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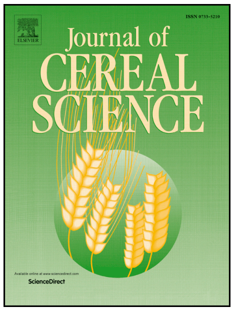

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Amylopectin chain length distribution in grains of japonica rice as affected by nitrogen fertilizer and genotype

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ABSTRACT

This study investigated the chain length distribution (CLD) of two japonica rice cultivars under six nitrogen (N) treatments by high performance size exclusion chromatography, with the aims to elucidate the effect of N on rice quality and its biological mechanism. Results showed significant influence of N on CLD. In comparison with low N rate, high N lowered the percentage of short amylopectin branches. Fitting with the CLD model of Wu-Gilbert, it suggested that relative activity of SBE to SS was lower at high N rate, thus producing fewer short amylopectin branches. Comparison of CLD between N rates and between cultivars revealed that decrease in short amlopectin branches or the relative ratio of short to long amylopcetin branches correlated with increase in flour gelatinization temperatures (T_o, T_p, and T_c) and decrease in pasting values (except PaT) and amylose-lipid gelatinization temperatures. In addition, quality traits of Wuyujing3, a cultivar with premium eating quality, expressed stably across N treatments compared with the high-yielding cultivar Wuyunjing7.

***Keywords*:** Japonica rice; Nitrogen; Amylopectin fine structure; Physicochemical properties

1. Introduction

Rice (*Oryza Sativa.* L) is growing in importance outside Asia as considerable growth in its consumption in Africa and South America (Muthayya et al., 2014). Generally, rice varieties in Asia are classified into two major types, indica and japonica. Japonica rice is preferred by people in northern Asia due to its sensory quality of soft texture and moderate stickiness (Sun et al., 2011). China is the largest producer and consumer of japonica rice. Along with rise in living standard, rice quality, eating and cooking quality in particular has become prime consideration of both rice producers and researchers like geneticists, breeders, and agronomists. However, our current knowledge of the physicochemical factors affecting quality of japonica rice is still incomplete, as is reflected by the persistence of benchmark varieties of Koshihikari (Fitzgerald et al., 2009) and Wuyujing3 (Yang et al., 2013) for many decades in spite of the grain yield achieved over decades.

Physicochemical foundation of rice eating and cooking quality has been investigated extensively. Results showed that chemical components including amylose, amylopectin, proteins, and lipids coordinately control physical properties of cooked rice and subsequently the eating quality (Bhattacharya et al., 2011). There has been an increasing awareness of the role of amylopectin fine structure in physicochemical properties of rice. Amylopectin contributes to swelling of starch granules and pasting, as measured by Rapid Visco Analyzer (RVA). Jane et al. (1999) reported that long chains of AP (DP>37) were positively associated with starch pasting temperature (PaT) but negatively with peak viscosity (PKV) and breakdown (BDV). By contrast, Yang et al. (2013) found no significant relationship between amylopectin structure and RVA parameters. Amylopectin also plays a substantial role in the process of starch gelatinization and retrogradation. Using differential scanning calorimeter (DSC), numerous studies showed that short amylopectin A chain (DP 6-12) was negatively correlated to the gelatinization temperature (T_o, T_p, T_c) and enthalpy (ΔH_g) during flour gelatinization (Nakamura et al., 2002; Vandeputte et al., 2003), while the long amylopectin chains exhibited the opposite trends (Jane et al., 1999). In addition, Wang and Wang (2002) investigated retrograding behavior of waxy rice starch, and attributed the low retrogradation tendency in waxy rice starches to a larger amount of A chain and a shorter exterior chain length of amylopectin.

Nitrogen (N), a primary element demand for rice grain, has obvious effect on eating and cooking quality of rice. It is well known that nitrogen application increase protein content and decrease amylose content in rice grain (Dong et al., 2007). Gunaratne et al. (2011) showed that nitrogen fertilizer application increased pasting onset temperature (PaT), cold paste viscosity (CPV) and setback viscosity (SBV), but lowered peak viscosity (PKV), breakdown viscosity (BDV), peak temperature (T_p). In contrast, Singh et al. (2011) reported that all gelatinization parameters were obviously enhanced. With respect to rice sensory quality, Champagne et al. (2009) reported that N fertilizer significantly worsen the palatability of cooked rice, causing it hard, rough and stick in texture as the results of increase in protein content and decrease in amylose content, as was also reported by Singh et al. (2011). On the other hand, Gunaratne et al. (2011) found that N fertilizer reduced the gel hardness of rice flour but increased the gel cohesiveness. Therefore, N effect on physical properties and sensory qualities of rice is still unclear. In addition, little is known concerning the effect of N on rice quality from perspective of amylopectin fine structure.

In Southern China, one of the main areas for japonica rice planting, rice production is characterized as being high yielding and high N input. For example in Jiangsu Province, the average N rate in rice is 270 kg/ha and even exceeds 300 kg/ha in many counties (Xue et al., 2013). Excessive use of nitrogen fertilizer has been a serious problem in this area, posing not only a threat to environment but also negative impact on rice eating and cooking quality. However, its physicochemical foundation is largely unknown, especially in knowledge of N influence on amylopectin fine structure and it biosynthesis mechanism.

Recently, Wu et al. (2013) developed a model of amylopectin fine structure (chain-length distribution, CLD) in cereal endosperm, with the aim to link CLD to concerted action of three key enzymes (starch synthase, SS; starch branching enzyme, SBE; and starch debranching enzyme, DBE). In the current study, CLD of two japonica rice cultivars under six N treatments was analyzed by high performance size exclusion chromatography (HPSEC) with refractive index detection, with the aims of (1) uncovering the variations of rice amylopectin structure with N and genotype, (2) clarifying the contribution of CLD to physicochemical properties of rice flour, and (3) exploring the biological mechanism of N effect on rice quality by the Wu-Gilbert model. We hope that these findings will further our understanding of physicochemical foundation of rice quality and be helpful for breeding and cultivation.

2. Materials and methods

2.1 Plant material and experiment design

Two japonica rice cultivars, Wuyujing3 and Wuyunjing7, were selected here. Wuyujing3 is famous for premium eating quality, while Wuyunjing7 is high-yielding but with medium eating quality (Suppl. Table S1 and Fig. S1) (Wei et al., 2011). Field experiment of nitrogen treatments was conducted at the experimental station of Nanjing Agricultural University (31°54′31″N, 119°28′21″E) in 2011. The soil type was clay soil, containing 1.48 g/kg total N, 10.37 mg/kg available P, and 49.53 mg/kg exchangeable K. The experiment was laid out in a split plot design with three replications. Six N treatments of two N rates and three timings of topdressing were conducted as follows: (1) LN1, LN2 and LN3, low N rate (75 kg/ha) and topdressing at initiation of panicle differentiation (as spikelet-promoting fertilizer), two weeks after initiation of panicle differentiation (as spikelet-sustaining fertilizer) and booting stage (as grain-filling fertilizer), respectively; (2) HN1, HN2 and HN3, high N rate (150 kg/ha). In addition, we use N0 as control, for which no N fertilizer was applied during the whole growth stage, except that P and K fertilizer (calcium superphosphate 140 kg/ha and potassium chloride 186 kg/ha) were applied before transplanting. To avoid leaching, each plot (11.5 m^2 in area) was separated by ridges mulched by plastic film. Rice was sown in seedbeds on May 25, 2011, and transplanted on June 20, 2011. At maturity, about 100 panicles with similar maturity were harvest, and then naturally dried and stored in -4℃.

2.2 Milled-rice appearance and chemical compositions

Grain weight (GW) was weighted using an analytical balance, and grain length and width ratio was determined using digital vernier calipers. The chalky rice ratio (CRR) was calculated as total grain number divided by chalky grain number. Head milled rice was ground into powder and stored in -20℃ for physicochemical analysis. Moisture content, total starch and crude lipids and apparent amylose content (AAC) were analyzed according to AACC procedures (AACC, 2000). Total protein content (TPC) was calculated as sum of the four fractions, albumin, globulin, prolamin, and glutelin, which were extracted and assayed according to the method reported by Ning et al. (2009).

2.3 Amylopectin chain length distribution

The preparation, debranching and analysis of amylopectin conducted by high-performance size-exclusion chromatography (HPSEC) were performed as detailed in our previous report (Yang et al., 2013). For quantitative analysis, the empirical division method were used here: firstly, the HPSEC data were transformed to the number distribution, and then the amylopectin chain length were subdivided into four categories: (1) A, $6 \leq DP \leq 12$; (2) B_1, $13 \leq DP \leq 24$; (3) B_2, $25 \leq DP \leq 36$; (4) B_3, $37 \leq DP \leq 60$; and which were described as short, medium, long, and very long, respectively. Consequently, the ratio of $(A+B_1)$ to (B_2+B_3) was used as index of short to long chains distribution of amylopcetin.

2.4 Modeling the amylopectin biosynthesis

As an alternative, the chain length distribution (CLD) of debranched amylopectin was fitted to Wu-Gilbert model, using a publicly available FORTRAN program package (Wu et al., 2013). The basic premise of the model is that the CLD of chains spanning two lamellae, short single-lamellar ($DP \leq 30$) and long translamellar ($30 \leq DP \leq 60$) branches, is controlled by two different enzyme sets. By fitting with this model, we get two categories of most informative categories of parameters. The first category of parameter is the ratio of activity of SBE to that of SS (β, β(i) &β(ii)). The second category of parameter is the relative contributions of each enzyme set. For example, h(ii/i), relative heights of the peak and shoulder in CLD number distribution, represents the relative contribution of enzyme set ii to that of set i. Before data fitting with Wu-Gilbert model, SEC weight distribution of debranched amylopectin $w_{de}(\log X)$ should be converted into number distribution $N_{de}(X)$ using the relation $w (\log X) = X^2 N(X)$ as described by Wang et al. (2014).

2.5 Starch particle size analysis

Milled rice of all the samples were grounded by mortar and pestle. Then proteins were isolated from rice flour by alkali extraction method and passed through 325 mesh sieve. Particle size distribution of the starches was measured by laser scattering on triplicate samples using a Satuen DigiSizer 5200 (Micromeritics, USA). The starch (100 mg) and distilled water (5 mL) was added into a tube and well mixed. The refractive index of starch and water used were 1.53 and 1.33, respectively. The sample was added to the sample port until the instrument read moisture 15% obscuration. The selected criteria were percent volume (% vol.) of granules with a diameter lower than 50 μm.

2.6 X-ray diffraction analysis

This was performed using an X-ray diffractometer (Tongda Science and Technology Co. Ltd., TD-3500 type, Dandong, China) operated at voltage of 20 kV and current of 30 mA. Rice flour samples (equilibrated at 75% relative humidity, at room temperature for a week) were packed tightly into the glass sample holder and diffraction data were collected over an angular range from 4° (2θ) to 30° (2θ) with a scanning speed of 2°/min. Relative crystallinity degree (%) was estimated according to the method of Wang et al. (2006).

2.7 RVA profiles

Pasting properties of rice flour samples were analyzed using a Rapid Visco Analyzer (RVA-3, Newport Scientific, Australia) following the method described by AACC procedure (AACC, 2000).

2.8 Thermal properties

Gelatinization and retrogradation properties of rice flour were analyzed by a differential scanning calorimeter (DSC1; Mettler-Toledo, Schwerzenbach, Switzerland) equipped with a thermal analysis data station named STARe11.0. A suspension of flour (20 weight %)in distilled water were prepared before thermal analysis, stirred and allowed to stand for 24 h at 4□. While heating, homogenized suspension weighted 20μl into a 40μl aluminum pan (Mettler, ME-26763), the samples were hermetically sealed. The DSC analyzer was calibrated using indium and empty pan was used as a reference. For testing the gelatinization, the sample pans were heated from 25°C ~120°C at a rate of 10/min. To determine the amylase-lipid melting peak, a reheating schedule was carried out according to the method of Iturriaga et al. (2004). The onset (T_o), peak (T_p), and conclusion (T_c) temperatures and gelatinization enthalpy (ΔH_g, J/g) of gelatinization were determined. For determination of retrogradation, the pans were stored at 4°C for 2 weeks, and were heated again. The peak temperature and the enthalpy (ΔH_r, J/g) associated with the retrograded starch melting peak appearing between 40°C and 70°C were calculated.

2.9 Statistical Analysis

All analyses were determined in triplicate. Analysis of variance (ANOVA) and Duncan's multiple comparison were conducted using SPSS20.0 statistical software (Statistical Graphics Corp., Princeton, NJ).

3. Results

3.1 N and genotype effects

3.1.1 Rice grain appearance and chemical composition

N fertilization showed significant effect on grain appearance and chemical composition, but the effect varied with rate and timing of N fertilization, and was also affected by genotype. For appearance quality, N rate had no significant influence on milled rice appearance of Wuyujing3 like grain weight, grain length/width and chalky rice ratio, while it slightly increased grain length/width of Wuyunjing7 (Table 1). Under high N rate, contents of starch and amylose for Wuyujing3 were slightly decreased, whereas those of total protein and crude fat were not significantly changed as compared to low N rate. In contrast, increases in total protein and decreases in crude fat were detected for Wuyunjing7.

With respect to N application timing, topdressing at two weeks after panicle differentiation (spikelet-sustaining fertilization) increased grain weight and chalkiness ratio of both cultivars in comparison with other timing of N application. The chalk rice ratio of Wuyujing3 was 40.5% for CK, and was increased to 61.5% and 60.3% for spikelet-sustaining fertilization at low rate (LN2) and high rate (HN2), respectively. Similarly, the chalk rice ratio of Wuyunjing7 was 24.3% for CK and it was increased to 46.5% for LN2 and 58.5% for HN2 (Table 1). In addition, spikelet-sustaining fertilizer slightly decreased total starch content of Wuyujing3, while increased total protein content of Wuyunjing7 (Table 1).

Wuyujing3 and Wuyunjing7 differed in grain appearance and chemical composition, with the former having smaller grain, larger chalky grain ratio, and higher contents of total starch, amylose, and crude fat. Further, genotypic differences in response to N treatments were noted. Wuyunjing3, the well-known cultivar for its good cooking and eating quality, was less sensitive to N treatments, with coefficients of variation (CVs) of chalk rice ratio, and amylose content, total protein and crude fat content across the 7 N treatments being 26.2%, 1.7%, 7.4%, and 17.6%, respectively. By contrast, Wuyunjing7, the high yielding cultivar with moderate eating quality, was prone to be affected by N fertilizer, with higher values of CVs (Table 1).

3.1.2 Amylopectin chain length distribution

Fig. 1(a) shows typical weight distribution of debranched amylopectin chains of rice flour determined by HPSEC and the empirical division results shown in Table 2. Wuyujing3 had lower percentage of short A chain and more long B_3 chain (Table 2). It has the average $(A+B_1)/(B_2+B_3)$ ratio of 3.68 across 7 N treatments, being lower than that of Wuyunjing7 (4.10). Significant influence of N fertilizer application on amylopectin chain length distribution was detected for both cultivars (Table 2). High

N rate lowered the percentage of short A chain, and consequently decreased ratio of $(A+B_1)/(B_2+B_3)$. In addition, long chains (B_2 and B_3) of Wuyunjing7 were increased significantly with N rate. Timing of N topdressing also significantly influenced the chain length distribution of amylopectin, but it depended on N rate. At low rate, ratio of $(A+B_1)/(B_2+B_3)$ of both cultivars were increased for topdressing at booting stage. However, it was reduced by spikelet-sustaining fertilizer at high N rate.

Further, the debranched amylopectin weight CLD Fig.1(b) was first transformed into number CLD Fig.1(c) and then fitted with the quantitative parameterized model of Wu-Gilbert (Wu et al., 2013), with all the features reproduced well in the fitted number CLD as illustrated in Fig. 1(d). The model provides information on the activities of the core starch synthesizing enzymes and gives insights into starch biosynthesis. The two linear region in the fitting plot Fig.1(d) demonstrated the contributions of two enzyme sets which controlled short single-lamellar region ($DP \leq 30$, enzyme set i) and long trans-lamellar region ($30 \leq DP \leq 60$, enzyme set ii), respectively. From the model fitting, three core parameters (βi, βii and hii/i) whose significance were were summarized here and they differed significantly among N treatments and between Wuyujing3 and Wuyunjing7 (Table 2). Spikelet-promoting N fertilizer at high rate significantly reduced β(i) of both cultivars. However, no clear trend was found in β(ii) for N rate and timing. Wuyujing3 showed lower β(i) (0.140-0.150) than that of Wuyunjing7 (0.140-0.158). In addition, h(ii/i) showed the opposite changing trend in comparison with βi, being increasing with N rate. The results suggested that enzymatic processes required for amylopectin biosynthesis were significantly affected by N and cultivar. By fitting to the biosynthesis-based model, the amylopectin CLD parameters obtained are much preferred over the parameters from the old empircal division method, which can results in arbitrarily chosen DP ranges and different results can be obtained if different ranges are chosen.

3.1.3 Starch pasting properties and granule size

The pasting parameters of flour peak viscosity (PKV), hot paste viscosity (HPV), breakdown viscosity (BDV), cool paste viscosity (CPV), setback viscosity (SBV) and pasting temperature(PaT) of Wuyujing3 and Wuyunjing7 under different N treatments were listed in Table 3. N fertilizer treatments significantly lowered the values of most parameters including PKV, HPV, BDV, and CPV. As the timing of topdressing postponed, HPV and CPV of Wuyujing3 were increased. However, no obvious effect of the timing of topdressing was detected for Wuyunjing7. On average, Wuyujing3 exhibited similar PKV, HPV, BDV, and PaT with those of Wuyunjing7. Notably, CPV and SBV of Wuyujing3 (2743cp, -29cp) were significantly lower than those of Wuyunjing7 (2886cp, 183cp), indicating a more soft texture of cooked rice of Wuyujing3, as is perceived by consumers. Regarding the genotypic difference in response to N treatments, Wuyunjing7 was more sensitive to N application than Wuyujing3, reflected by the higher CV values for most pasting parameters (Table 3).

The progress of starch pasting involves swelling and bursting of starch granule. Previous study by Zhang et al. (2013) has demonstrated that smaller starch granule size can result in lower viscosity as determined by RVA. No significant influence of N treatments on starch granule size was founded for both cultivars. In addition, no distinct difference was recognized between cultivars (Table 3 and Suppl. Fig. S2).

3.1.4 Gelatinzation, retrogradation and crystalline structure of starch

Table 4 shows the gelatinization parameters of rice flour from Wuyujing3 and Wuyunjing7 under N treatments. High input of N fertilizer significantly increased T_p and decreased gelatinization enthalpy (ΔH_g) for Wuyujing3. Similarly, the majority of gelatinization temperatures of Wuyujing7 were increased by high N rate of N. Timing of N topdressing had no significant influence on gelatinization parameters of Wuyujing3, but significantly altered the gelatinization temperatures of Wuyunjing7. On average, Wuyujing3 showed higher gelatinization temperatures and gelatinization enthalpy than those of Wuyunjing7. We also detected an endothermic peak at higher temperature around 95℃, which may be associated with the melting of the amylose-lipid complex (Iturriaga et al., 2004). High rate of N fertilizer significantly decreased the melting enthalpy (ΔH_g) of amylose-lipid complex for both cultivars. The largest gelatinization enthalpy of amylose-lipid complex was observed in Wuyunjing7 when treated with spikelet-sustaining fertilizer, as compared with other N application timing (Table 4). Averagely, Wuyujing3 had significantly lower gelatinization enthalpy of amylose-lipid complex than that of Wuyunjing7.

Retrogradation of gelatinized rice flour of samples was analyzed by DSC after stored at 4℃ for 1 week (Table 4). N application treatments showed no significant influence on retrogradation parameters of both cultivars. Except that there is a slight reduction in retrogradation enthalpy of Wuyujing3 with the increase in N rate. Wuyujing3 and Wuyungjing7 had similar retrogradation temperatures but different retrogradation enthalpy, on average being 2.67J/g and 1.56J/g for Wuyujing3 and Wuyunjing7, respectively (Table 4).

Crystallinity of rice flour closely related with gelatinizatioin and retrogradation. Calculation of relative crystallinity (RC) according to the methods of Wang et al. (2006) revealed no significant N effect. In addition, Wuyujing3 had lower RC value than that of Wuyunjing7 (Table 4 and Suppl. Fig. S2).

3.2 Relationships among rice amylopectin fine structure, chalky rice ratio, and physicochemical properties

Changes in starch fine structure can significantly alter flour physicochemical properties and rice quality. In this study, significant differences in amylopectin chain length distribution under various N treatments were observed. Correspondingly, changes in grain chalkiness and physicochemical properties of rice flour were founded as well. To reveal the potential relationships among rice amylopcetin fine structure and chalky rice ratio and flour physicochemical properties, we compared the amylopectin fine structure, grain appearance, and physicochemical properties of milled-rice flour between low and high N rates, and between cultivars of Wuyujing3 and Wuyunjing7 (Table 5), based on the results of VONVA in Table 1 to Table 4.

Comparison of amylopectin fine structure between N rates showed high N rate had a depressing effect on short amylopectin branches and consequently reduced the ratio of short to long amylopectin branches for both cultivars. Further comparison revealed similar influence of high N rate on PKV, CPV, and AM-lipid ΔH_g for both cultivars, indicating a synergetic effect between amylopectin fine structure and these parameters. On the other hand, high N rate exhibited promoting effect on PaT and flour T_p for both cultivars, suggesting a reverse relation between amylopectin fine structure and these parameters.

It is seen that Wuyujing3 had lower percentage of short amylopectin branches and lower ratio of short to long amylopectin branches than those of Wuyunjing7. The genotypic difference in amylopectin structure may be associated with the differences in CPV, SBV, gelatinization properties and retrogradation properties between Wuyujing3 and Wuyunjing7. In this study, Wuyujing3 showed significantly lower RC than that of Wuyunjing7, this maybe related with their amylopectin CLD difference. However, it has been revealed that the production of crystalline-amorphous lamellar structure beyond simple enzymatic control of branching, such as the strucutre of the whole amylopectin molecule or some enzymatic “shepherding” of the crystallization process or differences in the synthesis conditions due to botancial/verietal differences can significantly influence RC (Witt et al, 2012; 2014). The reason for RC difference between Wuyujing3 and Wuyunjing7 needs further research. In addition, our previous study (Yang et al., 2013) showed that japonica rice varieties with high chalk rice ratio have lower ratio of short/long chains, and similar results were detected in this study, implying the intrinsic relationship between amylopectin biosynthesis and chalkiness formation.

Collectively, comparison of the amylopectin fine structure, grain appearance, and physicochemical properties between N rates and between cultivars revealed that decrease in short amylopectin branches or the ratio of short to long amylopectin branches could cause lower values of pasting properties (except PaT), higher flour gelatinization temperatures (flour T_o, T_p, and T_c), and smaller AM-lipid gelatinization temperatures (Table 5).

4. Discussion

4.1 Relations between starch fine structure and pasting properties, thermal properties and chalkiness

Historically, amylose content was considered as predictor of rice eating and cooking quality. Emerging instrumental techniques have facilitated in-depth research, revealing the relation between the amylopectin fine structure and physicochemical properties of rice grain (Patindol et al. 2015). SBV is generally taken as an indicator of cooked rice texture, being positively correlated with amylose content or the percentage of extreme long chains of amylopectin (Wang et al., 2014). Conversely, our results showed that SBV increased with the increase percentage of short amylopectin branches, and similar change trend was found for CPV. Gilbert et al. (2013) stated that starches with greater amounts of amylopectin chains with DP 6-12 have lower pasting temperatures than those having more longer amylopectin chains (DP>12) within the same botanical origin. Similarly, in this study, at low rate of N application both cultivars showed more percentage of short amylopectin branches and consequently lower pasting temperatures. The amylopectin chain length distribution can influence starch crystal structure and thereby alter gelatinization and retrogradation properties. Wang et al. (2010) demonstrated that the percentage of short chains (DP 6-11) was negatively related to the transition temperatures (T_o, T_p, and T_c). However, Koroteeva et al. (2007) reported that high percentage of very long chains (DP≥37) facilitated the stabilization of the starch granular structure, causing a rising gelatinization temperature and enthalpy. In this study, decrease in short amylopectin branches was accompanied by increase in gelatinization temperatures and gelatinization enthalpy of rice flour. However, for Wuyujing3 under high rate of N fertilizer, decrease in short amylopectin branches was accompanied by concurrent decrease in gelatinization enthalpy, which may be explained as the result of reduction in amylose content, as argued by Biliaderis et al. (1986). Regarding retrogradation, Wuyujing3 showed higher retrogradation enthalpy than Wuyunjing7, being partly associated with fewer short amylopectin branches in Wuyujing3, as explained by Singh et al. (2012). Conversely, Wuyujing3 showed lower retrogradation enthalpy under high N rate, and this is due in part to the reduced amylose content as reported by Shi et al. (2009).

4.2 N effect on starch fine structure and its biological mechanism

To obtain high rice yield and quality, intensive studies have been undertaken to develop efficient methods of N fertilization. However, it is still unclear how N fertilization affects starch fine structure and what is the biological mechanism underlying it. In barley, Gous et al. (2014) observed that high rate of N fertilizer under drought stress can increase the proportions of short amylopectin branches and long amylose branches, while N showed no impact on starch structure under favorable conditions. However, in the current study, we found that high level of N panicle fertilizer, especially spikelet-sustaining fertilizer, reduced the percentage of short amylopectin branches significantly and thereby the ratio of short to long amylopectin branches. These discrepancies maybe due to that starch synthesis is very sensitive to various environmental factors, and thus resulted in different starch structure and functional properties (Patindol et al., 2015). For example, Chun et al. (2015) reported that as the rice ripening temperature increased, the amylose content and number of short amylopectin chains decreased. Therefore, while study the effect of N fertilizer application on rice amylopectin structure, further considerations of environmental factors are required.

Furthermore, by fitting with starch biosynthesis model, we can see that spikelet-sustaining fertilization reduced βi (SBE/SS) and significantly increased h(ii/i) (long chains/short chains), indicating that N may have depressing effect on SBE expression or promoting effect on SS during grain filling, leading to reduced short amylopectin branches and increased very long amylopectin branches. No clear trend was found in β(ii) for N rate and timing, which meant that the enzyme set i may be more sensitive to N treatment and had more significant contribution to the amylopectin CLD compared with enzyme set ii. In this fitting model, there is no direct relation between the CLD and some certain starch biosynthesis enzyme isoforms, thus further analysis on the genetics of these potential enzymes could prove these hypotheses, which may provide information for genetically modifying genes to produce rice with ideal quality.

4.3 Varietal differences in fine structure and other physiochemical traits

With globally rising demand for high quality rice, understanding its physicochemical foundation is of great importance. Lipids are important to rice quality, nutritionally and functionally. Rice cultivars with higher crude fat possessed better sensory quality (Yoon et al., 2012). The current study reveals higher crude fat content in Wuyujing3 than Wuyunjing7, which may be the reason for its good eating quality, as reported in our previous study (Gu et al., 2011). In addition, Wuyujing3 had significantly lower SBV, reflecting well its softer texture. Low amylose content was considered as the major factor contributing to soft texture of cooked rice. However, this study showed that Wuyujing3 had slightly higher apparent amylose content than Wuyunjing7. We speculate that the lack of short amylopectin branches in Wuyujing3 may contribute to its softer structure, and suggest amylopectin chain length distribution be considered as an indicator of cooked rice texture in genetic improvement in rice quality.

The stable expression of rice quality traits in response to nitrogen fertilizer and other environmental/cultural conditions is of significance for rice breeding, cultivating and its end-use in food industry. In this study, significant genotypic variations in quality traits in response to N panicle-fertilizers have been detected in most parameters tested. The premium rice cultivar Wuyujing3 is more stable in response the N-fertilizer application, whereas the medium rice cultivar Wuyunjing7 is more sensitive. This indicates that such genotype like Wuyujing3 can be used in rice quality improvement in future breeding work. However, the physiological and molecular mechanism contributing to this stability needs to be further investigated.

5. Conclusions

As shown by HPSEC, there was a substantial influence of N fertilization and genotype existed for starch fine structure of rice grain. High level of N panicle fertilizer, in particular spikelet-sustaining fertilizer, decreased the percentage of short amylopectin branches and the ratio of short to long amylopectin branches, which could be partly due to the lower relative activity of SBE to SS as explained by the Wu-Gilbert model. The relation between starch fine structure and physicochemical properties of rice grain were evaluated by comparison of CLD between N rates and between cultivars. Results showed that a decrease in short amylopectin branches or the ratio of short to long amylopectin branches correlated with smaller pasting values (except PaT), higher flour gelatinization temperatures (flour T_o, T_p, and T_c), and lower amylose-lipid gelatinization temperatures and enthalpy. Genotypic differences in CLD were recognized between the premium cultivar Wuyujing3 and high-yielding cultivar Wuyunjing7, with the former containing fewer short amylopectin branches. In addition, quality traits of Wuyujing3 were stable in response to N fertilization.

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Table 1 Effects of N rate and timing on appearance of milled rice and grain chemical composition (%, on dry basis)

Cultivar	Nitrogen fertilizer	Grain weight (mg)	Length/ Width	Chalky rice (%)	Total starch (%)	Amylose content (%)	Total protein (%)	Crude fat (%)
Wuyujing3	N0(CK)	21.3d	1.7a	40.5abc	90.88ab	18.45a	5.14b	0.40a
	LN 1	21.9bc	1.6b	45.8abc	90.63ab	18.99a	5.88ab	0.39a
	LN 2	22.7a	1.6b	61.5a	89.55bc	18.87a	6.41ab	0.37a
	LN3	21.1d	1.7a	29.3c	91.41a	18.85a	5.55ab	0.35a
	HN 1	22.00bc	1.7ab	57.7ab	90.09abc	18.43a	6.31ab	0.26a
	HN2	22.3ab	1.6ab	60.3a	88.85c	18.26a	6.87a	0.39a
	HN3	21.6cd	1.7ab	38.3bc	89.78bc	18.20a	6.44ab	0.27a
	Mean	21.8**	1.7**	47.6**	90.05*	18.60**	6.24^{NS}	0.34*
	CV (%)	2.6	3.3	26.2	1.0	1.7	7.4	17.6
VONVA	N_{rate}	0.1	3.2	1.8	6.7*	6.2*	3.2	1.7
	N_{timing}	22.2**	4.2*	10.4**	5.4*	0.2	1.5	0.7
	$N_{rate}×N_{timing}$	4.1*	2.6	0.6	0.8	0.0	0.2	0.9
Wuyunjing7	N0(CK)	22.4bc	1.7ab	24.3c	91.14a	18.75a	5.14c	0.23a
	LN 1	21.4c	1.6b	35.0bc	88.75a	17.65abc	5.56bc	0.32a
	LN 2	23.2ab	1.7a	46.5ab	87.55a	18.11ab	6.17b	0.34a
	LN3	22.3bc	1.6b	23.5c	87.22a	16.82bc	6.01b	0.36a
	HN 1	22.1c	1.8a	43.3b	89.39a	17.63abc	6.07b	0.17a
	HN2	23.4a	1.7a	58.5a	88.62a	17.21bcd	6.91a	0.27a
	HN3	22.3bc	1.7a	27.5c	87.21a	15.92c	7.13a	0.19a
	Mean	22.4	1.7	36.9	88.42	17.22	6.31	0.28
	CV (%)	3.0	4.1	35.5	1.0	4.5	9.4	29..0
VONVA	N_{rate}	1.3	12.8**	4.2	0.2	1.6	15.6**	5.4*
	N_{timing}	10.6**	1.8	15.6**	0.6	3.2	6.1*	0.4
	$N_{rate}×N_{timing}$	0.4	3.8	0.3	0.1	0.4	0.8	0.3

N0: control of fertilizer treatments, CK; HN: high nitrogen rate of 150 kg/ha; LN: low nitrogen rate of 75 kg/ha; 1: spikelet-promoting fertilizer applied at top fourth leaf stage; 2: spikelet-sustaining fertilizer applied at top second leaf stage; 3: top-dressing fertilizer at grain-filling stage. VONVA: Analysis of variance; CV: coefficient of variation. Mean values of Wuyujing3 with * and ** are significantly different with Wuyunjing7 at the 0.05 and 0.01 probability level, respectively. Values with different letters in the same column are significantly different at 0.05 levels. *F*-value in VONVA with* and ** indicates significance 0.05 and 0.01 probability level, respectively. For supporting data, refer to the Supplementary Tables S1.

Table 2 Effects of N rate and timing on grain amylopectin chain length distribution and the biosynthesis-model fitting parameters

Cultivar	Nitrogen fertilizer	Chain lengths distribution of amylopectin					Biosynthesis model fitting parameters		
		A (6≦DP≦12)%	B_1 (13≦DP≦24)%	B_2 (25≦DP≦36)%	B_3 (37≦DP≦60)%	$(A+B_1)/(B_2+B_3)$	β(i)	β(ii)	h(ii/i)
Wuyujing3	N0(CK)	41.16bc	37.15a	12.86a	8.83ab	3.62bc	0.146b	0.085cd	0.0292bc
	LN 1	40.19c	37.04ab	13.32a	9.45a	3.39c	0.141cd	0.083d	0.0319a
	LN 2	41.95b	36.68ab	12.69a	8.68ab	3.68b	0.147b	0.083cd	0.0284c
	LN3	43.73a	37.67a	11.64b	6.95c	4.38a	0.150a	0.094a	0.0232d
	HN 1	41.29bc	37.27a	13.27a	8.17c	3.66b	0.143c	0.089b	0.0283c
	HN2	38.28d	35.44b	12.84a	8.66ab	3.44bc	0.140d	0.086c	0.0314ab
	HN3	41.26bc	37.01ab	12.71a	9.06ab	3.60bc	0.143c	0.083d	0.0297abc
	Mean	41.12 **	36.89*	12.76**	8.53**	3.68**	0.144**	0.086**	0.0289**
	CV (%)	4.0	1.9	4.4	9.4	8.9	2.5	4.8	9.9
VONVA	N_{rate}	21.3**	1.6	2.9	1.4	30.1**	68.5**	2.3	16.2**
	N_{timing}	15.3**	3.3	8.1**	4.7*	43.0**	34.6**	12.5**	23.3**
	$N_{rate}\times N_{timing}$	15.5**	0.9	2.3	18.6**	44.7**	38.2**	73.9**	36.9**
Wuyunjing7	N0(CK)	43.16a	36.77b	11.88bc	8.19ab	3.99b	0.151bc	0.083c	0.0255b
	LN 1	42.90a	37.81a	11.86bc	7.44bc	4.20ab	0.149c	0.091ab	0.0243bc
	LN 2	44.20a	36.95b	11.39c	7.38bc	4.34ab	0.155ab	0.091ab	0.0238bc
	LN3	44.29a	37.66a	11.22c	6.92c	4.52a	0.152bc	0.089abc	0.0213c
	HN 1	43.11a	36.75b	12.31b	7.83bc	3.97bc	0.151bc	0.089abc	0.0264b
	HN2	40.13b	37.74a	13.17a	8.96a	3.53c	0.140d	0.085bc	0.0299a
	HN3	43.12a	37.51a	11.86bc	7.51bc	4.17ab	0.158a	0.093a	0.0246bc
	Mean	42.99	37.31	11.96	7.75	4.10	0.151	0.086	0.0251
	CV (%)	3.2	1.3	5.4	8.6	7.7	3.7	4.0	10.5
VONVA	N_{rate}	15.4**	7.9*	30.2**	10.2**	17.9**	3.5	0.6	20.4**
	N_{timing}	3.9	15.0**	6.3*	4.3*	4.7*	13.5**	1.2^{NS}	7.2**
	$N_{rate}\times N_{timing}$	9.2**	120.7**	5.7*	1.9	2.5	26.2**	4.4*	2.0

N0: control of fertilizer treatments, CK; HN: high nitrogen rate of 150 kg/ha; LN: low nitrogen rate of 75 kg/ha; 1: spikelet-promoting fertilizer applied at top fourth leaf stage; 2: spikelet-sustaining fertilizer applied at top second leaf stage; 3: top-dressing fertilizer at grain-filling stage. VONVA: Analysis of variance; CV: coefficient of variation. Values with different letters in the same column are significantly different at 0.05 levels. NS means no significant at P=0.05. *F-value significant at the 0.05 probability level. ** F-value significant at the 0.01 probability level. For supporting data, refer to the Supplementary Tables S2.

Table 3 Effects of N rate and timing on pasting properties of milled-rice flour and starch granule size

Cultivar	Nitrogen fertilizer	PKV (cP)	HPV (cP)	BDV (cP)	CPV (cP)	SBV (cP)	PaT(℃)	Starch granule size (μm)
Wuyujing3	N0(CK)	2904a	1777a	1128ab	2921a	16a	76.9b	4.681a
	LN 1	2774ab	1572c	1202a	2677cd	-98b	76.6b	4.991a
	LN 2	2777ab	1647bc	1130ab	2773bc	-3a	77.1b	5.081a
	LN3	2844ab	1715ab	1129ab	2827ab	-17ab	76.6b	4.672a
	HN 1	2693b	1564c	1129ab	2632d	-60ab	77.9ab	4.700a
	HN2	2709b	1619bc	1089b	2663d	-30ab	79.9a	4.811a
	HN3	2703b	1620bc	1099ab	2705cd	-15ab	78.7ab	4.958a
	Mean	2772	1645	1130	2743**	-29**	77.7	4.842
	CV (%)	2.9	4.7	3.2	3.8	129.7	1.6	3.5
VONVA	Nrate	5.3*	2.0	3.9	10.8**	0.0	9.79**	0.3
	Ntiming	0.3	3.7*	2.2	5.3**	4.2*	1.26	0.2
	Nrate×Ntiming	0.3	0.7	0.3	0.8	0.8	0.49	1.1
Wuyunjing7	N0(CK)	3058a	1872a	1186a	3231a	173a	74.7c	5.388ab
	LN 1	2785b	1645b	1140ab	2938bc	153a	77.2bc	4.603ab
	LN 2	2777b	1675b	1102ab	2972b	194a	78.0ab	5.152ab
	LN3	2792b	1649b	1144ab	2964b	172a	77.2bc	4.499b
	HN 1	2659b	1577b	1082ab	2837bc	178a	77.9ab	5.532a
	HN2	2620b	1580b	1040b	2839bc	219a	80.4a	4.450b
	HN3	2584b	1554b	1030b	2766c	183a	79.1ab	4.812ab
	Mean	2754	1613	1090	2886	183	77.8	4.919
	CV (%)	5.8	3.0	4.4	2.9	12.2	2.3	8.9
VONVA	Nrate	5.3*	5.3*	4.2*	7.3*	0.8	4.40*	0.5
	Ntiming	0.1	0.2	0.4	0.2	1.1	1.36	0.9
	Nrate×Ntiming	0.1	0.1	0.2	0.3	0.0	0.39	3.6

N0: control of fertilizer treatments, CK; HN: high nitrogen rate of 150 kg/ha; LN: low nitrogen rate of 75 kg/ha; 1: spikelet-promoting fertilizer applied at top fourth leaf stage; 2: spikelet-sustaining fertilizer applied at top second leaf stage; 3: top-dressing fertilizer at grain-filling stage. VONVA: Analysis of variance; CV: coefficient of variation. PKV: peak viscosity; HPV: hot paste viscosity; BDV: breakdown viscosity; SBV: setback viscosity; PaT: paste temperature. Values with different letters in the same column are significantly different at 0.05 levels. NS means no significant at P=0.05. *F-value significant at the 0.05 probability level. ** F-value significant at the 0.01 probability level. For supporting data, refer to the Supplementary Tables S3.

Table 4 Effects of N rate and timing on gelatinization and retrogradation properties of milled-rice flour and crystallinity

Cultivar	Nitrogen fertilizer	Gelatinization								Retrogradation				Relative Crystallinity (RC, %)
		Flour endoderm				Amylose-lipid complex endoderm								
		To /℃	Tp/℃	Tc/℃	ΔH_g (J/g)	To /℃	Tp/℃	Tc /℃	ΔH_g (J/g)	To /℃	Tp/℃	Tc /℃	ΔH_r (J/g)	
Wuyujing3	CK	59.09a	65.68ab	72.94ab	9.63a	87.61a	95.57a	102.03a	0.90a	39.66a	50.12a	58.67a	2.65ab	30.7a
	LN 1	59.90a	65.49b	72.22c	9.20ab	86.12b	94.93ab	100.75ab	0.96a	38.98a	49.86a	58.63a	2.40b	28.3a
	LN 2	59.54a	65.83ab	73.00ab	9.24a	86.90ab	95.52a	101.43a	0.92a	39.58a	49.79a	58.32a	3.16a	28.6a
	LN3	59.29a	65.58ab	73.56bc	9.48a	86.45b	95.22a	101.74a	0.99a	40.07a	49.74a	57.72a	2.97ab	27.4a
	HN 1	59.15a	65.77ab	72.59ab	8.70c	86.37b	94.58ab	101.15ab	0.88a	39.95a	50.37a	58.55a	2.38b	27.4a
	HN2	59.50a	65.99a	72.90ab	8.77bc	86.39b	94.44ab	100.96ab	0.71b	39.63a	50.37a	58.61a	2.44b	30.2a
	HN3	59.29a	65.94ab	73.21a	8.75bc	86.30b	93.83b	99.62b	0.84ab	39.75a	49.76a	58.12a	2.67ab	28.6a
	Mean	59.39**	65.75**	72.92**	9.11**	86.59	94.87	101.10	0.89**	39.66	50.00**	58.37	2.67**	28.7**
	CV (%)	0.5	0.3	0.6	4.1	0.6	0.7	0.8	10.4	0.9	0.6	0.6	11.3	4.4
VONVA	N_{rate}	0.7	4.2*	3.6	16.9**	0.2	6.2*	2.4	15.2**	0.7	3.5	0.4	4.9*	0.3
	N_{timing}	0.2	1.6	4.6*	0.5	0.5	0.5	0.4	3.0	0.9	1.4	1.5	3.2*	0.8
	$N_{rate}\times N_{timing}$	0.5	0.2	1.9	0.3	0.5	0.7	0.5	0.9	2.0	0.8	0.2	1.7	0.5
Wuyunjing7	CK	57.48cd	64.16c	70.89bc	8.69a	86.69a	94.95ab	101.45ab	1.32a	40.70a	50.35ab	58.28b	1.74a	29.4b
	LN 1	57.13d	63.97c	70.69c	8.39ab	86.56a	95.06a	101.66a	1.16b	39.19a	50.04b	58.81ab	1.64a	31.6ab
	LN 2	58.15ab	64.49b	71.17b	8.64a	85.65a	94.46b	101.20ab	1.22ab	40.46a	50.39ab	58.81ab	1.49b	31.5ab
	LN3	57.75bc	64.49b	71.03bc	8.62a	85.71a	94.87ab	101.27ab	1.20b	39.75a	50.49ab	58.66ab	1.56a	33.0a
	HN 1	57.11d	64.06c	70.81bc	8.46ab	86.46a	94.87ab	100.76b	0.91d	39.25a	50.50ab	59.01ab	1.65a	31.9ab
	HN2	58.25ab	65.00a	71.62a	8.24ab	86.26a	94.60ab	101.05ab	1.10bc	39.16a	50.54ab	59.94a	1.65a	31.5ab
	HN3	58.59a	65.04a	71.56a	7.95b	85.65a	94.46b	100.93ab	1.03c	40.08a	50.95a	58.64ab	1.22b	31.0ab
	Mean	57.78	64.46	71.11	8.43	86.14	94.75	101.19	1.13	39.8	50.47	58.88	1.56	31.4
	CV (%)	1.0	0.7	0.5	3.1	0.5	0.2	0.3	11.9	1.6	0.5	0.9	10.9	3.4
VONVA	N_{rate}	5.5*	20.1**	11.5**	3.7	0.3	1.0	14.7**	40.1**	0.5	4.6*	1.3	0.5	0.5
	N_{timing}	29.6**	55.3**	13.4**	0.3	3.6*	2.8	0.3	6.2**	1.1	2.5	1.3	3.9*	0.2
	$N_{rate}\times N_{timing}$	4.2*	3.3*	1.3	1.6	0.8	1.0	3.5*	1.8	1.5	0.4	0.9	3.6*	0.8

CK: control of fertilizer treatments; HN: high nitrogen rate of 150 kg/ha; LN: low nitrogen rate of 75 kg/ha; 1: spikelet-promoting fertilizer applied at top fourth leaf stage; 2: spikelet-sustaining fertilizer applied at top second leaf stage; 3: top-dressing fertilizer at grain-filling stage. VONVA: Analysis of variance; CV: coefficient of variation. Values with different letters in the same column are significantly different at 0.05 levels. NS means no significant at P=0.05. *F-value significant at the 0.05 probability level. ** F-value significant at the 0.01 probability level. For supporting data, refer to the Supplementary Tables S4.

Table 5 Comparison of amylopectin fine structure, grain appearance, and physicochemical properties of milled-rice flour between low and high N rates, and between cultivars of Wuyujing3 and Wuyunjing7

Parameters	**N rates**		**Cultivars**
	Wuyujing3	**Wuyunjing7**	**(Wuyujing3 vs Wuyunjing7)**
Amylopectin fine strucutre			
A chains	▼	▼	▼
B_1 chains	−	▼	▼
B_2 chains	−	▲	▲
B_3 chains	−	▲	▲
$(A+B_1)/(B_2+B_3)$	▼	▼	▼
Chalk rice ratio	−	−	▲
RC	−	−	▼
Pasting properties			
PKV	▼	▼	−
HPV	−	▼	−
BDV	−	▼	−
CPV	▼	▼	▼
SBV	−	−	▼
PaT	▲	▲	−
Gelatinization properties			
Flour To	−	▲	▲
Flour Tp	▲	▲	▲
Flour Tc	−	▲	▲
Flour ΔH_g	▼	−	▲
AM-lipid To	−	−	−
AM-lipid Tp	▼	−	−
AM-lipid Tc	−	▼	−
AM-lipid ΔH_g	▼	▼	▼
Retrogradation properties			
Flour To	−	−	−
Flour Tp	−	▲	▼
Flour Tc	−	−	−
Flour ΔH_r	▼	−	▲

▲: significantly higher for high N rate than low N rate, or for the cultivar Wuyujing3 than Wuyunjing7. ▼: significantly lower for high N rate than low N rate, or for the cultivar Wuyujing3 than Wuyunjing7. −: no significant difference between N rates or between cultivars.

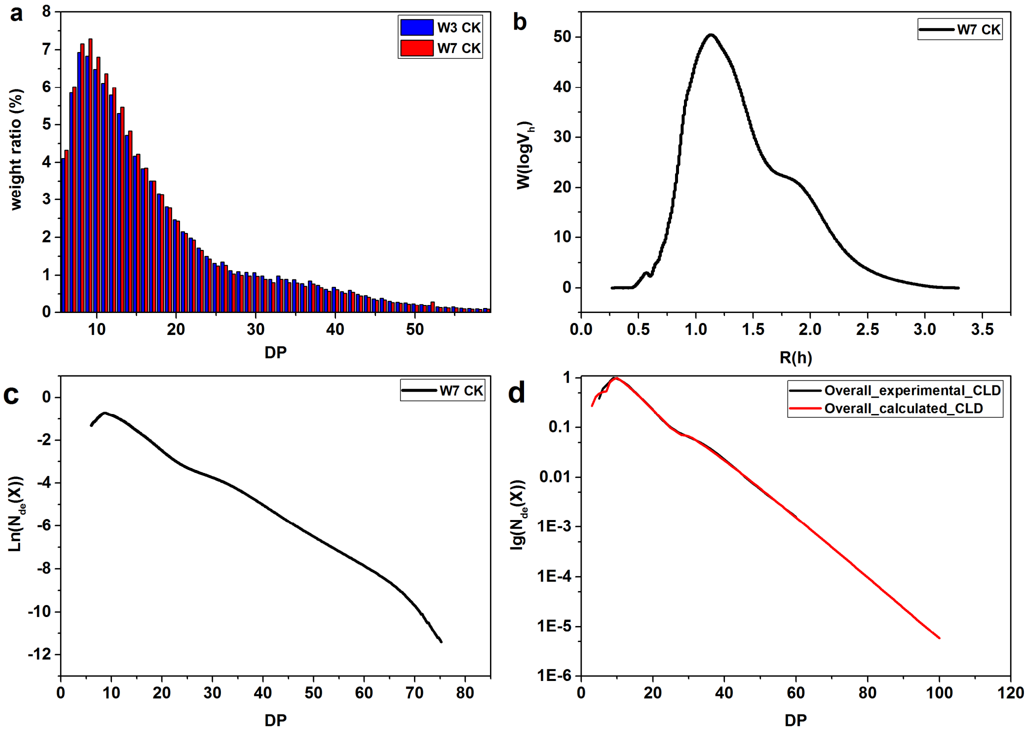

Fig.1 Representative distribution of rice debranched amylopectin. a) HPSEC-normalized number distribution variance, b) Starch size-weight distribution, c) Chain length number distribution, d) Model fitting of amylopectin biosynthesis

Highlights

- We aimed to elucidate N effect on chain length distribution of amylopectin;
- N lowered the ratio of short chains of amylopectin in rice grain;
- N decreased the relative activity of SBE to SS;
- Quality traits of premium rice cultivar expressed stably across N treatments.