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Three novel alleles of *FLOURY ENDOSPERM2* (*FLO2*) confer dull grains with low amylose content in rice

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ABSTRACT

Rice is a major food source for much of the world, and expanding our knowledge of genes conferring specific rice grain attributes will benefit both farmer and consumer. Here we present novel dull grain mutants with a low amylose content (AC) derived from mutagenesis of *Oryza sativa*, ssp. *japonica* cv. Taikeng 8 (TK8). Positional cloning of the gene conferring the dull grain phenotype revealed a point mutation located at the acceptor splice site of intron 11 of *FLOURY ENDOSPERM2* (*FLO2*), encoding a tetratricopeptide repeat domain (TPR)-containing protein. Three novel *flo2* alleles were identified herein, which surprisingly conferred dull rather than floury grains. The allelic diversity of *flo2* perturbed the expression of starch synthesis-related genes including *OsAGPL2*, *OsAGPS2b*, *OsGBSSI*, *OsBEI*, *OsBEIIb*, *OsISA1*, and *OsPUL*. The effect of the *flo2* mutations on the physicochemical properties of the grain included a low breakdown, setback, and consistency of rice, indicating a good elasticity and soft texture of cooked rice grains. The effects of *FLO2*, combined with the genetic background of the germplasm and environmental effects, resulted in a variety of different amylose content levels, grain appearance, and physicochemical properties of rice, providing a host of useful information to future grain-quality research and breeding.

1. Introduction

Rice is one of the most important cereal crops in the world. More than half of the world's population relies on rice as a primary food, and it is estimated that demand will have increased by 40% by 2030 due to the growing population of rice-consuming countries [1]. Besides yield increases, improvements to grain quality are one of the most important goals for rice breeders, due to consumer preference and market profits. Grain quality can be assessed in terms of grain appearance, milling quality, and eating and cooking quality. Each factor can be negatively impacted by either abnormal grain shape or non-compact arrangement of starch granules, both of which are controlled by interplay between genetics and environment [2]. There also remains the need for diversity in grain quality and the physicochemical properties of rice because of the variety of rice flours needed for multi-dimensional end-products. Elucidating the genetic factors regulating grain quality is essential in rice breeding to meet miscellaneous usages for end-products and to promote unit price and farmers' profits.

Up to 90% of the polished rice grain is starch. Starch grains are built up of growth rings, or semi-crystalline arrays of blocklets, which are in turn formed by alternating layers of semi-crystalline lamella, consisting of amylopectin chains, and amorphous lamella, which also incorporate amylose [3,4]. The amylose content (AC) of endosperm is thus the key determinant for rice grain appearance and physicochemical properties such as eating and cooking quality (ECQ). The gene responsible for amylose synthesis is *Waxy* (*Wx*), which encodes granule-bound starch synthase I (OsGBSSI). The functional allele Wx^a, present in *indica* rice, results in a high AC, ≥20%; the partially functional Wx^b allele present in *japonica* rice results in a low AC, 12–16%, due to a G-to-T single nucleotide polymorphism (SNP) in intron 1, resulting in deficient mRNA splicing. The null *wx* allele present in waxy rice produces almost no amylose [5,6]. Conversely, amylopectin is synthesized via the activities of branching enzyme (OsBE), soluble starch synthase (OsSS), and isoamylase (OsISA) and pullulanase (OsPUL), which in turn affect starch granule formation and other physicochemical properties of

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starch [3]. Starch synthase genes, *OsSSIIa* (also named *ALK*), mainly control gelatinization temperature and regulate gel consistency [7,8], and *OsISA*, *OsBEIIb*, and *OsPUL* have minor effects on these pasting properties of cooked rice [9–11]. Mutations in genes such as *OsGBSSI* (*Wx*), *OsBEIIb*, and *OsSSIIIa* (*Floury5*) alter starch granule formation, AC, and grain appearance, which in turn affects ECQ [12,13]. In addition, the transcription factor Rice Starch Regulator1 (RSR1) down regulates starch synthesis-related genes in seeds. As such, the null allele *rsr1* caused round and loosely packed starch granules due to a high AC and improper amylopectin formation [14].

Rice endosperm mutants have been used to elucidate how gene function can alter grain appearance. Waxy and dull rice grains have an opaque endosperm due to pores between and within starch granules. White-core floury grains, on the other hand, have this appearance due to irregular and loosely packed starch granules. Dull rice endosperm exhibits approximately half the AC of non-waxy *japonica*, whereas *indica* rice showed intermediate translucence and staining by iodine/potassium iodide [15–17]. More than a dozen "dull" loci were identified, including *du-1*, *du-4*, *du* (*2035*), *du* (*EM47*), *du* (*2120*), *du12(t) Du(t)*, *Du6a(t)*, and *Du7(t)* [18–23]. *Dull* genes regulate the expression of *Wx* by reducing its splicing efficiency [15,24]. *Dull1* and *Dull3* encode a Prp1 protein and the 20-kDa subunit of a cap-binding protein, respectively, which are crucial in pre-mRNA modification of *Wx* [25,26]. Floury endosperm results from mutation of *FLOURY* genes, regardless of whether these genes directly participate in starch synthesis. To date, five *FLOURY* genes have been isolated and their function identified. Three of them participate in starch metabolism pathways, including *Flo4*, which encodes a pyruvate orthophosphate dikinase; *Flo5* encodes starch synthesis enzyme OsSSIIIa, and *osagpl2-3* (also named *Flo6*) encodes the ADP-glucose (synthase) pyrophosphorylase OsAGPL2 [13,27,28]. The other two *FLOURY* genes encoded in the rice genome are involved in mediating protein-protein interactions in the regulation of starch synthesis-related genes including *FLO2*, which encodes a tetratricopeptide (TPR) domain-containing protein regulating *Wx* expression, and *FLO6*, which encodes the CBM 48 domain interacting isoamylase1 (ISA1) and binds to starch [29,30]. In addition to *FLOURY* genes, other genes in which a mutation can result in floury endosperm include *Grain Incomplete Filling1* (*GIF1*) encoding a cell wall invertase; *OsRab5a* encoding a small GTPase; and *OsSar1* encoding small GTP protein [31–33]. Thus, the genetic basis of rice grain quality relies not only on starch synthesis-related genes but also those involving carbon partition, protein accumulation and protein trafficking in developing seeds.

In this study, we identified nine dull grain mutants from thousands of mutants derived from ethyl methanesulfonate (EMS) mutagenesis of *Oryza sativa*, ssp. *japonica* cv. Taikeng 8 (TK8). One of nine dull mutants exhibiting the dull grain phenotype and also containing relatively low AC (11.9%) was further investigated, isolating the mutated gene by positional cloning. We found out that the gene conferring a dull grain phenotype was the floury gene, *FLO2* or *FLO(a)* [30,34]. FLO2, a TPR-binding protein, mediates protein-protein interaction and has been linked to heat tolerance during seed development. *FLO2* regulates starch synthesis-related genes *in trans*, and defective *FLO2* reduced starch and protein storage [30,35]. Subsequently, we identified a total of three novel *flo2* mutants from the nine dull mutants, which differed from major characters of floury mutants. The three mutants exhibited low AC and dull grains rather than normal AC and floury grains. The differences in the phenotypes associated with mutations in this gene may be influenced by genetic background and cultivation practice. For example, several dull genes transferred to *indica* IR36 by two generations of backcrossing conferred higher AC in dull mutants in an *indica* than a *japonica* background [16]. In addition, allelic variation in *Wx* and other starch synthesis-related genes results in different grain appearance and physicochemical properties [10,36–38]. Thus, we examined the uncovered possible roles of FLO2 and assessed allelic effects of *flo2* on the expression of starch synthesis-related genes to help elucidate their effects on grain appearance, starch synthesis and physicochemical properties of cooked rice grains. The discoveries in this study provide useful information for breeding of diverse rice grain qualities for multiple-usage end products.

2. Materials and methods

2.1. Plant materials

Opaque endosperm mutants were screened from *Oryza sativa* ssp. *japonica* cv. Taikeng 8 (TK8) induced by treatment with 0.1 M ethylmethane sulphonate (EMS) for 5 hr at 25 °C in 2001 [39]. Three seeds were randomly collected from 2000 M1 individuals and self-crossed to the M2 generation; the selection procedure was repeated for each generation until M6. Mutant lines of the M6 generation were screened for normal fertility and agronomic traits other than the presence of an opaque endosperm. The mutant lines exhibiting an opaque endosperm underwent high-throughput screening for low AC by staining with 0.02% iodine (I_2) and 0.2% potassium iodide (KI), and then the AC was consequently estimated following the protocol as previously described [40]. One line, CNY8-1, exhibited dull grains with 11.9% AC, and transverse-cut grains with little powdery flour were selected for grain quality, positional cloning undertaken to isolate the mutated gene, and expression of starch synthesis-related genes analyzed. The other eight mutants with low AC, varying from 10.1% to 11.9%, were used to determine allelic variation of the same locus conferring the low AC of CNY8-1. All seed stocks of mutant lines were maintained in Chiayi Agricultural Experiment Station (CAES), Taiwan Agriculture Research Institute, Chiayi, Taiwan. For genetic analysis and positional cloning, CNY8-1 was crossed to *O. sativa* ssp. *indica* cv. Taichung Sen 17 (TCS17) to generate an F_2 segregating population in August 2007. A total of 22 F_1 plants were grown in the paddy field of CAES in February 2008, and 4391 selfed seeds were harvested in June 2008.

2.2. Physicochemical properties of rice grains

Air-dried rice grains were ground into fine powdery flour to pass through a 60-mesh sieve. The paste viscosity profiles for polished grains were tested by a rapid visco analyzer (RVA, Model series 4, Newport Scientific, Australia), according to the AACC61-02 standard method released by the American Association of Cereal Chemists [41] and the detailed protocol was described by Hsu et al. [42]. RVA profiles were obtained by use of Thermocline for Windows 2.4 (Newport Scientific, Australia) and characterized for three parameters, peak viscosity (PKV), hot paste viscosity (HPV), and cool paste viscosity (CPV). Three derived parameters were determined as breakdown viscosity (BDV) = PKV-HPV, setback viscosity (SBV) = CPV-PKV, and consistency viscosity (CSV) = CPV-HPV.

2.3. Scanning electron microscopy (SEM) of starch granules

Rice grains of TK8, CNY8-1, and TCS17 were dried to 14% moisture content and transversely cut. The grain surface was coated with gold by use of the Hitachi Ion Sputter Coater E-1010 at 2.4 Kv, and structures and arrangements of starch granules were examined by use of SEM (Quanta Scanning Electron Microscope, FEI-Quanta 200) at 400× and 3000× magnification.