Coexpression Analysis Identifies Rice Starch Regulator1, a Rice AP2/EREBP Family Transcription Factor, as a Novel Rice Starch Biosynthesis Regulator1[W][OA]

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Starch biosynthesis is important for plant development and is a critical factor in crop quality and nutrition. As a complex metabolic pathway, the regulation of starch biosynthesis is still poorly understood. We here present the identification of candidate regulators for starch biosynthesis by gene coexpression analysis in rice (Oryza sativa). Starch synthesis genes can be grouped into type I (in seeds; sink tissues) and type II (in vegetative tissues; source tissues), and 307 and 621 coexpressed genes are putatively involved in the regulation of starch biosynthesis in rice seeds and vegetative tissues, respectively. Among these genes, Rice Starch Regulator1 (RSR1), an APETAL2/ethylene-responsive element binding protein family transcription factor, was found to negatively regulate the expression of type I starch synthesis genes, and RSR1 deficiency results in the enhanced expression of starch synthesis genes in seeds. Seeds of the knockout mutant rsr1 consistently show the increased amylose content and altered fine structure of amylopectin and consequently form the round and loosely packed starch granules, resulting in decreased gelatinization temperature. In addition, rsr1 mutants have a larger seed size and increased seed mass and yield. In contrast, RSR1 overexpression suppresses the expression of starch synthesis genes, resulting in altered amylopectin structure and increased gelatinization temperature. Interestingly, a decreased proportion of A chains in rsr1 results in abnormal starch granules but reduced gelatinization temperature, whereas an increased proportion of A chains in RSR1-overexpressing plants leads to higher gelatinization temperatures, which is novel and different from previous reports, further indicating the complicated regulation of starch synthesis and determination of the physicochemical properties of starch. These results demonstrate the potential of coexpression analysis for studying rice starch biosynthesis and the regulation of a complex metabolic pathway and provide informative clues, including the characterization of RSR1, to facilitate the improvement of rice quality and nutrition.

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Rice (Oryza sativa) provides a staple food source in the diet of a majority of the world’s population. Breeding of high-yield and high-quality rice is the main goal of rice geneticists and breeders. There is great demand for the improvement of grain quality to meet the requirements of increased standards of living.

As the predominant component (90% dry basis) of milled rice, the physicochemical properties of starch, including apparent amylose content (AAC), amylopectin structure, gelatinization temperature, and pasting viscosity, determine the eating, cooking, and milling qualities of rice (Bao et al., 2008). Many efforts have been made to identify the genetic mechanisms controlling the physicochemical properties of rice starch, especially the diversity of rice starch synthesis genes. The Waxy gene, which encodes a granule-bound starch synthase I (GBSSI), plays a key role in amylose synthesis and is responsible for the biosynthesis of extra-long-unit chains of amylopectin (Hanashiro et al., 2008). Starch synthase IIα (SSIIα) is involved in the modification of rice starch quality and differentiates amylopectin structure and starch quality between japonica and indica varieties (Nakamura et al., 2005). Other starch synthesis genes in rice, including OsSSI, OsSSIⅢa, isoamylase1 (OsISA1), pullulanase (OsPUL), and branching enzyme (OsBEI and OsBEⅢb; Nishi et al., 2001; Fujita et al., 2003, 2006, 2007; Satoh et al., 2003; Li et al., 2009), have also been reported to regulate starch structure and seed physicochemical properties.

The expression of starch synthesis genes is finely regulated and is closely related to plant development. These genes are differentially expressed in plant sink and source organs (Ohdan et al., 2005) and show diurnal variation. The rice GBSSII coding gene is regulated by a circadian rhythm (Dian et al., 2003), and Suc and abscisic acid can cooperatively stimulate the expression of a large subunit of the ADP-Glc pyrophosphorylase coding gene (OsAPL3; Rook et al.,...
2001; Akihiro et al., 2005). Some transcription factors (TFs) regulate the expression of starch synthesis genes. A MYC (for v-myc avian myelocytomatosis viral oncogene homolog) protein and an EREBP (for ethylene-responsive element binding protein) can interact to enhance the transcription of the *Waxy* gene (Zhu et al., 2003). ABA INSSENSITIVE4, an *Apetala2*-type TF, mediates the abscisic acid- and sugar-induced expression of OsAPL3 (Rook et al., 2001). To date, most studies have focused on the expression profiles and effects of single starch synthesis genes on starch biosynthesis, but no systematic study of the regulatory mechanisms of starch biosynthesis, especially the whole metabolic pathway, has been reported. Coexpression analysis, which is based on the assumption that genes with similar expression patterns are more likely to be functionally associated, has proven to be a powerful tool for identifying regulatory factors in transcriptional networks, especially those related to a specific biological process, in different organisms such as yeast (*Saccharomyces cerevisiae*), Arabidopsis (*Arabidopsis thaliana*), and human (Ihmels et al., 2004; Lee et al., 2004; Persson et al., 2005; Aoki et al., 2007; Hirai et al., 2007). In plants, this strategy has been used to identify factors regulating several metabolic pathways, including two genes involved in cellulose synthesis (Persson et al., 2005) and two MYB (for v-myb avian myeloblastosis viral oncogene homolog) TFs that regulate the aliphatic glucosinolate biosynthesis pathway (Hirai et al., 2007).

To systematically identify the genes that regulate rice starch biosynthesis, genome-wide coexpression analysis was performed. The results show that 928 genes are putatively associated with starch biosynthesis, providing an informative resource for studying rice starch synthesis regulation and improving rice starch quality and nutrition by regulating starch metabolism throughout the pathway. Further functional studies show that one of the identified factors, Rice Starch Regulator1 (RSR1), an *APETALA2* (AP2)/EREBP-type TF, negatively regulates starch biosynthesis and is important for starch content and structure, starch granule morphology, and gelatinization properties.

**RESULTS**

**Identification and Functional Annotation of Genes Coexpressed with Rice Starch Synthesis Genes**

Twenty-seven rice starch synthesis genes, including genes encoding AGPase, SS, GBSS, BE, DBE, PHO (starch phosphorylase), and DPE (disproportionating enzyme), were selected as “guide genes” (Supplemental Table S1; Ohdan et al., 2005) to identify the coexpressed genes using expression data from 171 GeneChip Rice Genome Array chips. Any two genes with an absolute value of the Pearson correlation coefficient (PCC) greater than 0.6 between their expression profiles were considered as coexpressed genes (Aoki et al., 2007). We first analyzed the intercorrelation of these guide genes, and results showed that most of them were coexpressed closely (Fig. 1A). Specifically, 23 of the 27 guide genes could be divided into two relatively independent groups (types I and II, containing 15 and eight genes, respectively), while the other four genes have less correlation with others. Surveys and summaries of the expression patterns of these guide genes in various tissues, including root, seedling, leaf, ovary, and...
developing endosperm and embryo (Xue et al., 2009), showed that type I guide genes were preferentially expressed in developing endosperm, whereas type II guide genes were preferentially expressed in vegetative tissues and embryos (Fig. 1B). These findings were consistent with the differentiation of these genes in starch biosynthesis in sink and source organs (Ohdan et al., 2005).

Next, we calculated the PCC of all genes on the chips with each guide gene. With a PCC cutoff of 0.6, putatively correlated genes were selected and classified into the two groups, including 3,853 type I genes with an average of 750 per guide gene and 6,808 type II genes with an average of 2,200 per guide gene. Based on the number of correlated guide genes, genes that correlated with more than eight type I guide genes or more than six type II guide genes were classified as coexpressed genes that might be involved in regulating starch biosynthesis. In total, 307 out of 3,853 type I and 621 out of 6,808 type II coexpressed genes were identified.

The coexpressed genes were then annotated using the Gene Ontology biological process (http://www.tigr.org). The results showed that many of the top-ranked genes were directly involved in starch biosynthesis. Genes involved in starch biosynthetic and metabolic processes were enriched among type I coexpressed genes, and those involved in photosynthesis and carbohydrate metabolism were enriched among type II genes (Supplemental Tables S2 and S3). Genes involved in lipid storage and aromatic amino acid biosynthesis were also enriched among type I coexpressed genes, indicating the close relationship of starch, lipid, and amino acid metabolism pathways. In addition, some type I coexpressed genes were also involved in the response to gibberellin and abscisic acid stimuli, suggesting the potential role of hormones in starch regulation (Supplemental Table S2). Among type II coexpressed genes, those involved in Fru metabolism, vitamin E biosynthesis, and fatty acid metabolism were also enriched (Supplemental Table S3), suggesting that multiple metabolic pathways are correlated with starch biosynthesis.

Many genes involved in regulating gene expression at the transcriptional and/or posttranscriptional level were identified, among which we are particularly interested in TFs. Forty-five and 16 TFs were included among type I and type II coexpressed genes, respectively (Supplemental Tables S4 and S5). Most type I coexpressed TFs were members of the AP2/EREBP, bZIP (for basic Leu zipper), MYB, or NAC (for NAM, ATAF, and CUC) families, whereas most type II coexpressed TFs were members of the bHLH (for basic helix-loop-helix), homeobox, or SET [for Su(var)-3-9, Enhancer-of-zeste, and Trithorax] families. Members of the CCAAT_HAP2 (for heme activator protein 2), CCAAT_HAP3, and CCAAT_HAP5 families, which may form a complex in plant cells (Gusmaroli et al., 2001), were found being coexpressed with both type I and type II starch synthesis genes.

RSR1 Is Negatively Correlated with Type I Starch Synthesis Genes

Among the identified TFs, a member of the AP2/EREBP family, designated as RSR1, was the only one that negatively coexpressed with type I starch synthesis genes (Fig. 1A). A putative T-DNA insertion mutant of RSR1 in the SHIP (for Shanghai T-DNA Insertion Population) stock (http://ship.plantsignal.cn;Fu et al., 2009) was then identified for further analysis. RSR1 belongs to the euAP2 group of the AP2/EREBP family, which is defined by the presence of the AP2 domain (Kim et al., 2006). In rice, there are five members of the euAP2 group. Some euAP2 members or their orthologs in maize (Zea mays) and Arabidopsis have been demonstrated to play roles in the boundaries of floral meristem identity, floral organ
Figure 3. **RSR1** negatively regulates the expression of rice starch synthesis genes in seeds. qRT-PCR analysis of the expression of type I starch synthesis genes in 6-DAP seeds of **rsr1** mutant, transgenic **rsr1** mutant with complementary expression of **RSR1** (**rsr1 pRSR1::RSR1**: three lines), and **RSR1**-overexpressing (**pRSR1::RSR1**: three lines) lines. The relative expression of the examined genes indicates the ratio (log2) to the expression in the control. For the **rsr1** mutant, Zhonghua 11 (wild type) was used as a control; for transgenic **rsr1** mutant with complementary expression of **RSR1** (**rsr1 pRSR1::RSR1**), and **RSR1**-overexpressing (**pRSR1::RSR1**) lines, the transgenic plants transformed with empty vector were used as a control. All data are presented as means ± SD from three replicates.
identity, or the control of floral organ number (Kim et al., 2006; Lee et al., 2007).

Analysis of expression pattern by quantitative real-time reverse transcription (qRT)-PCR showed that RSR1 is highly expressed in root, seedling, and leaf and expressed to a lesser extent in stem, panicle, and seeds at different developmental stages (Fig. 2A). This expression pattern is almost opposite to that of type I starch synthesis genes, which are preferentially expressed in endosperm and expressed to a lesser extent in vegetative tissues (Fig. 1B), consistent with the negative correlation between RSR1 and type I starch synthesis genes.

The T-DNA insertion mutant rsr1 (SHIP_ZSF6068), in which the T-DNA putatively inserts at the sixth intron of the RSR1 gene (Fig. 2B), was confirmed by PCR analysis, and Southern-blot analysis revealed the presence of a single locus of T-DNA in rsr1. qRT-PCR analysis of RSR1 transcription revealed a deficient expression of RSR1, indicating that rsr1 is a knockout mutant (Fig. 2C). The rsr1 mutant with complementary expression of RSR1 or RSR1-overexpressing lines (Zhonghua 11 background) were generated by transferring the genomic fragment containing the whole RSR1 gene (including the promoter) to rsr1 mutant and the wild type (Zhonghua 11), respectively, through Agrobacterium tumefaciens-mediated transformation (Hiei et al., 1994), and three independent lines were confirmed by qRT-PCR analysis (Fig. 2C).

Coexpression analysis indicated a negative correlation between RSR1 and type I starch synthesis genes, so we analyzed the expression of type I starch synthesis genes in rsr1. Indeed, qRT-PCR analysis showed that all type I starch synthesis genes were distinctly up-regulated in rsr1 seeds 6 d after pollination (DAP) in comparison with the wild type (Zhonghua 11); some of them (OsISA1, OsAGPL2, and OsBEI) increased by up to approximately 16-fold (Fig. 3). Complementary expression of RSR1 into rsr1 mutant results in the recovered expression of starch synthesis genes, and as expected, the suppressed expression trend of type I starch synthesis genes was observed in RSR1-overexpressing lines (Fig. 3), further confirming the negative regulatory effect of RSR1 on type I starch synthesis genes. Further examination of the expression pattern of starch synthesis genes in rsr1 and the wild type at different developmental stages of seeds (including 3, 6, 9, and 12 DAP) confirmed that the expression of most starch synthesis genes was enhanced in the rsr1 mutant throughout seed development, although the extent of increase varied in genes or developmental stages (Fig. 4), which may result in the enhanced starch metabolism in the rsr1 mutant.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Expression profiles of type I rice starch synthesis genes during seed development of Zhonghua 11 (wild-type [WT]) and the rsr1 mutant. Total RNA was extracted from seeds at 3, 6, 9, or 12 DAP. For each gene, the expression of wild-type seeds at 3 DAP was set as a control. All the data are shown as means ± SD from three replicates.
RSR1 Affects Starch Content, Grain Morphology, and Yield

Observation of growth of the rsr1 mutant or RSR1-overexpression lines revealed no obvious visible phenotype at the vegetative stage, except that plants with RSR1 overexpression showed delayed flowering (approximately 10 d). Because type I starch synthesis genes are primarily responsible for starch biosynthesis in seeds, we focused on starch accumulation in seeds. Measurement of seed starch content showed that AAC in the rsr1 mutant was significantly increased compared with the wild type (Fig. 5A), which may be due to the 7-fold up-regulation of OsGBSSI in rsr1. The AAC of seeds in RSR1-overexpressing plants and total starch content in rsr1 and RSR1-overexpressing lines were comparable with those in the wild type (Fig. 5, A and B).

Observations of grain morphology showed that rsr1 seeds, in comparison with the wild type, were larger in seed size and had significantly increased 1,000-grain weight. In contrast, seeds of RSR1-overexpressing plants were much smaller and had reduced 1,000-grain weight (Fig. 5, C and D). In addition, the seeds of rsr1 exhibited a white-cored chalky appearance. Cross-sections of mature seeds revealed a chalky interior endosperm and normal exterior of rsr1 seeds (Fig. 5D).

As endosperm starch quality is closely related to yield, and rsr1 showed increased seed mass, we further examined the yield-related traits of the rsr1 mutant continuously for four generations to confirm that the observed phenotypes were stably inherited. The rsr1 and wild-type plants were planted in a paddy field under the same natural environment. The number of panicles per plant, number of grains and filled grains per panicle, and filled grain percentage of rsr1 were the same as those of the wild type; however, the 1,000-grain weight of rsr1 was much increased, and the yield per plant was increased accordingly (Table I). We estimate that the yield of rsr1 was increased by about 7.2% compared with the wild type.

Effects of RSR1 on the Fine Structure of Amylopectin in Endosperm

Blue value (BV) and maximum absorbance ($\lambda_{max}$) are two characteristics of starch representing its ability to combine with iodine. Greater values of BV and $\lambda_{max}$ indicate the greater fractions of long chains (Hizukuri, 1986). To examine whether RSR1 affects the molecular structure of starch, the BV and $\lambda_{max}$ of amylopectin were first analyzed, and results showed that both BV and $\lambda_{max}$ of the amylopectin-iodine complex were higher in rsr1 than in the wild type, whereas both values were lower in RSR1-overexpressing lines (Fig. 6A), indicating that RSR1 deficiency or overexpression leads to larger or smaller fractions of long-branched starch chains, respectively.

To further examine the change in fine structure of amylopectin caused by altered RSR1, the endosperm starch of wild-type, rsr1 mutant, and RSR1-overexp-
RSR1 overexpression was distinctly different from that of the wild type and almost opposite to that of rsr1. Under RSR1 overexpression, chains with DP in the ranges of 5 to 8 and 22 to 42 were reduced, whereas those with DP in the range of 9 to 21 were significantly increased. Although the extent of changes in chain length distribution differed among three RSR1-overexpressing lines, all lines showed the same trend (Fig. 6, B and C).

Amylopectin includes chains of the types A, B1, and B2-3, with size ranges of DP ≤ 17 to 18, DP = 18 to 28, and DP ≥ 28, respectively (Bertoft and Koch, 2000). Calculation of the ratio of each type in the amylopectin of wild-type, rsr1, and RSR1-overexpressing plants showed that short chains (A) were much less common in rsr1 and more common in RSR1-overexpressing plants than in the wild type, whereas intermediate chains and long chains (B1 and B2-3, respectively) were much more common in rsr1 and long chains (B2-3) were less common in RSR1-overexpressing plants (Table II).

Deficiency of RSR1 Results in Altered Morphology and Gelatinization Properties of Endosperm Starch Granules

Amylopectin structure is crucial for starch granule formation and morphology. To test whether the starch granules in endosperm were affected by the altered RSR1, seed cross-sections were examined using scanning electron microscopy. In wild-type endosperm, the starch granules formed similarly sized, compactly arranged polygonal granules with sharp edges; in contrast, the starch granules of the rsr1 mutant were round in shape and loosely packed in the endosperm cell (Fig. 7A). Enhanced expression of RSR1 did not cause obvious changes in starch granule morphology (Supplemental Fig. S1).

Because both the fine structure of amylopectin and the morphology of starch granules were changed in the rsr1 mutant, we examined the gelatinization properties of the starch granules in terms of solubility in urea solution (Nishi et al., 2001). Powdered starch from rsr1 and the wild type were mixed with urea solutions (0-9 M), and we observed a slightly lower onset concentration of urea gelatinization in rsr1 starch than in the wild type (Fig. 7B). The supernatant of starch solubilized by 5 M urea solution, in which the difference in swelling between rsr1 and the wild type was most distinct (Fig. 7B, right panel), was then reacted with iodine solution. Measurement of the absorbance spectra of starch-iodine complexes showed that absorbance values between 480 and 700 nm were greater in rsr1 than in the wild type, and the λmax of rsr1 starch (558 nm) was also much greater than that of the wild type (531 nm; Fig. 7C). These results suggest that the starch of rsr1 endosperm is more readily gelatinized in urea.

Furthermore, the thermal gelatinization temperatures of endosperm starch from wild-type, rsr1, and RSR1-overexpressing plants were analyzed by differential scanning calorimetry to confirm the effects of...
RSR1 on the physicochemical properties of starch. The onset (To), peak (Tp), and conclusion (Tc) temperatures of gelatinization of endosperm starch in the rsr1 mutant were all about 10°C lower than those in the wild type, whereas the To of endosperm starch in RSR1-overexpressing plants was 13°C higher than that in the wild type and the Tp and Tc of RSR1-overexpressing plants showed no significant difference (Table II). These results indicate that the differences in fine structure of amylopectin caused by RSR1 alteration modulated the gelatinization properties of starch.

**DISCUSSION**

Coexpression analysis has been successfully exploited to identify functional transcription regulators in many organisms (reported studies are performed using Arabidopsis as material in plants; Persson et al., 2005; Hirai et al., 2007). In this study, coexpression analysis is exploited to our knowledge for the first time, to study a multiple-gene metabolic pathway in rice while corroborating the results with physiological studies, resulting in the identification of a TF, RSR1, that negatively regulates endosperm starch biosynthesis and is involved in determining starch quality and physicochemical characteristics of seeds by modulating the expression of starch synthesis genes. Characterization and further analysis of other candidate genes will provide more information to facilitate understanding of the regulatory network of rice starch biosynthesis. Additionally, our results also demonstrate the potential of coexpression analysis to explore the regulatory mechanisms of metabolic pathways or biological processes and thus expedite the discovery and characterization of molecular regulatory networks during plant development.

Our results show that rice starch synthesis genes form two relatively independent modules (Fig. 1A). The preferential expression of these two groups of genes strongly supports their primary roles in sink and source organs (Ohdan et al., 2005). Accordingly, Gene Ontology analysis shows very different functions for the two types of coexpressed genes. Type I is primarily associated with starch biosynthetic and metabolic processes, and type II is primarily associated with photosynthesis. Among type I coexpressed genes, those responding to gibberellin or abscisic acid stimuli are enriched (Supplemental Table S2), consistent with the fact that these two hormones influence starch biosynthesis at different levels. Indeed, OsAPL3 expression and starch content in cultured cells are cooperatively induced by changes in both Suc and abscisic acid concentrations (Akihiro et al., 2005), and the application of gibberellin to cultured maize endosperm cells affects starch accumulation (Cao and Shannon, 1997). Additionally, several genes involved in lipid storage are also found among type I genes, consistent with the close links between lipid fluxes and starch biosynthesis in seeds. Interestingly, some type I genes are related to responses to low temperatures, water deprivation, and bacterial infection, suggesting that biotic or abiotic stresses may influence starch accumulation.

Most type II coexpressed genes are involved in photosynthesis through regulating chloroplast organization and chlorophyll biosynthetic processes. This is reasonable because starch is a primary product of photosynthesis in leaves. Rice HAP3 regulates chloroplast biogenesis (Miyoshi et al., 2003) and is found to coexpress with type II starch synthesis genes. In addition, two genes involved in ethylene biosynthesis are found among type II genes (Supplemental Table S3), suggesting the involvement of ethylene in starch metabolism and accumulation, which is consistent with a recent report that ETR2, a subfamily II ethylene receptor, promotes starch accumulation in rice internodes (Wuiziyanghan et al., 2009).

**Table II. Chain distribution of amylopectin in different fractions and gelatinization temperature of endosperm starch**

| Lines        | A %  | B1 %  | B2,3 % | To °C | Tp °C | Tc °C |
|--------------|------|-------|--------|-------|-------|-------|
| Wild type    | 53.15 ± 0.26 | 32.45 ± 0.22 | 14.39 ± 0.12 | 54.10 ± 2.33 | 69.06 ± 0.77 | 72.91 ± 1.34 |
| rsr1         | 50.36 ± 0.55** | 34.47 ± 0.17** | 15.17 ± 0.16** | 44.94 ± 0.34** | 57.64 ± 1.55** | 63.46 ± 1.31* |
| pRSR1::RSR1-L1 | 55.18 ± 0.25** | 32.60 ± 0.30* | 13.22 ± 0.20** | 67.61 ± 0.28** | 71.98 ± 0.14 | 76.75 ± 0.21 |
| pRSR1::RSR1-L2 | 55.75 ± 0.58** | 32.25 ± 0.10 | 12.00 ± 0.11** | 66.83 ± 0.42** | 72.65 ± 0.07 | 75.82 ± 0.14 |
| pRSR1::RSR1-L3 | 55.12 ± 0.26** | 31.91 ± 0.08 | 12.97 ± 0.07** | 68.25 ± 0.35** | 71.25 ± 1.77 | 77.34 ± 0.14 |

* P < 0.05, ** P < 0.01.

The onset (To), peak (Tp), and conclusion (Tc) temperatures of gelatinization of endosperm starch in the wild type and the Tp and Tc of RSR1-overexpressing lines was 13°C higher than that in the wild type and the Tp and Tc of endosperm starch in the rsr1 mutant were all about 10°C lower than those in the wild type.
RSR1 Regulates Rice Starch Biosynthesis

...to the relatively small extent of down-regulation of OsGBSSI). BEs, DBEs, and SSs cooperatively determine the fine structure of amylopectin in a highly complex manner. BEs are responsible for the generation of branching chain, which is then extended by SSs, and DBEs selectively remove the branches that are inappropriately positioned (Ohdan et al., 2005). Among them, BEIIb specifically transfers the short chains and SSII then extends them to DP 8 to 12 to generate short A chain (Nishi et al., 2001; Fujita et al., 2006), whereas BEI cooperates with SSIIa and SSIIa to synthesize intermediate B1 chains and long B2–3 chains, respectively (Satoh et al., 2003; Nakamura et al., 2005; Fujita et al., 2007). During this process, the synthesis of long B2–3 chains is by using B1 and A chains as substrates, and the synthesis of intermediate B1 chains is by using A chains as substrates. In the rsr1 mutant, genes encoding OsBEI, OsBEIIb, OsSSI, OsSSIIa, and OsSSIIa are all up-regulated, resulting in the stimulated synthesis of both B chains and A chains, and may finally lead to the increased proportions of B1 and B2–3 amylopectin chains and decreased proportions of short A chains. In contrast, enhanced expression of RSR1 leads to the down-regulation of SS, BE, and DBE genes and thus the increased proportions of “substrate” A chains and decreased proportions of “product” B chains of amylopectin (Table II). The changes of amylopectin in the rsr1 mutant (in which OsSSI and OsBEIIb were up-regulated) are similar to those in OsSSI or OsBEIIb, and the changes in RSR1-overexpressing lines (in which OsISA1 and OsPUL were down-regulated) are opposite to those in OsISA1 and OsPUL mutants, indicating that the changes in the chain length distribution of rsr1 or RSR1 overexpression lines were caused by multiple starch synthesis genes and not by the single gene, and similar effects in the determination of starch composition can be achieved by different ways.

In the rsr1 mutant, the RSR1 deficiency stimulated the expression of type I starch synthesis genes throughout seed development; however, these genes were significantly up-regulated in 3 or 6 DAP and slightly in 9 and 12 DAP (Fig. 4), implying that RSR1 mainly functions at the early stages of seed development. This is indeed consistent with the expression profile of RSR1 in the developing seeds at 3, 6, 9, or 12 DAP. On the other hand, different starch synthesis genes may interact and influence the expression of each other at different stages of developing seeds, leading to the differential changes of these genes.

The altered structure of amylopectin will cause specific changes in the crystalline structure of the starch granule and consequently affect the gelatinization property of starch. It seems that A chains play an important role in the formation of the crystalline structure (Jane et al., 1999), and changes in short A chains within clusters will result in changes in the gelatinization property of starch. In the rsr1 mutant, the altered starch granule morphology and gelatinization properties of starch cooperatively.

It is interesting that enhanced expression of starch synthesis genes in rsr1 did not change the starch content but enlarged the seed size and increased the 1,000-grain weight/yield, which was consistent with...
the previous report of maize AGPase. Transforming rice with a modified maize AGPase gene increased the seed yield and plant biomass but with no changed starch content (Smidansky et al., 2003), indicating the leading role of sink strength in grain filling. In Arabidopsis, AP2 affects seed mass and seed yield, which may act through regulating sugar metabolism to promote an extended period of cell division (Jofuku et al., 2005; Ohto et al., 2005). Previous studies in rice have also suggested that rice productivity and yield are primarily sink limited in source-sink relationships during seed development (Okita et al., 2001). Thus, deficiency of RSR1 may result in enhanced starch biosynthesis and stimulated cell division, which in turn leads to an enhanced capacity to utilize the initial photosynthetic product, resulting in increased yield. Meanwhile, the floral organs of the rst1 mutant or RSR1-overexpressing lines show no abnormalities except delayed flowering time, suggesting the possible functional conservation and diversity of AP2 family genes.

RSR1 negatively regulates the expression of starch synthesis genes; however, whether it regulates the downstream genes by a direct or indirect way is still unknown. Analysis of the promoter regions of the starch synthesis genes shows that many of them contain the DRE, GCC, or CAACA box, which can be recognized by EREBP, a subfamily of AP2/EREBP with a single AP2 domain (Xie, 2006). There is still no evidence indicating that the RSR1-like euAP2 subfamily, which contains two AP2 domains, can interact with these motifs. Further in vivo studies will help us understand the regulatory mechanism of RSR1 on starch biosynthesis.

Improving rice grain quality is essential to rice consumers; however, the formation of seed quality is a highly complex process. Starch synthesis-related proteins cooperate with each other to define the correlation of different properties, and selection for any single one in breeding would be insufficient (Tian et al., 2009). We provide candidate regulators of starch biosynthesis by coexpression analysis and show that RSR1 can simultaneously regulate all the starch synthesis genes and enhance the whole starch biosynthesis pathway. This may help to increase the seed mass and yield and decrease the starch gelatinization temperature in rice breeding, although the higher AAC and the white-cored appearance are not beneficial.

MATERIALS AND METHODS

Microarray Analysis Processes

The 171 microarray data (platform GPL2025 in Gene Expression Omnibus) were downloaded from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/geo/), including GSE3053, −4438, −4471, −10857, −10872, and −12069. The raw data were normalized by the GeneChip robust multiarray analysis algorithm (Wu et al., 2004). The probes on the array with very low expression level (marked as M/A using the MAS5.0 method) were filtered out, and 46,284 of total 57,381 probes were used for the analysis. Twenty-seven rice (Oryza sativa) starch synthesis genes were considered as the guide genes (Supplemental Table S1; Ohdan et al., 2005). For each guide gene, the expression data in all the microarrays were compared with all other probes, and the PCC between them were calculated. If the absolute value of a PCC is greater than 0.60, the probe will be considered as associated with the guide gene. The genes that associated with more than eight type I guide genes or more than six type II guide genes were classified as coexpressed genes. The coexpression network among RSR1 and guide genes was illustrated using the program Cytoscape (Shannon et al., 2003).

Plant Material and Genetic Analysis

The homozygous T-DNA insertion mutant rsr1 (SHIP_ZSF6068; http://ship.plantsignal.cn; Fu et al., 2009) was confirmed by PCR amplification using the T-DNA-specific primer LB (5′-ATAGGTTTCCCTAGTGAGGACG-3′) and RSR1 gene-specific primers T1 (5′-CCGATATGGGAAGGATTGTCG-3′) and T2 (5′-CCGATGATGATGGTTGTCG-3′).

To generate the complementary lines of rsr1 mutant and RSR1-overexpressing lines, an approximately 11.5 kb DNA fragment containing the whole RSR1 gene was digested with Xhol from bacterial artificial chromosome clone OSJN86015N12 (Arizona Genomics Institute) and subcloned into the Xhol site of pCAMBIA2300 vector and pCAMBIA1300 vector (CAMBIA), respectively. Agrobacterium tumefaciens-mediated transformation was performed as described previously (Hiei et al., 1994), and positive T0 transformants were identified by RT-PCR. The homozygous T2 lines were obtained and used for further analysis. All the plants including the wild type (japonica cv Zhonghua 11) were planted in a paddy field under natural agricultural conditions for the analysis of panicle-related traits.

qRT-PCR Analysis

Total RNAs were extracted, treated with RNase-free DNasel, and used for cDNA synthesis. qRT-PCR was performed by using the SYBR Green Realtime PCR Master Mix (Toyobo). The OsACT2 gene was amplified with primers 5′-GAATCTGTATGCTAACGGCTG-3′ and 5′-ACACGGAGCTCTGTTAAG-3′ and used as a positive internal control. The RSR1 gene was amplified with primers R1 (5′-GATAGCCGTCCTCCCTG-3′) and R2 (5′-TGCGCTTCACCTCCATTACA-3′). Type 1 starch synthesis genes were amplified with the primers described previously (Ohdan et al., 2005). The copy number of the tested genes in each sample was calculated by Rotor-Gene 6 software (Corbett Robotics) using the standard curve (Gauchen et al., 2004).

To study the expression pattern of RSR1, roots of 1-week-old seedlings, young leaves (first and second leaves of 2-week-old seedlings), old leaves (the topmost leaf during the tillering stage), stem and young panicle at the booting stage, old panicle at the heading stage, and developing seeds at 3, 6, 9, and 12 DAP were collected. To analyze the expression of starch synthesis genes, seeds of 6 DAP from wild-type, rsr1 mutant, and RSR1-overexpressing lines were used.

Starch Contents, 1,000-Grain Weight, and Amylopectin Structure Determination

Embryo and pericarp were removed from the dehulled grains, and the endosperms were ground to powder. The AAC of the sample was measured by the iodine colorimetric method (Juliano, 1971). To determine the total starch content, 50 mg of powder was washed two to three times by using 80% (v/v) ethanol and then extracted with 9.2 and 4.6 M perchloric acid in order. The supernatant was collected and diluted to 50 mL with water. An aliquot of this solution was analyzed for starch content by the anthrone method (Turner and Turner, 1960).

The 1,000-grain weight was determined by counting 10 replicates of 100-grain samples independently on an electronic balance. Data are shown as means ± se.

To measure the BV and λmax of amylopectin, a modified alkaline steeping method (Cai et al., 2006) was used to fractionate starch, and 15 mg of separated amylopectin powder was diluted to 100 mL with water. The absorbance spectrum of the starch-iodine complexes was recorded from 500 to 700 nm, and the wavelength at λmax was determined. The BV was λmax.

To determine the chain length distributions of amylopectin, 5 mg of rice powder was digested with Pseudomonas amylolovora isoamylase (Sigma-Aldrich) and then analyzed by HPAEC-PAD (model DX-500 [Dionex]; Naganome and Komae, 1996; Nishi et al., 2001).
Observation of Starch Granules of Endosperm

Rice seeds were dried completely under low pressure and cut across the short axis with a razor blade. The cross-sections were first photographed to show the white-core floury appearance, and the surface was sputtered coated with gold and observed by scanning electron microscopy (JEOL JSM-6360LV).

Measurement of the Gelatinization Properties

The gelatinization and swelling modes of endosperm starch in urea solution were measured according to a previous report (Nishi et al., 2001). For the gelatinization temperature analysis, 3 mg of rice powder was placed in an aluminum sample cup, mixed with 9 mL of distilled water, and sealed, and then the samples were analyzed by a differential scanning calorimeter (DSC 200PC; Netzsch). The heating rate was 10°C min⁻¹ over a temperature range of 30°C to 110°C.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AF46517 (RSR1, LOC_Os05g03040).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Morphology of starch granules of endosperm from wild-type and RSR1-overexpressing lines.

Supplemental Table S1. Rice starch synthesis genes that are used as “guide genes” for coexpression analysis.

Supplemental Table S2. Gene ontology biological process annotation of coexpressed genes correlated with type I rice starch synthesis genes.

Supplemental Table S3. Gene ontology biological process annotation of coexpressed genes correlated with type II rice starch synthesis genes.

Supplemental Table S4. Coexpressed TF coding genes correlated with type I rice starch synthesis genes.

Supplemental Table S5. Coexpressed TF coding genes correlated with type II rice starch synthesis genes.

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