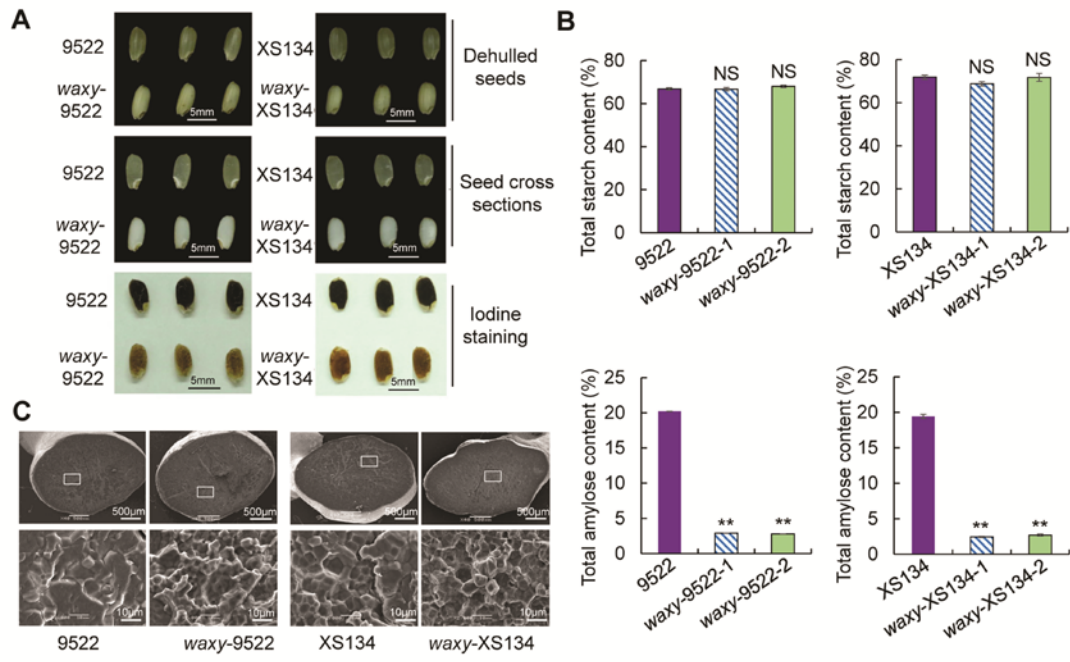
Figure 2.　Grain phenotypes, total starch and total amylose content and scanning electron micrographs of endosperms in mature seeds of *waxy* mutants and corresponding WT lines

(**A**) Grain phenotypes of CRISPR-waxy mutants and their corresponding WTs. Upper row, phenotype of the dehulled seeds. Middle row, endosperm phenotypes in seed cross-sections. Bottom row, iodine-staining of endosperm in cross sections of seeds. (**B**) Total starch and total amylose content in CRISPR-waxy mutants and their corresponding WTs. Data is presented as means ± sd. n=4; **P < 0.01, two-tailed, two-sample t-test; NS: no significant. (**C**) Scanning electron micrographs of endosperms in CRISPR-waxy mutants and their corresponding WTs.

Figure 1

Figure 2