Resistant starch formation in rice: Genetic regulation and beyond

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ABSTRACT

Resistant starch (RS), a healthy dietary fiber, is a particular type of starch that has attracted much research attention in recent years. RS has important roles in reducing glycemic index, postprandial blood glucose levels, and serum cholesterol levels, thereby improving and preventing many diseases, such as diabetes, obesity, and cardiovascular disease. The formation of RS is influenced by intrinsic properties of starch (e.g., starch granule structure, starch crystal structure, and amylose-to-amylopectin ratio) and non-starch components (e.g., proteins, lipids, and sugars), as well as storage and processing conditions. Recent studies have revealed that several starch-synthesis-related genes (SSRGs) are crucial for the formation of RS during seed development. Several transcription factors and mRNA splicing factors have been shown to affect the expression or splicing of SSRGs that regulate RS content, suggesting their potential roles in RS formation. This review focuses mainly on recent research progress on the genetic regulation of RS content and discusses the emerging genetic and molecular mechanisms of RS formation in rice.

Keywords: rice, resistant starch, resistant starch formation, genetic regulation, starch-synthesis-related genes

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INTRODUCTION

Resistant starch (RS) refers to the portion of starch that cannot be digested and absorbed in the small intestine of healthy human beings (Englyst et al., 1982; Asp and Björck, 1992). RS is also defined as a dietary fiber that is beneficial to health because it cannot be digested in the gastrointestinal tract (Haralampu, 2000; De Vries, 2003). However, RS can be fermented in the colon by the gut microbiota, thereby producing a mass of short-chain fatty acids (SCFAs) (e.g., butyrate, acetate, and propionate) that are beneficial to intestinal health (Nugent, 2005; Hu et al., 2016). RS with unique functional properties has a number of significant biological effects, such as improvement of fermentable properties and bacterial activities in the colon, reduction of colon cancer risk, prevention of gall stone formation, promotion of mineral absorption, and improvement of gut health (Govers et al., 1999; Brown, 2004; Bauer-Marinovic et al., 2006; Sajilata et al., 2006; Birt et al., 2013; Keenan et al., 2015). Thus, RS is gradually being recognized as a functional food that is beneficial for various conditions, such as inflammatory bowel disease, insulin resistance and type 2 diabetes, glycemic index, energy and weight management, cholesterol levels, and coronary heart disease (Higgins et al., 1996, 2011; Jacobasch et al., 1999; Martinez-Flores et al., 2004; Park et al., 2004; Han et al., 2005; Morita et al., 2005; Behall et al., 2006; Regina et al., 2006; Zhang et al., 2007). Increasing daily intake of RS is becoming an important target for improving public health. Therefore, RS, an emerging healthy food, has received continuous research attention in recent years.

Starch, a major storage carbohydrate, is a polysaccharide that is composed of single sugar units linked together with α-1,4- and α-1,6-glycosidic bonds (Hizukuri et al., 1989). Based on its in vitro digestibility rate, starch is classified into three categories: rapidly digestible starch (RDS), slowly digestible starch (SDS), and RS (Englyst and Hudson, 1996). RDS refers to the portion of starch that can be completely digested and absorbed in the small intestine; it is rapidly hydrolyzed and converted to single glucose molecules within 20 min by enzymatic digestion (Englyst...
et al., 1992). SDS is a fraction of starch that requires more time for digestion but can be completely digested after 120 min of enzymatic digestion (Englyst et al., 1992). RS is defined as the starch fraction that cannot be digested by enzymes within 120 min (Englyst et al., 1992).

Based on its origin and resistance to enzymatic hydrolysis, RS is further classified into five subtypes: RS1, RS2, RS3, RS4, and RS5 (Figure 1) (Brown, 1996; Englyst et al., 1992; Guo et al., 2021; Raigond et al., 2015). RS1 is physically inaccessible to digestion, mainly because it is enclosed by cell walls or other tissues that prevent contact and reaction with amylase (Figure 1A) (Oladele, 2016). RS1 is unstable, and fine milling or deep processing will destroy its structure; it is generally found in partly milled grains, seeds, or tubers. RS2 refers to native starch granules with a compact structure that are relatively dehydrated (Figure 1B) (Sajilata et al., 2006). RS2 is tightly packed in a radial pattern in raw starch granules, restricting the accessibility of digestive enzymes and the majority of amylases. RS2 exists in raw potatoes, peas, and green bananas. RS3 is composed of retrograded starches that are principally recrystallized amylase produced after cooking and cooling of starchy foods (Figure 1C). RS3 belongs to the physically modified starches that are primarily formed via the gelatinization and retrogradation process during food processing and manufacturing (Haralampu, 2000). RS4 generally refers to chemically modified starch with altered molecular structure that contains new chemical bonds rather than \( \alpha-1,4 \)- or \( \alpha-1,6 \)-glycosidic bonds and thus exhibits increased resistance to amylolytic enzymes (Figure 1D) (Raigond et al., 2015). RS5 is a complex of amylose and lipids that forms a helical structure, preventing amylase digestion (Figure 1E) (Hasjim et al., 2010). The lipids bind to the amylose in the starch granule, preventing its expansion and producing resistance to enzymatic hydrolysis. Such amylose–lipid complexes are usually generated from high-amylose starch cereals.

Different crops contain distinct RS contents. For example, RS is most abundant in beans, ranging from 32% to 36%, compared with only 0.1% to 3.2% in cereal crops (Ragaei et al., 2006; Ambigaipalan et al., 2011). In recent years, several genes involved in starch biosynthesis have been reported to influence RS content in rice and other crops (Butardo et al., 2012; Matsumoto et al., 2012; Yang et al., 2012; Zhou et al., 2016; Guo et al., 2020). Breeding elite varieties with high RS content has become a new goal, owing to the significant biological effects of RS on human health. Several excellent reviews have provided very detailed information about the structure, classification, physiological effects, applications, and
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geneic improvement of RS (Nugent, 2005; Sajilata et al., 2006; Raigond et al., 2015; Ma and Boye, 2017; Xia et al., 2018; Bello-Perez et al., 2020; Jiang et al., 2020; Jukanti et al., 2020; Tian and Sun, 2020; Chang et al., 2021). In this review, we focus mainly on recent research progress on the genetic regulation of RS content in rice, including direct and potential genetic factors that influence RS formation, and we discuss how current knowledge may be used to improve RS content in crops.

FORMATION OF RESISTANT STARCH

Because RS is a special kind of starch that cannot be digested in the small intestine, RS formation is closely associated with starch biosynthesis. Based on the types of RS, the formation of RS is influenced by the intrinsic properties of starch (e.g., starch granule structure, starch crystal structure, and amylose-to-amylopectin ratio) and non-starch components (e.g., proteins, lipids, and sugars), as well as by storage and processing conditions (e.g., temperature, milling, and baking) (Sajilata et al., 2006; Raigond et al., 2015; Tian and Sun, 2020).

Intrinsic properties of starch influence RS formation

Structure of the starch granule influences RS formation

The size and structure of starch granules significantly affect the content of RS. Several studies have reported that the size of starch granules was negatively correlated with starch digestibility (Singh et al., 2010; Asare et al., 2011). Starch granules in a high-RS rice variety (TRS) were large, voluminous, and non-angular rounded bodies compared with those in a normal rice variety (TQ) (Wei et al., 2010a), indicating that the size of starch granules is related to RS content in rice. In general, starch granules with a large size and lower surface-to-volume ratio are more difficult to digest. The size of starch granules varies dramatically among different crops and organs. For instance, the diameter of starch granules in rice grains is generally less than 5 μm, whereas the diameter of starch granules in potato is more than 75 μm (Chakraborty et al., 2020). Therefore, potato starch is more resistant to digestion than most cereal starches, such as rice and wheat starches (Holm et al., 1988; Ring et al., 1988).

Crystallinity of starch influences RS formation

Based on X-ray diffraction characteristics, starch crystal structure can be divided into four types: A-, B-, C-, and V-type (Imberty et al., 1991; Buleon et al., 1998; Cheetham and Tao, 1998). The A-type starch crystal is usually present in cereal crops. Amylopectin with short lateral chains and closed branching points mainly determines the structure of A-type starch crystals (Buleon et al., 1998). The B-type starch crystal generally exists in raw potato and banana. Amylopectin with long side chains and distant branching points predominantly determines the structure of B-type starch crystals (Imberty et al., 1991). The C-type starch crystal is a mixture of A- and B-type and exists in smooth-seeded peas and beans (Bogracheva et al., 1998). It is generally accepted that the B-type starch crystal is more resistant to digestion than the A- and C-type crystals, and the C-type starch crystal shows more resistance to digestion than the A-type crystal (Ma and Boye, 2017). Consistent with this feature, raw potatoes with a high proportion of the B-type crystalline form have a higher RS content than cereal crops (Lunn and Buttriss, 2007). Starch granules in the high-RS rice line Goami 2 contain B-type crystals (Kang et al., 2003). It should be pointed out that chemical and physical treatments (such as cooking with high temperature) will eliminate the crystalline structure of this type of starch and lead to a reduction in RS content (Englyst and Cummings, 1986; Björck et al., 1989; Schweizer et al., 1990). Therefore, the crystallinity of starch influences its digestibility.

Amylose-to-amylopectin ratio influences RS formation

Starch consists of amylose and amylopectin. Amylose is a linear polymer of glucose residues mainly linked by α-1,4-glycosidic bonds. In general, amylose accounts for 0%–30% of the total starch in cereals. However, amylopectin is a highly branched glucose polymer linked by α-1,4-glycosidic bonds in linear chains and α-1,6-glycosidic bonds at branch points. Starch with a higher percentage composition of amylose has low digestibility, suggesting a positive correlation between amylose content (AC) and RS content (Berry, 1986; Sievert and Pomeranz, 1989). Consistent with this notion, several high-amylose rice mutants show high RS contents (Mizuno et al., 1993; Nishi et al., 2001; Itoh et al., 2003; Zhou et al., 2016; You et al., 2022). High amylose is also associated with high RS content in other cereals, such as wheat, maize, and barley (Granfeldt et al., 1995; Regina et al., 2006; Carciofi et al., 2012). It is generally accepted that a high amylose-to-amylopectin ratio leads to a high RS content, owing to the specific structure of amylose (Ahuja et al., 2013; Xia et al., 2018; Tian and Sun, 2020; Chang et al., 2021). The access of small intestine β-amylases to the two terminal glucose units of the amylose chain is limited because of the straight chains of amylose. By contrast, the highly branched structure of amylopectin provides numerous terminal glucose units that are conducive to access by β-amylases (Ahuja et al., 2013). Therefore, the amylose-to-amylopectin ratio is a major factor that affects the formation of RS2 and RS3 (Sajilata et al., 2006). Furthermore, the retrogradation of amylose also contributes to the formation of RS3 (Berry, 1986; Björck et al., 1990). In most cereals, retrograded amylose was found to be highly resistant to amylases owing to the formation of RS3 (Haralampu, 2000). Notably, varieties with similar AC can nonetheless differ in starch digestibility and RS content, suggesting that AC is an important factor—but not the only factor—in RS formation (Frei et al., 2003; Leeman et al., 2006).

Chain length of amylopectin influences RS formation

Chain length is another factor that affects the formation of RS. It is generally recognized that the medium and long chains of amylopectin are positively correlated with RS content (Ramadoss et al., 2019). This is mainly because long chains form much more stable helices in the starch crystal structure, thereby reducing digestibility (Lehmann and Robin, 2007). Consistent with this scenario, the percentage of medium and long amylopectin chains was increased in the high-RS rice mutants RSML 184 and RSML 278, whereas the content of short-chain amylopectin was reduced (Ramadoss et al., 2019). The opposite result was observed in the low-RS mutant RSML 352 (Ramadoss et al., 2019). A study showed that amylopectin retrogradation was affected by both external chain length and inter-block chain length (Vamadevan and Bertoft, 2018). Similarly, an increase in amylopectin with medium-length chains enhances retrogradation in maize, thereby increasing the RS content (Wu et al., 2017). These
results suggest that medium- and long-chain amylopectin contributes to the formation of RS.

**External factors influence RS formation**

*Non-starch components influence RS formation*

Interactions of starch with different food components, such as proteins, lipids, dietary fibers, ions, and sugars, have been known to affect the formation of RS. Proteins can pack tightly around the surface of starch granules and prevent their accessibility to digestive enzymes, thereby resulting in increased RS1 content (Escarpa et al., 1997). By contrast, proteins can form hydrogen bonds with amylose during the retrogradation of starch, thereby influencing starch retrogradation and decreasing the yield of RS3 (Escarpa et al., 1997).

Lipids also significantly affect the formation of RS, as they can attach to the surfaces of starch granules and form an amylose–lipid complex, resulting in increased resistance to digestion (Hasjim et al., 2010). Consistent with this scenario, removal of lipids from rice flour can increase the digestibility of starch (Ye et al., 2018). A recent study showed that lipid content is positively associated with total RS content and negatively related to digestibility and glucose index (GI) in rice (Shen et al., 2021). In high-lipid mutants, the extent of digestion was significantly enhanced after non-starch-lipid removal, suggesting a decrease in RS content. However, this phenomenon was not observed in a low-lipid rice variety (Shen et al., 2021). In addition, during the retrogradation of starch, free lipids may competitively bind amylose to form amylose–lipid complexes, causing reductions in recrystallizable AC and RS3 yield (Eliasson et al., 1988; Eerlingen et al., 1994; Escarpa et al., 1997). Similarly, the presence of lipids on the surface or inside the starch granules may inhibit the gelatinization and retrogradation of starch in wheat, thereby decreasing the formation of RS3 (Wang et al., 2016). Although lipids can decrease the formation of RS3 during the retrogradation of starch, they can increase RS5 content due to the formation of amylose–lipid complexes.

Furthermore, calcium and potassium ions can also reduce the content of RS because these ions may prevent the formation of hydrogen bonds between amylose and amylopectin (Escarpa et al., 1997). Interaction between sugars and starch chains can increase the gelatinization temperature (GT) of starch, affecting the recrystallization of amylose and reducing the content of RS (Ianson et al., 1990; Kohyama and Nishinari, 1991). In addition, dietary fibers (e.g., polyphenols, phytic acid, tannic acid, and lectins) have been reported to affect the formation of RS (Thompson and Yoon, 1984; Björck and Nyman, 1987; Escarpa et al., 1997).

**Storage and processing conditions influence RS formation**

The content of RS is significantly affected by storage conditions such as temperature and time. In general, low-temperature storage is conducive to the formation of RS. Low-temperature storage has been reported to increase RS content in various cereals such as rice and wheat (Johansson et al., 1984; Perdon et al., 1999; Dhitai et al., 2010; Yu et al., 2010). Moreover, the longer time that fully gelatinized starch paste is stored at 4°C, the more RS is produced (Chung et al., 2006), mainly owing to increased amylose retrogradation, which results in increased RS3.

Various treatments during food processing, such as milling, baking, extrusion cooking, frying, and autoclaving, affect the RS content of food (Siljestrom and Asp, 1985; Björck and Nyman, 1987; Siljestrom et al., 1989; Muir and O’Dea, 1992; Rabe and Sievert, 1992). Processing techniques affect the formation of RS by influencing the retrogradation processes of starch. During food processing, water and temperature are crucial factors that affect the formation of RS. The recrystallization of starch takes place within a certain range of moisture content (20%–90%), and the degree of recrystallization reaches a peak at 50% moisture content (Longton and Legrys, 1981). Cooking with high moisture and temperature conditions can substantially reduce RS contents by disrupting the crystalline structure. By contrast, extrusion and cooling can increase the yield of RS owing to the induction of amylose recrystallization (Haralampu, 2000). For example, the RS content was higher in parboiled rice than in raw rice because of the recrystallization of amylose (Marsono and Topping, 1993). In addition, stewing and baking affect the formation of RS. Compared with ordinary baking conditions, bread baked for a long time at a low temperature contains more RS (Lijieberg et al., 1996).

**GENETIC REGULATION OF RS CONTENT**

RS content is genetically controlled by a number of genes that are mainly involved in starch synthesis (Figure 2). Mutations in the genes encoding granule-bound starch synthase (GBSS), soluble starch synthase (SS), and starch-branching enzyme (SBE) have been reported to influence RS contents in rice, maize, and wheat (Zhou et al., 2016; Baysal et al., 2020; Li et al., 2021; Liu et al., 2021). These genetic factors that influence RS formation in rice are listed in Table 1. In addition, several transcription factors, mRNA splicing factors, and epigenome modifiers regulate the expression of starch-synthesis-related genes (SSRGs), potentially influencing RS content.

**Genetic factors that determine RS content**

**Wx alleles**

GBSS is the key enzyme for amylose biosynthesis and catalyzes the conversion of individual ADP-glucose molecules into linear chains of glucose residues through α,1,4-glycosidic bonds (Tetlow, 2011). GBSSI, encoded by the Waxy (Wx) gene, is a key enzyme for amylose biosynthesis in the endosperm (Shure et al., 1983; Wang et al., 1990). The Wx gene has been reported to regulate RS contents in rice (Bao et al., 2017; Biselli et al., 2019; You et al., 2022). Several studies revealed that starch with a higher percentage of amylose has lower digestibility, indicating a positive correlation between AC and RS content in rice (Berry, 1986; Sievert and Pomeranz, 1989; Chen et al., 2017b). Consistent with this notion, the RS content of rice with the Wxδ allele that produced a high AC was significantly higher than that of rice with the wx allele (ito et al., 2003; You et al., 2022). Moreover, a high amylose level is also associated with a high RS content in other cereals, such as wheat and maize (Li et al., 2008, 2020; Guzman and Alvarez, 2016; Lin et al., 2016). In rice, the broad diversity of AC is due mainly to allelic variation at the Wx locus (Tian et al., 2009; Zhang et al., 2019). In cultivated rice, there are two major functional Wx alleles, Wxδ and Wxδ, which are primarily found in indica and japonica cultivars, respectively (Sano, 1984; Sano et al., 1986; Hirano
Compared with the Wxa allele, the Wxb allele contains a G-to-T single base mutation at the 5' splice site of the first intron, which results in lower mRNA and protein levels of Wx (Sano et al., 1986; Cai et al., 1998; Isshiki et al., 1998). In general, rice cultivars with the Wxa allele produce a large amount of amylose, whereas rice cultivars with the Wxb allele generate a small amount of amylose (Sano et al., 1986; Hirano and Sano, 1998; Isshiki et al., 1998; Larkin and Park, 2003).

Figure 2. Regulatory network of RS formation in the rice endosperm.
RS formation is a complicated process that incorporates a number of complex and highly regulated reactions. AGPase is a key rate-limiting enzyme that catalyzes the first step of starch biosynthesis, the production of adenosine diphosphoglucose (ADP-Glc) from glucose-1-phosphate (Glc-1-P). ADP-Glc, mainly synthesized in the cytosol and transported to the amyloplast by Brittle-1 (BT1), is the basic substrate for amylose and amylopectin synthesis. In the amyloplast, GBSSI interacts with GBSS-binding protein (OsGBP), which facilitates the localization of GBSSI to the surface of starch granules and promotes the synthesis of amylose. During amylopectin synthesis, SSⅠ, SSⅡ, and SSⅢ are commonly responsible for the elongation of α-1,4-glycosidic bonds in amylopectin, whereas SSⅣ is responsible for the initiation of starch granules. SBEs catalyze the production of α-1,6-glycosidic bonds and form branches. Isoamylase (ISA) and pullulanase (PUL) hydrolyze α-1,6-glycosidic bonds and remove incorrect branches to guarantee the orderly synthesis of amylopectin. SSREs (underlined) are direct regulators that are involved in RS formation. Starch granules enclosed by cell walls or other tissues are known as RS1. B-type starch granules with compact crystalline structure are known as RS2. Amyloses and lipids form amylose–lipid complexes (RS5). At the transcriptional level, several transcription factors that promote or suppress the expression of direct regulators (GBSSI, SSs, and SBEs) of RS formation are potential regulators. At the posttranscriptional level, Du genes (Du1, Du2, and Du3) have been reported to regulate amylose synthesis by specifically affecting the efficient splicing of the WxΔ pre-mRNA. At the posttranslational level, phosphorylation regulates starch synthesis by affecting the formation of multi-enzyme complexes. Blue lines represent RS formation and starch biosynthetic processes. Gray lines represent potential regulations of RS formation.
Consistent with this pattern, indica cultivars with the Wx\(^a\) allele have higher RS than japonica cultivars with the Wx\(^b\) allele (Bao et al., 2017). The increased RS in the Wx\(^a\) background is mainly due to high AC that promotes the formation of RS3 and RS5. Moreover, several other natural alleles of Wx and genome-edited Wx mutants have been shown to influence the AC of rice (Liu et al., 2009; Ma et al., 2015; Zhang et al., 2019, 2021; Huang et al., 2020; Zeng et al., 2020; Zhou et al., 2021), but their effects on RS content have not been reported. Considering that AC is strongly related to RS content, it is possible that these natural Wx alleles and genome-edited mutants may also affect RS content in rice. It will be interesting to investigate the effects of these Wx natural alleles/mutants on RS content in the future.

### SS family genes

SSs, which are mainly responsible for the extension of amylopectin chains, include four members (SSI, SSII, SSIII, and SSIV) with different substrate specificities in rice (Ohdan et al., 2005). SSI, SSII, and SSIII are commonly responsible for the elongation of α-1,4-glycoside bonds in amylopectin, whereas SSIV is required for the initiation of starch granules (Hirose and Terao, 2004; Dian et al., 2005; Ohdan et al., 2005; Jeon et al., 2009). SSs, which are mainly responsible for the extension of amylopectin chains, include four members (SSI, SSII, SSIII, and SSIV) with different substrate specificities in rice (Ohdan et al., 2005). SSI, SSII, and SSIII are commonly responsible for the elongation of α-1,4-glycoside bonds in amylopectin, whereas SSIV is required for the initiation of starch granules (Hirose and Terao, 2004; Dian et al., 2005; Ohdan et al., 2005; Jeon et al., 2009).

| Gene | Mutant/Allele | Accession number | RS content | Key references |
|------|--------------|-----------------|------------|----------------|
| GBSSI | Wx\(^a\) | LOC_Os06g04200 | Increased | Cai et al. (1998); Isshiki et al. (1998); Itoh et al. (2003); You et al. (2022) |
| SSI | Os-076 | LOC_Os06g06560 | Increased | Raja et al. (2017) |
| | Os-631 | LOC_Os06g06560 | Increased | |
| | Os-678 | LOC_Os06g06560 | Increased | |
| SSIla | SSIla\(^i\) | LOC_Os06g12450 | Increased | You et al. (2022) |
| | b10 | LOC_Os08g09230 | Increased | Zhou et al. (2016) |
| | ss3a-1/e1 | LOC_Os08g09230 | Increased | Fujita et al. (2007) |
| | ss3a-2 | LOC_Os08g09230 | Increased | |
| SBEIIb | sbe2b | LOC_Os02g32660 | Increased | Butardo et al. (2011) |
| | sbell | LOC_Os02g32660 | Increased | Guo et al. (2020) |
| | E15 | LOC_Os02g32660 | Increased | Baysal et al. (2020) |
| | be2b/ae | LOC_Os02g32660 | Increased | Nishi et al. (2001); Asai et al. (2014); Miura et al. (2021) |
| | sbell | LOC_Os02g32660 | Increased | Sun et al. (2017) |
| | EM10 | LOC_Os02g32660 | Increased | Nishi et al. (2001); Asai et al. (2014) |
| | EM129 | LOC_Os02g32660 | Increased | Mizuno et al. (1993); Wada et al. (2018) |
| | sbe3-rs | LOC_Os02g32660 | Increased | Yang et al. (2012); Yang et al. (2006) |
| GBSSI SBEIIb | wx ae | LOC_Os06g04200/Loc_Os02g32660 | Increased | Matsumoto et al. (2012) |
| SSI SSIla | ss1\(^i\) ss3a | LOC_Os06g06560/Loc_Os08g09230 | Increased | Hayashi et al. (2015) |
| SSI SBEIIb | ss1\(^i\) be2b | LOC_Os06g06560/Loc_Os02g32660 | Increased | Abe et al. (2014) |
| SSIla SSIla | γ278 | LOC_Os06g12450/Loc_Os08g09230 | Increased | Guranathan et al. (2019) |
| SSIla SSIVb | ss3a ss4b | LOC_Os08g09230/Loc_Os05g45720 | Increased | Toyosawa et al. (2016) |
| SSIla SBEI | ss3a be1 | LOC_Os08g09230/Loc_Os06g51084 | Increased | Tsuiki et al. (2016) |
| SSIla SBEIIb | ss3a be2b | LOC_Os08g09230/Loc_Os02g32660 | Increased | Asai et al. (2014) |
| SBEI SBEIIb | be1 be2b | LOC_Os06g51084/Loc_Os02g32660 | Increased | Miura et al. (2021) |
| | TRS | | | Wei et al. (2010a, 2010b); Zhu et al. (2012) |
| Unknown | Goami 2/Goamy 2/ Suweon 464 | | Increased | Butardo et al. (2012); Kang et al. (2003); Kim et al. (2004); Kim et al. (2005a, 2005b); Lee et al. (2006); Nakaya et al. (2013) |
| | RSML 184 | | Increased | Ramadoss et al. (2019) |
| | RSML 278 | | Increased | Ramadoss et al. (2019) |
| | RSML 352 | | Decreased | Ramadoss et al. (2019) |
| | RS111 | | Increased | Shu et al. (2006); Yang et al. (2006) |
| | RS4 | |Increased | Shu et al. (2009) |

Table 1. Direct regulators of RS formation in the rice endosperm.
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SSIIa has been reported to affect RS formation in rice. SSIIa, which is encoded by the alkali degeneration (ALK) gene, is the major enzyme that controls GT in rice (Gao et al., 2003). Based on three key SNPs in exon 8 of SSIIa, three ALK alleles have been identified in rice cultivars. The ALKα and ALKβ alleles (named SSIIaα and SSIIaβ) are found mainly in japonica cultivars and encode the SSIIa enzyme with about 10% activity, whereas the ALKγ allele (named SSIIaγ) is found mainly in indica cultivars and encodes the active SSIIa enzyme (Nakamura et al., 2005; Waters et al., 2006; Gao et al., 2011; Chen et al., 2020). Recently, the novel ALK allele ALKδ was identified, which can increase GT and improve retrogradation properties (Zhang et al., 2020). A recent study showed that the SSIIaα allele produces higher RS content in indica cultivars than does the SSIIaβ allele in japonica cultivars (You et al., 2022). Furthermore, the functional SSIIa produces high-crystallinity starch that is more resistant to enzymatic digestion, leading to a higher RS content (Bao et al., 2017). In rice, SSIIα is responsible for elongating short A and B1 chains of amylopectin (6 ≤ degree of polymerization [DP] ≤ 12) to long-type B1 chains (13 ≤ DP ≤ 24) (Nakamura et al., 2003), resulting in a higher accumulation of B1 chains in indica rice than in japonica rice. These results suggest that SSIIα regulates the formation of RS, possibly by increasing the number of medium amylopectin chains. Consistent with this notion, downregulation of SSIIα in rice significantly increases the short amylopectin chains (6 ≤ DP ≤ 10) and reduces the medium amylopectin chains (12 ≤ DP ≤ 24) (Zhang et al., 2011; Butardo et al., 2020). Moreover, the SSIIα-deficient mutant EM204 (ss2a) produced higher AC and shorter branch chains of amylopectin (DP ≤ 12) and lower GT than the wild type (Miura et al., 2018). The SSII-3 single mutant ss3, which was generated using CRISPR-Cas9 technology, also showed a significant increase in short amylopectin chains (5 ≤ DP ≤ 12) but a significant decrease in mid-length amylopectin chains (13 ≤ DP ≤ 24) (Huang et al., 2021a). In wheat and barley, the absence of SSIIα caused a significant increase in RS content, which was accompanied by significant increases in the AC and short branch chains of amylopectin (6 ≤ DP ≤ 10) and a decrease in the mid-length chains of amylopectin (11 ≤ DP ≤ 24) (Morell et al., 2003; Bird et al., 2004; Shimbata et al., 2012; Schoen et al., 2021; Yang et al., 2022). These results reveal that SSIIα can influence RS formation by changing the AC and the distribution of amylopectin chain length in rice and wheat.

SSIII consists of SSIIIa and SSIIIb, which are important for the formation of RS in rice. SSIIIa is mainly expressed in developing rice endosperm, whereas SSIIIb is primarily expressed in leaves (Ohdan et al., 2005). The b10 mutant, which was isolated from the indica variety R7954, contains a G-to-A mutation at the 3’ splice site of the fifth intron in SSIIIa, which leads to a 4-base pair (bp) deletion in the coding sequence and introduces a premature stop codon (Zhou et al., 2016). The b10 mutant had significantly higher RS content compared with its parental line, R7954. Genetic analyses showed that the effect of the b10 mutant on RS content depends in part on the functional Wx gene (Wxα). Consistent with this finding, RS contents were only slightly higher in two transfer DNA (T-DNA) insertion mutants of SSIIIa in the japonica background (Wxδ) (Ryoo et al., 2007; Zhou et al., 2016). During starch biosynthesis, a large complex is formed from a series of enzymes, including ADP-glucose pyrophosphorylase (AGPase), pyruvate orthophosphate dikinase (PPDK), SSIIα, SSIIβ, and SBEIIb (Hennen-Biervliet et al., 2009; Crofts et al., 2015). Loss of function of SSIIα disrupts this protein complex, which promotes the biosynthesis of lipids, and deficiency in SSIIα decreases amylopectin synthesis and promotes amyllose synthesis. Consequently, the increase in amyllose and lipids in b10 results in an increase in the amyllose–lipid complex RS5S, thereby increasing the RS content (Zhou et al., 2016). Similarly, several other ss3a mutants have been reported to exhibit high RS content (Fujita et al., 2007; Ryoo et al., 2007; Tsuiki et al., 2016). Interestingly, rice ss2a ss3a double mutants have a higher RS content than the wild type and the ss2a and ss3a single mutants (Gurunath et al., 2019), suggesting that SSIIα and SSIIIa may function redundantly to control RS content. In wheat and maize, mutations in SSII or SSIII dramatically increase the AC and change the distribution of amylopectin chain lengths, suggesting that they may also influence the content of RS (Zhang et al., 2004; Shimbata et al., 2012; Zhu et al., 2013).

SBE family genes

SBEs consist of three functional members, SBEI, SBEIIa, and SBEIIb, which catalyze the formation of α-1,6-glycosidic bonds and form branches in amylopectin (Han et al., 2007; Tian et al., 2009). SBEI and SBEIIb are specifically expressed in the developing endosperm, and SBEIIa shows a constitutive expression pattern in different rice tissues (Ohdan et al., 2005). Previous studies showed that SBEI is required mainly for the formation of medium and long amylopectin chains, whereas SBEIIb plays an important role in forming short amylopectin chains (Stitt and Zeeman, 2012; Sawada et al., 2018). Remarkably, SBEIIa is predominantly responsible for the formation of intermediate chains and partially compensates for the function of SBEI rather than the function of SBEIIb (Sawada et al., 2018). In rice, SBEIIb is a key gene that controls the formation of RS, but the roles of SBEI and SBEIIa in RS formation have not been reported. Several studies have shown that a deficiency in SBEIIb activity can significantly increase the RS content in the rice endosperm (Butardo et al., 2011; Baysal et al., 2020; Guo et al., 2020; Miura et al., 2021). In rice and maize, amylose extender (ae) mutants contain mutations in SBEIIb. In the japonica background, the ae mutant (EM10) showed a significant increase in RS content in raw rice flour, accompanied by a 2-fold increase in amyllose and a marked increase in GT (Mizuno et al., 1993; Nishi et al., 2001; Asai et al., 2014; Miura et al., 2021). Similarly, another ae mutant (EM129) that exhibited
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similar starch properties to EM10 has been used to breed a rice cultivar, Chikushi-kona 85, with an RS content as high as 17.4% (Mizuno et al., 1993; Wada et al., 2018). The sbe3-rs mutant with a single amino acid mutation in SBEIIb is a mutant of SBEIIb, which contributes to high RS (11.6%) in Jiangtangdao 1 (Yang et al., 2006, 2012). In sbeIIb mutants generated by CRISPR-Cas9 in the japonica cultivar Kitaake, the RS content was dramatically enhanced because of the increase in AC and the high proportion of long de-branched amylopectin chains (Sun et al., 2017). Recently, the high-RS/low-glutelin rice germplasm sbeIIb/Lgc1 was generated by CRISPR-Cas9-induced site-specific mutations of SBEIIb in an elite low-glutelin japonica rice cultivar; it had a high RS content (6.36%) and a 1.8-fold increase in AC (Guo et al., 2020). In addition, downregulating the expression level of SBEIIb in rice endosperm by RNAi produced a high RS content (4.8%) in cooked rice, as well as an increase in long and intermediate amylopectin chains and AC (41.2%) (Butardo et al., 2011). Moreover, complete inhibition of SBEIIb activity produced increases of 0.2% to 17.2% in RS content and 19.6% to 27.4% in AC in the rice endosperm (Baysal et al., 2020). Interestingly, the significantly increased AC in the sbe2b mutant was not due to an increase in the relative proportion of amylose chains. Instead, it occurred because of an increase in long and intermediate de-branched amylopectin chains (Butardo et al., 2011). Furthermore, the increased proportion of long amylopectin chains led to the formation of B-type starch crystals in the sbe2b mutant endosperms. The absence of SBEIIb also caused significant increases in sugar, lipid, and protein contents. Thus, all these changes contribute to the high RS content in the sbe2b mutant. In both winter and spring wheat, knockout of the three copies of SBEIIb by CRISPR-Cas9 resulted in significant increases in RS content (from 1.2% to 8.7%) and AC (from 22.6% to 38.7%) (Li et al., 2021). These results highlight the conserved function of SBEII in the control of RS content in cereal crops.

SSRG coordinators

Several SSRGs, such as GBSSI, SSIla, SSIIda, and SBEIIb, are known to influence the formation of RS. Moreover, combinatorial effects of SSRGs on RS content have been reported in rice. AMF18 is a wx ae double mutant that was generated by a cross between the wx mutant EM21 and the ae/sbe2b mutant EM16 (Kubo et al., 2008). The RS content in the wx ae mutant was as high as 27.8%, higher than that in each single mutant and the wild type (Matsumoto et al., 2012; Nakayama et al., 2013). Although starches in the wx ae double mutant lacked amylose, they contained abundant long-unit chains of amylopectin that were conducive to the production of B-type starch crystals, which may contribute to high RS content in the double mutant (Kubo et al., 2010). This result strongly suggests that long amylopectin chains and starch structure also play important roles in promoting RS formation in rice.

The ss1 be2b double mutant generated by a cross between the leaky ss1 mutant and the ae mutant EM10 had approximately 35% RS content, much higher than that of the single mutants (Abe et al., 2014; Tsuiki et al., 2016). The ss3a be2b double mutant derived from a cross between the ss3a mutant e1 and the ae mutant EM10 exhibited high RS content (15%) (Asai et al., 2014; Tsuiki et al., 2016). Recently, a novel be1 be2b double mutant was generated by a cross between be1 (EM557) and be2b (EM10), resulting in a higher RS content (28.4%) than that of the be2b mutant (Nishi et al., 2001; Satoh et al., 2003; Miura et al., 2021). Similarly, simultaneous downregulation of both SBEI and SBEIIb expression led to a higher RS content (14.6%) compared with the wild type (Wei et al., 2010b; Zhu et al., 2012). In wheat, repressing the expression of both SBEIIa and SBEIIb caused a significant increase in RS content (16.6%) (Regina et al., 2015). Similar results have also been observed in durum wheat (Hazard et al., 2015). These studies reveal that combinations of different loss-of-function alleles of SSRGs can be used to generate ultra-high RS materials for RS breeding in rice and other crops.

Potential genetic factors controlling RS content

SSRGs that potentially influence RS content

In addition to the SSRGs such as GBSI, SSIla, and SBEIIb that directly influence the formation of RS, other SSRGs may potentially affect RS formation. Several studies revealed that downregulation of SSIIda leads to significant decreases in AC and mid-length amylopectin chains, as well as an increase in short amylopectin chains, resulting in large starch granule size in the rice endosperm (Huang et al., 2021a; Li et al., 2018b; Xu et al., 2020). Moreover, suppression of SSIIda expression in both Wx∂ and Wxδ backgrounds results in a decrease in AC and changes the distribution of amylopectin chain lengths, leading to better eating and cooking quality (Huang et al., 2021a). AC, amylopectin chain distribution, and starch granule size were markedly changed in the ss2b mutant, suggesting a potential role for SSIIda in RS formation. SSIIV is mainly responsible for the initiation of starch granules in plants. To date, the function of SSIIV in RS formation has not been reported. However, the RS content of the ss3a ss4b double mutant is higher than that of the wild type and ss3a, and the starch granules in the ss3a ss4b double mutant are small and spherical compared with those in the wild type and ss3a (Toyosawa et al., 2016; Tsuiki et al., 2016). These findings suggest the possibility that SSIIVb might influence the formation of RS.

Transcription factors that potentially influence RS content

Several transcription factors have been reported to promote or suppress the expression of SSRGs that directly influence RS formation, such as basic leucine zipper (bZIP), nuclear factor-Y (NF-Y), myelocytomatisos (MYC), and MADS-box proteins (Zhu et al., 2003; Bello et al., 2019; Wang et al., 2020a; Niu et al., 2020, 2021; Feng et al., 2022), suggesting possible roles for these transcription factors in RS formation. In rice, the bZIP family member OsbZIP33 can associate with the ACGT motif in the promoter regions of Wx and SBEI genes and promote their expression (Yang et al., 2001; Cai et al., 2002). Another bZIP transcription factor, OsbZIP58, that interacts with the DNA binding with one finger (DoF) family transcription factor RPBF (rice prolamin box binding factor) can promote the expression of OsAGPL3, Wx, SSIla, SBEI, SBEIIb, and IS2A by directly binding to their promoter regions. The osbzip58 mutant had low total starch, AC, and lipid contents and showed a significant decrease in short amylopectin chains and an increase in long chains (Kawakatsu et al., 2009; Wang et al., 2013; Xu et al., 2020a; Yamamoto et al., 2006).

The dehydration-response element-binding (DREB)-type transcription factor SALT-RESPONSIVE ERF1 (SERF1) represses the expression of both Wx and RPBF by binding to their promoter regions. The contents of total starch and proteinoigenic amino acids were clearly increased in the serf1 mutant (Schmidt et al., 2022).
is possible that DNA methylation levels of major plant growth and development. A previous study showed that epigenetic modification plays a crucial role in the regulation of epigenetic modifications that potentially influence RS content. Other genetic factors that potentially influence RS content

Starch has been proposed to interact with different components, such as proteins, lipids, dietary fibers, ions, and sugars, to affect the formation of RS. Basically, genes involved in protein, lipid, and sugar biosynthesis and metabolism could influence RS content. However, researchers have not measured the effects of these genes on RS content in rice. As the second component of the rice endosperm, protein content is negatively related to starch content. In general, an increase in protein content is accompanied by a decrease in starch content, suggesting that genes related to protein synthesis may potentially influence RS formation. GPA3 (gliadin precursor accumulation 3) encodes a regulator of post-Golgi vesicular trafficking that interacts with the Rab5a-GTPase exchange factor VPS9 to form a complex with Rab5a via VPS9. This complex regulates dense vesicle-mediated post-Golgi trafficking in rice. In the gpa3 mutant, AC was significantly reduced, whereas protein and lipid contents were increased (Ren et al., 2014). OsAAP10, an amino acid permease, is responsible for amino acid loading in the rice endosperm. The mutation of OsAAP10 led to decreases in both protein content and AC (Wang et al., 2020). A recent study showed that lipid content is positively related to RS content in rice (Shen et al., 2021), further supporting the possibility that genetic modification of lipid-related genes might influence RS content. In addition, several genes involved in carbon metabolism influence starch and lipid contents. For example, rice OsPPDKB gene contains two neighboring CpG islands that may contribute to its level of DNA methylation, and the low DNA methylation level in its promoter is closely associated with high AC in the rice endosperm (Anacleto et al., 2019). These results indicate that DNA methylation may potentially affect RS formation. Recently, DNA methylation of SSRGs was also discovered in the maize endosperm, and the coding regions of SSRGs with low expression were highly methylated (Hu et al., 2021). Thus, these epigenetic modifications that are involved in starch synthesis could provide a novel strategy for fine-tuning RS content in rice.

**Posttranslational modifications that potentially influence RS content**

Phosphorylation, an important posttranslational protein modification, can modify the catalytic activities of starch-synthesis-related enzymes (SSREs) in cereals, thus influencing starch synthesis in the endosperm (Chen et al., 2016; Pang et al., 2018). In rice, oligomerization of GBSSI/Wx is crucial for its enzymatic activity, promoting amylase synthesis in the endosperm. However, phosphorylation represses the oligomerization of GBSSI (Liu et al., 2013). Moreover, nine phosphorylated sites were identified in GBSSI, and the Ser415 site is a key phosphorylated site that is strongly associated with the enzyme activity of GBSSI. The substitution of Ser415 with Pro415 in Wx<sup>α</sup> results in high GBSSI activity compared with that of Wx<sup>α</sup>, leading to high AC in the rice endosperm (Zhang et al., 2019). This result suggests the possibility that phosphorylation might influence RS formation by changing the AC of the rice endosperm.

Another study showed that lipid content is positively related to RS content in rice. As the second component of the rice endosperm, protein content is negatively related to starch content. In general, an increase in protein content is accompanied by a decrease in starch content, suggesting that genes related to protein synthesis may potentially influence RS formation. GPA3 (gliadin precursor accumulation 3) encodes a regulator of post-Golgi vesicular trafficking that interacts with the Rab5a-GTPase exchange factor VPS9 to form a complex with Rab5a via VPS9. This complex regulates dense vesicle-mediated post-Golgi trafficking in rice. In the gpa3 mutant, AC was significantly reduced, whereas protein and lipid contents were increased (Ren et al., 2014). OsAAP10, an amino acid permease, is responsible for amino acid loading in the rice endosperm. The mutation of OsAAP10 led to decreases in both protein content and AC (Wang et al., 2020). A recent study showed that lipid content is positively related to RS content in rice (Shen et al., 2021), further supporting the possibility that genetic modification of lipid-related genes might influence RS content. In addition, several genes involved in carbon metabolism influence starch and lipid contents. For example, rice OsPPDKB gene contains two neighboring CpG islands that may contribute to its level of DNA methylation, and the low DNA methylation level in its promoter is closely associated with high AC in the rice endosperm (Anacleto et al., 2019). These results indicate that DNA methylation may potentially affect RS formation. Recently, DNA methylation of SSRGs was also discovered in the maize endosperm, and the coding regions of SSRGs with low expression were highly methylated (Hu et al., 2021). Thus, these epigenetic modifications that are involved in starch synthesis could provide a novel strategy for fine-tuning RS content in rice.

**Epigenetic modifications that potentially influence RS content**

Epigenetic modification plays a crucial role in the regulation of plant growth and development. A previous study showed that DNA methylation levels of major SSRGs in rice endosperm were frequently lower than those in other tissues, suggesting that DNA methylation is crucial for starch synthesis (Zemach et al., 2010). Consistent with this, the promoter region of the Wx gene contains two neighboring CpG islands that may contribute to its level of DNA methylation, and the low DNA methylation level in its promoter is closely associated with high AC in the rice endosperm (Anacleto et al., 2019). These results indicate that DNA methylation may potentially affect RS formation. Recently, DNA methylation of SSRGs was also discovered in the maize endosperm, and the coding regions of SSRGs with low expression were highly methylated (Hu et al., 2021). Thus, these epigenetic modifications that are involved in starch synthesis could provide a novel strategy for fine-tuning RS content in rice.
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encodes a PPK and regulates carbon metabolism and flow for starch and fat biosynthesis in the rice endosperm. Mutations in OsPPDKb caused significant decreases in both total starch and AC and an increase in lipid content (Kang et al., 2005; Zhang et al., 2018). To further understand the roles of genes involved in protein, lipid, and sugar biosynthesis, metabolism, and transport in RS formation, it will be important to measure RS content using mutants or transgenic lines in future research.

CHALLENGES AND PERSPECTIVES

In recent years, research on RS has attracted significant scientific attention because of its promising physiological benefits for human health. Recent studies have identified several key genes that encode enzymes involved in RS formation, but our knowledge of RS formation in rice and other crops remains limited. To date, the genetic and molecular mechanisms of RS formation have not been well dissected in plants. In addition to these well-characterized SSREs, several studies have shown that other factors potentially regulate the formation of RS, including transcription factors, mRNA splicing factors, and protein posttranslational modifications (Isshiki et al., 2000, 2008; Zeng et al., 2007; Zhang et al., 2019; Xu et al., 2020b).

However, the effect of these potential factors on RS content is still unclear. Therefore, a straightforward strategy is to test whether these potential genetic factors are really involved in RS formation by measuring the RS content of their loss-of-function mutants or overexpression lines. These studies will help us understand genetic and molecular mechanisms and the ways in which transcriptional, posttranscriptional, or posttranslational regulation of SSRGs determine RS formation. It is also necessary to identify novel genetic factors that determine RS content in rice and other crops using genetic screens, genome-wide association studies (GWAS) (Bao et al., 2017; Parween et al., 2020), and quantitative trait loci (QTL) mapping. Meanwhile, it is important to explore modern high-throughput methods, such as transcriptomics, proteomics, and metabolomics, to elucidate the genetic and molecular mechanisms by which these genes influence RS formation. The future challenge will be to construct the genetic and molecular regulatory network that underlies RS formation in crops.

Breeding novel rice varieties with high RS content has become a focus of breeders. Rice breeders are utilizing the SSRGs, such as SBEIIb, to cultivate new varieties with high RS content (Yang et al., 2012; Wada et al., 2018). Several studies revealed that RS content was significantly increased by combining different loss-of-function alleles of the SSRGs, as in the double mutants wx ae, ss1I be2b, and be1 be2b (Matsumoto et al., 2012; Nakaya et al., 2013; Abe et al., 2014; Tsuiki et al., 2016; Miura et al., 2021). It will be promising to explore genome editing technology to simultaneously edit multiple genes that are known to control RS content and generate their different mutant combinations for improving RS content in the future.

Increasing the RS content of rice usually causes decreases in grain yield, cooking and taste quality, and appearance quality (Butardo et al., 2011; Sun et al., 2017; Li et al., 2018b). Currently, a number of mutants and transgenic lines with high RS content have been developed, but their utilization is still limited because of decreased yield and taste quality (Crofts et al., 2012; Miura et al., 2018; Ramadoss et al., 2019; Baysal et al., 2020; Butardo et al., 2020). For example, suppression of SBEIIb or both SBEI and SBEIIb led to a significant increase in RS content and AC but caused a reduction in grain yield and poor cooking and eating quality (Zhu et al., 2012; Sun et al., 2017; Miura et al., 2021). Overexpression of GBSS or repression of SS and/or SBE causes high RS content but results in decreased starch granule size, stickiness, grain weight, and yield and increased grain chalkiness and hardness (Itoh et al., 2003; Zhang et al., 2011; Zhou et al., 2016). Obviously, seed yield and eating quality have undergone artificial selection during crop domestication. Therefore, it is promising to explore natural variations and find beneficial alleles that can increase both RS content and cooking and taste quality. Furthermore, fine-tuning the expression of SSRGs in the endosperm may be an effective way to generate elite varieties with high RS content and good quality. Creation of some weak alleles by genome editing technology and identification of upstream regulators and epigenetic modifications of SSRGs will help to fine-tune their expression. Therefore, the main challenge we now face is to understand the genetic and molecular mechanisms that control the balance between RS content, grain yield, and eating quality and to apply this knowledge for the improvement of RS, yield, and quality in crops.

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