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Characterization of high-yielding rice cultivars with different grain-filling properties to clarify limiting factors for improving grain yield

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ABSTRACT

Recently developed high-yielding rice varieties with extra-large sink capacity often have unstable grain filling. Therefore, understanding the factors that limit grain filling is essential for further improvement of rice grain yield. Because grain-filling is determined by the complex sink–source balance, grain-filling ability is very difficult to evaluate. In this study, we compared grain-filling-related traits of three high-yielding cultivars with high sink capacity. We found that the translocation of non-structural carbohydrates (NSC) from stem to panicle during early ripening and grain filling was significantly lower in Momiroman than in Hokuriku 193 and Teqing, whereas dry matter accumulation of the whole plant did not differ among the cultivars throughout ripening. The NSC-components, sucrose and starch were both remained higher in stems of Momiroman than other cultivars. ADP-glucose pyrophosphorylase (AGPase; EC 2.7.7.27) activity was not enhanced and α-Amylase (EC 3.2.1.1) and β-Amylase (EC 3.2.1.2) activities were not impeded in the stems. These data suggested lower sucrose translocation is responsible for lower NSC translocation in Momiroman, rather than too high starch synthesis and too low starch degradation activities. At early ripening, grain growth of the superior spikelets was slow in Momiroman even if carbon supply was increased by spikelet-thinning treatments. These results raise the possibility that low sink strength determines low grain filling in Momiroman, which delays grain growth and decreases sucking force of NSC from the stem.

1. Introduction

Rice (*Oryza sativa* L.) is one of the world's most important crops, and its yield must be improved to feed the increasing global population. In Japan, the demand for high-yielding rice for animal feed has increased; to match it, many high-yielding cultivars have been developed. Although they commonly have extra-large sink capacity (total number of spikelets per unit area × filled grain weight), the grain-filling ability of different cultivars varies considerably (Yoshinaga et al., 2013). Chinese high-yielding cultivars, called 'super rice', also have unstable grain filling (Yang and Zhang, 2010).

The poor grain filling of high-yielding rice often occurs in inferior spikelets, which are located on the secondary branchs in the lower part of a panicle (Yang and Zhang, 2010; Yoshinaga et al., 2013). Some studies showed that removing superior spikelets of large-panicle cultivars improves grain filling of the inferior ones, suggesting that grain filling of the inferior spikelets is restricted by the supply of assimilated carbon (Kato, 2004; Kobata et al., 2013; You et al., 2016). Other studies suggested that the low starch-synthesis rate of inferior spikelets, caused by low enzyme activity or hormone imbalances, leads to poor grain filling (Yang et al., 2006; Wang et al., 2015; Zhang et al., 2012). Whether the carbohydrate supply (source ability) or starch-synthesis rate (sink strength) restricts grain filling may depend on the cultivar, location and field conditions. To understand the grain-filling properties and factors that limit rice cultivars with high yield potential, it is essential to analyse them in the same environment for further improvement of rice grain yield.

Grain-filling ratio is determined by the complex sink–source

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balance. High sink capacity requires high source ability for stable grain filling. Because translocation of non-structural carbohydrates (NSC) from stem to panicle is indispensable for stable grain filling (Yoshida 1972; Okamura et al., 2013), source ability for grain-filling is affected not only by the ability of carbon assimilation in leaves, but also by that of carbon translocation from stems to panicles. Therefore, to evaluate differences in grain filling, we must understand the differences and relationships of many traits such as sink size, dry matter accumulation and stem-carbon metabolism.

Momiroman is a Japanese high-yielding cultivar that showed one of the highest sink capacities but low grain-filling ratio in Tsukubamirai, Ibaraki, in the Kanto region of Japan (Hirabayashi et al., 2010; Yoshinaga et al., 2013). Another Japanese high-yielding cultivar Hokuriku 193 (H193) showed the highest yield with a relatively high grain-filling ratio at the same location (Goto et al., 2009; Yoshinaga et al., 2013). We compared the grain-filling properties and stem-carbon metabolism of Momiroman and H193 in terms of the sink–source balance. We also tested a Chinese high-yielding *indica* cultivar, Teqing. Based on these analyses, we deduced the possible factors limiting grain filling of high-yielding cultivars, which could be a target trait for achieving high yield potential.

2. Materials and methods

2.1. Plant materials and growth conditions

The rice cultivar Momiroman, H193 and Teqing were planted in the Yawara experimental paddy field of the National Agriculture and Food Research Organization (NARO) at Tsukubamirai, Ibaraki, Japan (36°00′N, 140°02′E, altitude above sea level: 10 m) in 2014–2016. The climate condition was shown in Supplementary Fig. S1. Momiroman and H193 are Japanese *indica-japonica* hybrid high-yielding cultivars and their major genetic backgrounds are assumed to be *japonica* and *indica*, respectively (Yonemaru et al., 2014). Teqing is a Chinese high-yielding *indica* cultivar. Seedlings (20–24 days old) were transplanted on 15 May 2014, 21 May 2015 and 19 May 2016. The plants were grown at a density of 22.2 hills m^{-2} (spacing of 15 cm × 30 cm), with one plant per hill. The plot size was 6.50 m^2 in 2014, 47.25 m^2 in 2015 and 10.80 m^2 in 2016. The plots were arranged in a randomized block design with three replicates, except for two replicates of Teqing in 2014. Controlled-release fertilizer (equal parts LP40, LPs100 and LP140; JCAM Agri, Tokyo, Japan) containing 16 g N m^{-2} was applied as a basal nitrogen dressing. LP40, LPs100 and LP140 release 80% of their total nitrogen content within 40, 100 and 140 days, respectively, after application, at 25 °C. Inorganic fertilizers containing 15 g P_2O_5 m^{-2} and 15 g K_2O m^{-2} in 2014 and 2015 and 20 g P_2O_5 m^{-2} and 15 g K_2O m^{-2} in 2016 were applied as a basal phosphorus and potassium dressing.

2.2. Yield and yield components

At maturity, when approximately 85% of grains became yellow, 20 plants were harvested in 2014 and 40 in 2015 and 2016; plants were air-dried for more than 2 weeks. The panicles were counted and then threshed; paddy (whole grains with hull) were weighed. A 30–60 g aliquot of paddy was separated using a Sample Divider (Fujikinzoku, Tokyo, Japan) for spikelet counting. Grains were counted on an auto-counter. Half of the paddy were hulled and weighed to rough (whole) brown rice yield. Then the rough brown rice was sieved with a grain sorter with a sieve size of 1.6 mm and retained grains were weighed to calculate actual brown rice yield. A 20–40 g aliquot of retained grains was counted to calculate 1000-g weight, number of filled spikelets, and the moisture content was measured using a grain moisture tester (Riceter f, Kett, Tokyo, Japan). Rough paddy yield, rough brown rice yield, brown rice yield and 1000-grain weight were adjusted to 15% (w/w) moisture content. Sink capacity was estimated by multiplying single grain yield by the number of spikelets per area, assuming all the spikelets were completely filled (Yoshinaga et al., 2013). Harvest index was calculated by dividing dry weight of sieved brown rice by shoot dry weight measured as described below.

2.3. Dry weight and non-structural carbohydrate content

At full heading, at about 20 days after heading (DAH) and at maturity, 10 plants per plot were harvested. The heading date and full-heading date were defined as the dates when about 50% and 80%, respectively, of panicles had emerged. The sampling dates for each cultivar and stage are shown in Supplementary Table S1. Two representative plants among 10 harvested plants with an average number of panicles were separated into panicles, leaf sheaths + culms (stems), and leaf blades. Those and the other 8 plants were dried on the same day at 80 °C for at least a week and the dry weights were determined. The total shoot weight per m^2 of the 10 plants was calculated. The weight of each part was calculated from that of the entire shoot multiplied by the ratio of the part in the 2 representative plants. These stems were powdered in a vibrating sample mill (TI-1001, CMT Co., Tokyo, Japan) for measurement of stem NSC content. The contents of starch, sucrose, glucose and fructose in the powdered samples were measured according to Okamura et al. (2016) with glucoamylase (Toyobo, Osaka, Japan), an F-kit #716260 (J.K. International, Tokyo, Japan) and a microplate reader (Sunrise, Tecan, Männedorf, Switzerland, or Epoch 2, BioTek, Winooski, VT, USA). The NSC content was calculated as the sum of the contents of these carbohydrates. The apparent NSC translocation (ΔNSC) is estimated from the difference of NSC content between full heading and 20 DAH. Apparent translocation ratio of NSC (Ratio of ΔNSC) was estimated by dividing ΔNSC by NSC content at Full heading.

2.4. Heading and flower-opening days, and spikelet thinning

We classified spikelets into six groups by their position within a panicle as follows (see also Supplementary Fig. S2a). First, panicles were divided into upper and lower parts so that the number of primary branches was the same (the middle branch was placed in the upper part when the number of primary branches was odd). Thereafter, spikelets in each part were divided into (A) those on the primary branch, (B) those on the secondary branch except its tip and (C) those on tip of the secondary branch. On 9–13 August 2015, the panicles that had just started heading were labelled. Immediately after labelling or the next day, at least five opening spikelets of the upper A group of H193 and Teqing were marked with colour markers. Two days after labelling, those of Momiroman were marked. Four days after labelling, the opening spikelets of the lower B group in the labelled panicles of H193 and Teqing were marked. Six days after labelling, those of Momiroman were marked. In addition, spikelets in some panicles were thinned out (Supplementary Fig. S2b, c) by marking three upper A or lower B opening spikelets on the same primary branch of a labelled panicle and removing all other spikelets on the same primary branch (spikelets on the other primary branches remained intact). The numbers of harvested panicles and grains are shown in Supplementary Table S2.

2.5. Carbohydrate contents and AGPase and amylase activities in stems

On 31 July 2015 (11 days before anthesis) and at 0, 7, 14, 21 and 28 days after spikelet marking (days after anthesis, DAA), the third internodes from the uppermost internodes and the sheaths of the third leaves from the flag leaves, which had the highest NSC contents among elongating internodes in Momiroman and H193 (our unpublished observations), were harvested, immediately frozen in liquid N_2 and ground under cryogenic conditions in a ShakeMaster Auto (Bio Medical Science, Tokyo, Japan). Starch, sucrose, glucose and fructose contents of the ground samples were measured as described in 2.3. ADP-glucose

Table 1
Yield and yield components.

Year	Cultivar	Panicle number (m^{-2})	Spikelets per panicle	Spikelet number ($\times 10^3 m^{-2}$)	1000-grain weight (g)	Ratio of filled grains (%)	Rough paddy yield ($g m^{-2}$)	Rough brown rice yield ($g m^{-2}$)	Brown rice yield ($g m^{-2}$)	Sink capacity ($g m^{-2}$)	Harvest index (%)
2014	Momiroman	213 ± 4	250 ± 9	53.4 ± 1.0	23.1 ± 0.1	54.8 ± 1.5	1103 ± 3	762 ± 6	674 ± 4	1233 ± 28	31.2 ± 0.3
	H193	225 ± 8	223 ± 4	50.0 ± 2.2	22.7 ± 0.0	79.9 ± 2.3	1261 ± 47	927 ± 41	907 ± 43	1135 ± 49	37.2 ± 0.4
	Teqing	220 ± 18	247 ± 9	54.3 ± 2.6	21.0 ± 0.0	74.6 ± 5.0	1195 ± 31	905 ± 6	848 ± 16	1140 ± 54	41.4 ± 2.5
2015	Momiroman	248 ± 6	174 ± 6	43.1 ± 2.0	22.2 ± 0.6	45.1 ± 2.5	792 ± 53	482 ± 48	434 ± 42	960 ± 67	17.9 ± 2.1
	H193	233 ± 5	205 ± 3	47.7 ± 1.3	22.0 ± 0.5	83.7 ± 2.5	1177 ± 47	887 ± 37	880 ± 37	1051 ± 23	33.9 ± 0.5
	Teqing	260 ± 9	239 ± 16	62.1 ± 4.0	21.1 ± 0.3	69.8 ± 2.8	1264 ± 31	947 ± 24	911 ± 20	1311 ± 75	44.6 ± 0.2
2016	Momiroman	223 ± 5	228 ± 2	50.8 ± 1.1	24.4 ± 0.3	57.8 ± 1.4	1057 ± 18	756 ± 10	715 ± 10	1238 ± 16	32.9 ± 2.1
	H193	202 ± 9	227 ± 17	45.6 ± 1.6	23.1 ± 0.1	85.9 ± 0.6	1176 ± 38	912 ± 29	905 ± 29	1054 ± 40	38.0 ± 1.1
	Teqing	210 ± 9	269 ± 11	56.5 ± 0.2	22.5 ± 0.1	73.1 ± 0.6	1247 ± 5	969 ± 7	931 ± 8	1272 ± 0	45.1 ± 2.9
mean	Momiroman	228a	217b	49.1b	23.2a	52.6c	984b	666b	608b	1144b	27.3c
	H193	220a	218b	47.8b	22.6b	83.2a	1205a	909a	897a	1080b	36.4b
	Teqing	230a	252a	57.6a	21.5c	72.5b	1235a	940a	897a	1241a	43.7a
ANOVA	Year (Y)	**	**	**	**	*	**	**	**	n.s.	**
	Cultivar (C)	n.s.	**	**	**	**	**	**	**	**	**
	Y × C	n.s.	*	**	n.s.	*	**	**	**	**	**

Ratio of filled grains = filled spikelet number ÷ total spikelet number. Sink capacity = spikelet number × 1000-grain yield ÷ 1000. Ratio of unfilled brown rice = weight of brown rice that passed through the sieve ÷ rough grain weight. Harvest index = dry weight of sieved brown rice ÷ to dry weight of shoot × 100. Values are means ± SE ($n = 3$). Different letters indicate statistically significant differences between cultivars ($P < .05$, Tukey's test). $^*P < .05$, $^{**}P < .01$ (ANOVA). n.s., not significant.

pyrophosphorylase (AGPase; EC 2.7.7.27) activity was measured according to Okamura et al. (2013). In brief, the extracted proteins were assayed in a reaction mixture containing 106 mM HEPES·NaOH (pH 7.5), 20 mM 3-phosphoglyceric acid, 2 mM ADP glucose, 3.2 mM pyrophosphoric acid, 5 mM $MgCl_2$ and 10 mM DTT at 30 °C for 10 min. The amount of glucose 1-phosphate produced by AGPase activity was measured by the enzymatic method on a microplate reader (Epoch 2, BioTek). α-Amylase (EC 3.2.1.1) activity was measured according to Sugimura et al. (2015) using a Ceralpha assay kit (Megazyme, Co. Wicklow, Ireland). β-Amylase (EC 3.2.1.2) activity was measured according to Hirano et al. (2016) using a Betamyl-3 assay kit (Megazyme). Both methods were slightly modified as follows. Ground samples (~100 mg) were extracted with the extraction buffer containing 50 mM MOPS-KOH (pH 7.5), 20 mM $MgCl_2$, 2 mM $CaCl_2$, 2 mM EDTA, 0.1% bovine serum albumin, 2% (w/v) polyvinylpolypyrrolidone and 0.1% (v/v) 2-mercaptoethanol. Samples were centrifuged at 20,000 × *g* for 10 min, and 25 µL of the supernatant was combined with 25 µL of substrate solution containing blocked *P*-nitrophenyl maltoheptaoside (BPNPG7) for α-amylase or *P*-nitrophenyl-β-maltotrioside (PNPβ-G3) for β-amylase and 100 µL of the reaction buffer containing 50 mM sodium acetate (pH 7.5), 2 mM EDTA, 0.1% bovine serum albumin and 0.1% (v/v) 2-mercaptoethanol. After 15 min, 500 µL of 1% (w/v) Tris·HCl (pH 8.5) was added and the absorbance was measured at 400 nm on a microplate reader (Epoch 2).

2.6. Distribution of grain weight

At maturity, two panicles from three plots (total six panicles) with marked upper A spikelets were harvested from each cultivar. Panicles were freeze-dried in vacuo; marked spikelets were kept for the analysis of grain growth rate and the other spikelets were divided into six groups: upper A–C and lower A–C (see Section 2.4). Grains with hull of each group were weighed individually using an automatic counting and weighing system (QWCALC, NK-Systems, Aichi, Japan).

2.7. Grain growth rate

At 0, 7, 14, 21 and 28 DAA and at maturity, panicles were harvested and immediately frozen in liquid N_2. The numbers of harvested panicles and spikelets are shown in Supplementary Table S2. Panicles were freeze-dried in vacuo, and from the marked spikelets were weighed individually using QWCALC. The mean grain weight data were fitted to a flexible sigmoid model considering the flower-opening day as the moment at which grain growth begins (Yin et al., 2003):

$$W = W_b + (W_m - W_b)\left(1 + \frac{t_e - t}{t_e - t_m}\right)\left(\frac{t}{t_e}\right)^{\frac{t_e}{t_e - t_m}} \quad (1)$$

where W is the grain weight (mg), W_b and W_m are the initial and maximum grain weights, respectively, t is the time (days after spikelet opening), t_e is the end of the growth period and t_m is the time when the maximum growth rate is achieved. W_m (except for those of the inferior spikelets of Momiroman and Teqing), t_e and t_m were calculated by the Gauss–Newton method in SAS v. 9.4 software (SAS Institute Inc., Cary, NC, USA). Grain weight measured on the spikelet-opening day was considered as W_b. Because the grain weight of Momiroman and Teqing did not reach a plateau even at maturity, the W_m of the inferior (lower B) spikelets (W_{mi}) was calculated from that of the superior (upper A) spikelet (W_{ms}), W_b of the superior spikelet (W_{bs}) and W_b of the inferior spikelet (W_{bi}):

$$W_{mi} = \frac{W_{bi}}{W_{bs}} W_{ms} \quad (2)$$

The maximum growth rate (C_m) was calculated as:

$$C_m = \frac{2t_e - t_m}{t_e(t_e - t_m)}\left(\frac{t_m}{t_e}\right)^{\frac{t_m}{t_e - t_m}}(W_m - W_b) \quad (3)$$

2.8. Statistical analysis

Statistical analysis was conducted in R software (R Core Team, 2017). Following analysis of variance (ANOVA) with cultivar and year as fixed factors, cultivars were compared by Tukey's test.

3. Results

3.1. Grain yield and yield components

Spikelet number per panicle was higher in Teqing than in Momiroman and H193 (Table 1). As there was no difference in panicle numbers among the cultivars, total spikelet number was higher in

Table 2
Shoot dry weight and shoot growth rate during ripening.

Year	Cultivar	Shoot dry weight (g DW m^{-2})			Shoot growth rate (g DW m^{-2} day^{-1})		
		Full heading	20 DAH	Maturity	FH–20	20–M	FH–M
2014	Momiroman	1082 ± 96	1468 ± 129	2142 ± 103	19.3 ± 3.9	20.4 ± 4.4	20.0 ± 3.2
	H193	1240 ± 104	1490 ± 115	2231 ± 18	12.5 ± 10.6	22.4 ± 3.8	18.7 ± 1.7
	Teqing	1384 ± 565	1490 ± 609	1966 ± 817	5.3 ± 0.8	14.0 ± 3.9	10.8 ± 2.5
2015	Momiroman	1579 ± 49	1618 ± 34	2073 ± 79	1.7 ± 1.4	9.3 ± 2.2	6.9 ± 1.4
	H193	1679 ± 26	1927 ± 27	2211 ± 116	11.3 ± 2.3	6.4 ± 2.0	8.1 ± 2.1
	Teqing	1372 ± 26	1683 ± 59	1737 ± 38	14.1 ± 2.6	1.3 ± 1.1	5.7 ± 0.3
2016	Momiroman	1489 ± 40	1698 ± 25	1865 ± 119	9.9 ± 1.9	5.2 ± 4.5	7.1 ± 2.7
	H193	1511 ± 62	1839 ± 74	2030 ± 108	16.4 ± 4.6	6.4 ± 1.5	10.4 ± 2.7
	Teqing	1582 ± 38	1599 ± 21	1771 ± 134	0.8 ± 2.2	4.7 ± 3.0	4.1 ± 2.3
mean	Momiroman	1383a	1595b	2027ab	10.3a	11.6a	11.3ab
	H193	1477a	1752a	2157a	13.4a	11.7a	12.4a
	Teqing	1446a	1591b	1825b	6.7a	6.7a	6.9b
ANOVA	Year (Y)	**	**	*	n.s.	***	**
	Cultivar (C)	n.s.	*	**	n.s.	n.s.	*
	Y × C	**	n.s.	n.s.	n.s.	n.s.	n.s.

Shoot growth rate = difference of shoot dry weight ÷ number of days. DAH: days after heading. FH–20: full heading to 20 DAH. 20–M: 20 DAH to maturity. FH–M: full heading to maturity. Values are means ± SE (n = 3). Different letters indicate statistically significant differences between cultivars ($P < .05$, Tukey's test). *$P < .05$, **$P < .01$ (ANOVA). n.s., not significant.

Teqing than in Momiroman and H193. Momiroman had the highest 1000-grain weight and Teqing had the lowest. H193 had the highest ratio of filled grains and Momiroman had the lowest. For this reason, the rough paddy yield, rough brown rice yield, and the brown rice yield of Momiroman was significantly lower than those of H193 and Teqing on 3-year average. Sink capacity was highest in Teqing, and there was no difference between Momiroman and H193. Harvest index was highest in Teqing and lowest in Momiroman.

3.2. Dry matter accumulation and non-structural carbohydrates in stems

Shoot dry weights at full heading and maturity, and shoot growth rate during ripening of Momiroman were not different from those of H193 and Teqing (Table 2). Shoot dry weights at 20 DAH and maturity were lower in Teqing than in H193. Panicle dry weight at 20 DAH and panicle growth rate from full heading to 20 DAH of Momiroman were significantly lower than those of H193 and Teqing (Table 3). There was no difference among the cultivars in panicle growth rate from 20 DAH to maturity. Stem NSC content at full heading was lower in Momiroman than in H193 and Teqing, whereas contents at 20 DAH and maturity were higher in Momiroman and H193 than in Teqing (Table 4). Therefore, the decrease in NSC content from full heading to 20 DAH, which appears to indicate the amount of NSC translocated from stem to panicle, was much smaller in Momiroman than in H193 and Teqing. Stem NSC content at maturity was much lower and its decrease from full heading to maturity was much greater in Teqing than in the other two cultivars. Apparent translocation ratio of NSC (Ratio of ΔNSC) from full heading to 20 DAH was highest in Teqing and lowest in Momiroman.

3.3. Stem carbohydrate contents and activities of enzymes related to starch metabolism

To investigate whether starch metabolism in stems is responsible for

Table 3
Panicle dry weight and shoot growth rate during ripening.

Year	Cultivar	Panicle dry weight (g DW m^{-2})			Panicle growth rate (g DW m^{-2} day^{-1})		
		Full heading	20 DAH	Maturity	FH–20	20–M	FH–M
2014	Momiroman	116 ± 13	447 ± 4	1127 ± 43	16.5 ± 0.8	20.6 ± 1.2	19.1 ± 1.1
	H193	146 ± 9	585 ± 83	1157 ± 35	22.0 ± 4.6	17.3 ± 3.6	19.1 ± 0.5
	Teqing	222 ± 91	644 ± 263	1192 ± 493	21.1 ± 2.1	16.1 ± 6.3	18.0 ± 5.0
2015	Momiroman	264 ± 11	543 ± 29	793 ± 66	12.7 ± 1.8	5.1 ± 1.9	7.4 ± 0.8
	H193	220 ± 13	704 ± 26	1117 ± 73	22.0 ± 0.7	9.4 ± 1.3	13.6 ± 1.0
	Teqing	230 ± 9	759 ± 22	966 ± 61	24.0 ± 0.6	4.9 ± 1.1	11.5 ± 0.8
2016	Momiroman	257 ± 9	632 ± 11	948 ± 102	17.9 ± 0.7	9.9 ± 2.0	13.1 ± 0.9
	H193	219 ± 17	805 ± 56	966 ± 90	29.3 ± 2.4	5.4 ± 1.8	14.9 ± 1.8
	Teqing	241 ± 4	776 ± 21	1046 ± 43	26.8 ± 0.8	8.0 ± 2.0	17.5 ± 2.2
mean	Momiroman	212ab	541b	956a	15.7b	11.9a	13.2b
	H193	195b	698a	1080a	24.4a	10.7a	15.9a
	Teqing	231a	726a	1068a	24.0a	9.7a	15.7ab
ANOVA	Year (Y)	**	**	**	**	**	**
	Cultivar (C)	*	**	n.s.	**	n.s.	*
	Y × C	**	n.s.	n.s.	n.s.	n.s.	n.s.

Panicle growth rate = difference of panicle dry weight ÷ number of days. DAH: days after heading. FH–20: full heading to 20 DAH. 20–M: 20 DAH to maturity. FH–M: full heading to maturity. Values are means ± SE (n = 3). Different letters indicate statistically significant differences between cultivars ($P < .05$, Tukey's test). *$P < .05$, **$P < .01$ (ANOVA). n.s., not significant.

Table 4
NSC content of stems during ripening.

Year	Variety	NSC content (g m^{-2})			ΔNSC (g m^{-2})		Ratio of ΔNSC (%)
		Full-Heading	20 DAH	Maturity	FH-20	20-M	FH-20
2015	Momiroman	139.4 ± 4.1	59.0 ± 3.5	155.1 ± 30.3	−80.5 ± 3.8	96.2 ± 27.3	57.7 ± 2.1
	H193	193.1 ± 10.8	44.7 ± 4.2	68.9 ± 5.2	−148.4 ± 8.2	24.2 ± 6.9	76.9 ± 1.4
	Teqing	161.5 ± 0.9	14.4 ± 5.8	24.4 ± 5.5	−147.1 ± 5.6	10.0 ± 1.9	91.1 ± 3.6
2016	Momiroman	167.9 ± 3.4	77.2 ± 6.1	74.7 ± 17.5	−90.7 ± 4.6	−2.5 ± 11.9	54.1 ± 3.1
	H193	274.6 ± 7.1	86.5 ± 21.1	117.7 ± 23.5	−188.1 ± 23.3	31.2 ± 10.6	68.4 ± 7.7
	Teqing	301.3 ± 8.8	34.6 ± 2.3	23.1 ± 3.3	−266.7 ± 10.9	−11.5 ± 4.2	88.4 ± 1.1
mean	Momiroman	153.7b	68.1a	114.9 a	−85.6a	46.9a	55.9c
	H193	233.9 a	65.6a	93.3 a	−168.3b	27.7ab	72.6b
	Teqing	231.4 a	24.5 b	23.8 b	−206.9c	−0.8b	89.8a
ANOVA	Year (Y)	**	**	n.s.	**	**	n.s.
	Cultivar (C)	**	**	**	**	*	**
	Y × C	**	n.s.	*	**	**	n.s.

ΔNSC (apparent NSC translocation) is the difference in NSC content. Ratio of ΔNSC = ΔNSC ÷ NSC content at Full heading × 100. DAH: days after heading. FH–20: full heading to 20 DAH. 20–M: 20 DAH to maturity. Values are means ± SE (n = 3). Different letters indicate statistically significant differences between cultivars ($P < .05$, Tukey's test). *$P < .05$, **$P < .01$ (ANOVA). n.s., not significant.

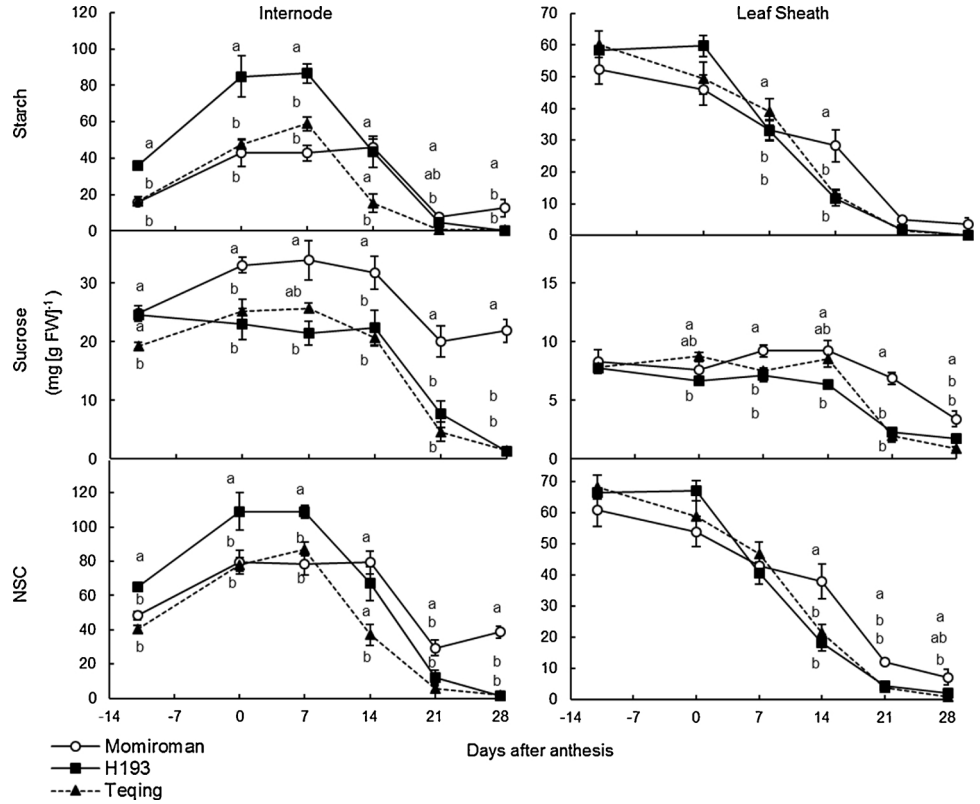

Fig. 1. Carbohydrate contents of internodes and leaf sheaths. The third internodes from the uppermost internodes and the sheaths of the third leaves from the flag leaves were analysed. Values are means ± SE in 2015 (n = 6). Different letters indicate statistically significant differences between cultivars ($P < .05$, Tukey's test).

the difference in NSC translocation efficiency among cultivars, we measured the contents of starch and sucrose, which are primary components of stem NSC, and activities of enzymes involved in starch metabolism in internodes and leaf sheaths harvested in 2015. Starch and NSC contents in the internodes of Momiroman and Teqing were significantly lower than in those of H193 from −11 to 7 DAA (Fig. 1). Those of H193 and Teqing then rapidly decreased, but that of Momiroman did not decrease until 14 DAA. These results resembled the data on NSC contents of whole stems (Table 4). Notably, the sucrose content in the internodes of Momiroman was constantly higher than in those of H193 and Teqing from 0 to 28 DAA. There was no difference in the dynamics of sucrose content between H193 and Teqing. The slower starch decrease and higher sucrose content were also observed in the leaf sheaths of Momiroman, although the difference among the cultivars was smaller in the leaf sheaths than in internodes.

The activity of AGPase, a key regulatory enzyme of starch synthesis (Tetlow et al., 2004), was highest in the internodes of all three cultivars at 0 DAA and then decreased gradually (Fig. 2). However, the maximum activity varied greatly and was highest in H193 and lowest in Momiroman. The activities of α-amylase and β-amylase, which are involved in starch degradation in leaf sheaths during ripening (Sugimura et al., 2015; Hirano et al., 2016), showed no consistent differences among the cultivars from −11 DAA to 28 DAA (Fig. 2).

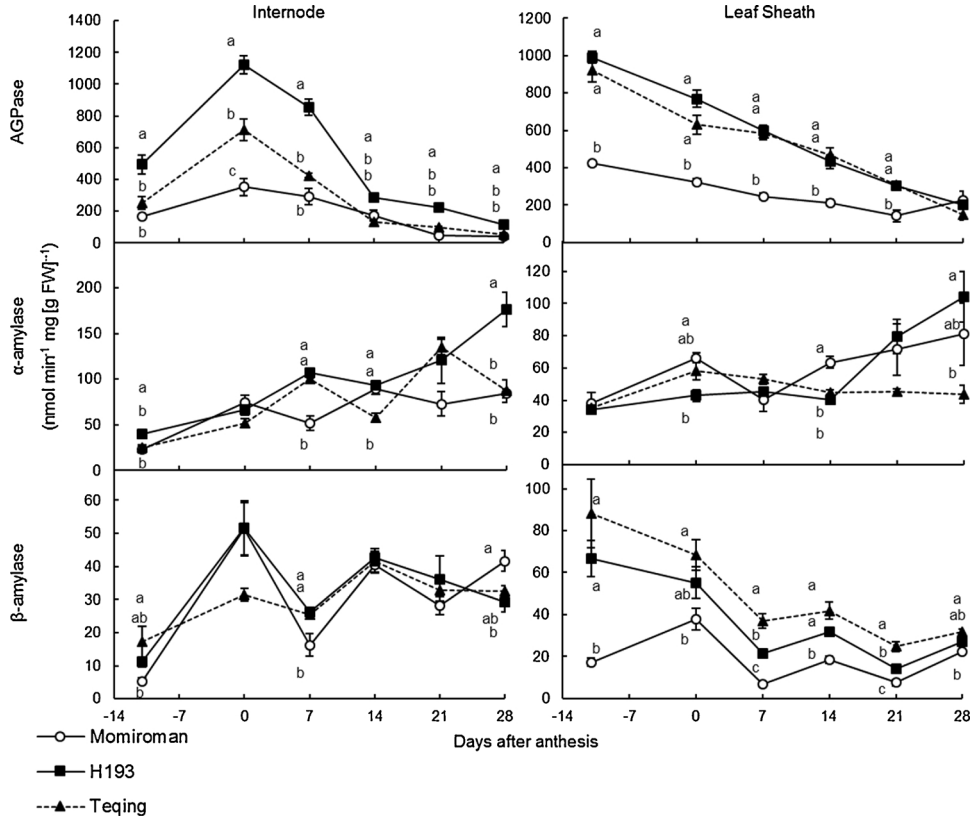

Fig. 2. Activities of AGPase, α-amylase and β-amylase in internodes and leaf sheaths. The third internodes from the uppermost internodes and the sheaths of the third leaves from the flag leaves were used. Values are means ± SE in 2015 (n = 6). Different letters indicate statistically significant differences between cultivars (P < .05; Tukey's test).

3.4. Differences in grain filling according to position in a panicle

Distribution of grain weight in a panicle at maturity is shown in Fig. 3. Momiroman had a broad peak at 28 mg and had many grains that were relatively light but not completely empty, even among the upper A (superior) grains. We classified grains by weight into filled (> 18.5 mg), fertile but unfilled (6.0–18.5 mg) and sterile for the most part (< 6.0 mg). The weight distribution of grains in each position is shown in Table 5. The occurrence of > 18.5-mg grains in Momiroman was notably low, even in the upper A group. That of the 6.0–18.5-mg upper A grains was higher in Momiroman than in H193 and Teqing, whereas there was no difference in those of < 6.0 mg. The occurrence of > 18.5-mg upper B grains was higher and that of > 18.5-mg lower B grains tended to be higher in H193 than in the other two cultivars.

3.5. Effect of spikelet thinning on grain-filling rate

The dynamics of grain weight fitted with the flexible sigmoid model (Yin et al., 2003) are shown in Fig. 4, and corresponding parameter values are listed in Table 6. The model accurately described the dynamics of grain weight, with $R > 0.91$ (Table 6). t_m was longer and C_m was smaller in superior spikelets of Momiroman than in those of H193 and Teqing. C_m was also smaller in inferior spikelets of Momiroman than in those of H193 and Teqing.

To increase carbohydrate supply to grains, we thinned spikelets on the flower-opening day. Overall, the effect of thinning on superior spikelets of Momiroman seemed to be smaller than on those of H193 and Teqing; t_m of H193 was shortened by thinning and C_m of H193 and Teqing was increased. The effects of thinning on inferior spikelets tended to be similar regardless of the cultivar (t_m was shortened and C_m was increased), although the effects were smaller in Momiroman.

4. Discussion

4.1. Relationships between yield, grain-filling and stem NSC

We found that the ratio of filled grains was much lower in Momiroman than in H193 throughout the 3 years, and that of Teqing was intermediate (Table 1), while there was no difference in the average of sink capacity (defined as single grain yield × number of spikelets per area) over the 3 years between Momiroman and H193; that of Teqing was highest (Table 5). Because the shoot growth rate during ripening in Momiroman was not lower than those of the other cultivars (Table 2), not the lower ability of carbon assimilation in leaves, but the lower grain-filling ability might be responsible for poorer grain-filling rate of Momiroman.

The panicle growth rate and ΔNSC were smaller in Momiroman than H193 and Teqing until 20 DAH (Tables 3 and 4), suggesting that the lower grain-filling rate at early ripening due to lower NSC translocation is a possible reason of poor grain-filling of Momiroman. Although NSC accumulation in the stem of Momiroman at full-heading was smaller, a lot of NSC remained in Momiroman in stems at 20 DAH. The ratio of ΔNSC of Momiroman was smallest among three cultivars (Table 4), indicating a very low efficiency of NSC translocation. Grain filling may be affected more directly by low translocation efficiency than by low NSC accumulation.

4.2. Starch metabolisms in stems of Momiroman

To understand the physiological reason of low NSC translocation efficiency in the stems of Momiroman, we further investigated NSC components and activities of enzymes involved in starch metabolism. Although they were conducted only in 2015, the similar results can be expected in the other two years because the trends of yield and stem NSC contents were highly constant over the three years. Low

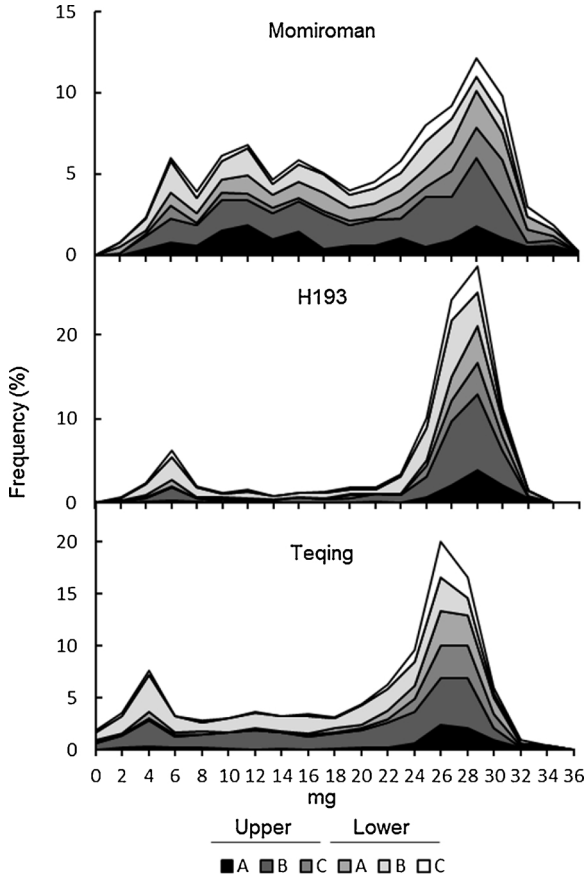

Fig. 3. Distribution of grain weight per panicle. "Upper", "Lower", "A", "B" and "C" mean position of grains in a panicle and see the Materials and Methods for the details. Values are means of six panicles from six plants (one panicle per plant) in 2015.

translocation efficiency might be caused by slow starch degradation or low efficiency of sucrose transport. Sucrose content in the internodes and leaf sheaths of Momiroman tended to be higher than H193 and Teqing after heading (Fig. 1). Because the synthesis of sucrose and its loading into the phloem follow starch degradation in NSC translocation, these results suggest that not starch degradation but sucrose translocation was the major cause of the lower rate of NSC decrease in Momiroman stems. This assumption is supported by the fact that the activities of AGPase in the internodes and leaf sheaths of Momiroman were not higher and those of α-amylase and β-amylase were not lower than those of H193 and Teqing during ripening (Fig. 2).

4.3. Sink strength in Momiroman

Sink limitation and stem carbohydrate metabolism may affect stem NSC metabolism, as Hirose et al. (2017) reported that genetic limitation of spikelet number using a mutation in the gene *SP1* (*Short-Panicle 1*; Os11g0235200) increased starch content in stems. Since there was no difference among the cultivars in the occurrence of sterile (< 6 mg) grains, sterility was not the main cause of poor grain filling in Momiroman (Table 5, Fig. 3). The occurrence of 6.5–18.5 mg grains was higher in Momiroman than in H193 and Teqing regardless of whether the spikelets were superior (upper A and C) or inferior (lower B) (Table 5). Other studies regarded low grain-filling rate to be limited to inferior spikelets (Kobata et al., 2013; Yoshinaga et al., 2013; You et al., 2016). Here, however, we observed differences among cultivars also in superior spikelets, and the calculated maximum growth rate (C_m) was lower in Momiroman than in H193 and Teqing in both superior and inferior spikelets (Fig. 4, Table 6). The C_m of inferior spikelets seemed to be improved by thinning regardless of the cultivar, suggesting that grain growth rate in inferior spikelets is restricted by carbon supply, in agreement with previous reports (Kobata et al., 2013; You et al., 2016). In superior spikelets, the C_m of Momiroman seemed not to be affected by thinning, but those of H193 and Teqing were improved. This result indicates that grain growth in the superior spikelets of Momiroman was restricted not by the shortage of carbohydrate supply, but probably by sink strength.

These results led us to hypothesise that low sink strength is the major cause of low grain filling in Momiroman, which delays grain growth and decreases the sucking force of NSC from stem. Although the existence of such a force based on Münch's hypothesis of convective bulk flow has long been proposed, there are neither direct evidence nor established model which can explain this (Chang and Zhu, 2017). Our results strongly support the theory from a physiological aspect. Sink strength is determined by both the rate of sucrose breakdown and starch synthesis. Many enzymes and transporters are involved in them (Thitisaksakul et al., 2012), which are controlled by many genes, hormones and signalling molecules, including sugars (Yang et al., 2003; Sun et al., 2015; Inukai, 2017). These facts would make it difficult to confirm the causes of low sink strength of Momiroman at the metabolite level, and a technical breakthrough would be needed to enable understanding of the whole process of grain filling.

4.4. Factors limiting grain yield in H193 and Teqing

Although the average grain yields of H193 and Teqing over 3 years were almost the same (Table 1), the factors limiting grain yield might be different. H193 showed a higher ratio of filled-grains (Table 1). More NSC remained at maturity in stems of H193 than those of Teqing (Table 4). These results suggest that the sink capacity of H193 was

Table 5
Distribution of grains by grain weight (%).

		Upper			Lower			Total
		A	B	C	A	B	C	
> 18.5 mg	Momiroman	45.8 ± 4.7b	51.7 ± 6.1b	74.7 ± 48b	55.6 ± 5.2b	38.9 ± 10.8a	73.4 ± 5.1a	53.1 ± 4.0b
	H193	90.4 ± 3.7a	82.5 ± 4.2a	89.6 ± 3.6a	87.0 ± 4.1a	68.8 ± 8.8a	82.4 ± 5.6a	80.5 ± 4.4a
	Teqing	80.6 ± 3.2a	54.7 ± 4.3b	90.8 ± 3.1a	81.6 ± 3.1a	44.9 ± 8.5a	85.7 ± 2.8a	64.3 ± 4.2b
6.0–18.5 mg	Momiroman	43.2 ± 5.1a	36.1 ± 5.0a	16.5 ± 4.9a	33.0 ± 4.7a	39.9 ± 8.0a	17.9 ± 5.4a	33.8 ± 2.4a
	H193	3.8 ± 1.5b	8.6 ± 3.1b	6.0 ± 2.6ab	4.9 ± 2.0b	12.0 ± 4.3b	6.1 ± 3.4a	8.4 ± 2.1c
	Teqing	9.3 ± 2.5b	28.6 ± 3.4a	3.5 ± 1.8b	10.5 ± 2.7b	30.1 ± 4.2ab	5.9 ± 2.7a	20.2 ± 2.4b
< 6.0 mg	Momiroman	10.9 ± 2.2a	12.2 ± 2.2ab	8.8 ± 2.0a	11.4 ± 1.5a	21.2 ± 4.3a	8.7 ± 2.5a	13.1 ± 1.7a
	H193	5.8 ± 2.2a	8.9 ± 1.7b	4.4 ± 1.2a	8.1 ± 3.1a	19.2 ± 6.1a	11.5 ± 2.9a	11.1 ± 2.7a
	Teqing	10.1 ± 3.9a	16.7 ± 2.4a	5.7 ± 3.3a	8.0 ± 1.5a	25.0 ± 4.7a	8.4 ± 1.0a	15.6 ± 1.8a

Values are means ± SE in 2015 (n = 6). "Upper", "Lower", "A", "B" and "C" mean position of grains in a panicle and see the Materials and Methods for the details. Different letters indicate statistically significant differences between cultivars (P < .05, Tukey's test).

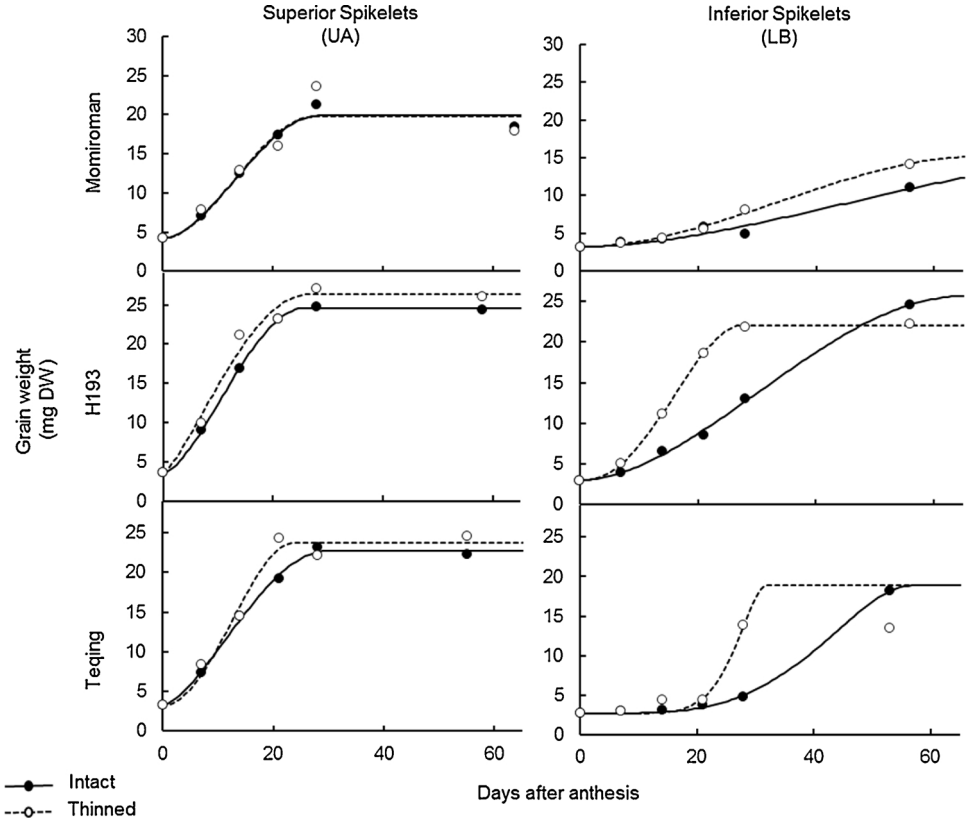

Fig. 4. The dynamics of grain weight. Circles indicate mean values of all harvested grains in 2015. Grain number in each plot is shown in Supplementary Table S2. Lines are regression curves fitted with the flexible sigmoid model (Yin et al., 2003).

Table 6
Correlation coefficients and estimated parameter values of the fitted sigmoid model shown in Fig. 4.

			R	$W_m m$	$t_e e$	t_m	C_m
Superior spikelets (Upper A)	Momiroman	Intact	0.9839**	19.9	28.2	13.1	0.82
		Thinned	0.9151*	19.7	27.2	13.2	0.85
	H193	Intact	0.9997***	24.6	25.8	10.2	1.18
		Thinned	0.9787**	26.3	26.5	7.0	1.25
	Teqing	Intact	0.9970***	22.7	29.6	11.2	0.95
		Thinned	0.9806**	23.7	23.6	12.5	1.33
Inferior spikelets (Lower B)	Momiroman	Intact	0.9698**	15.2[a]	97.6	44.4	0.18
		Thinned	0.9975***	15.2[a]	67.5	33.6	0.27
	H193	Intact	0.9987***	25.6	64.8	30.3	0.51
		Thinned	0.9999***	22.1	27.5	16.0	1.11
	Teqing	Intact	0.9995***	18.9[a]	56.7	43.9	0.64
		Thinned	0.9595**	18.9[a]	31.9	28.0	1.85

R: correlation coefficient. W_m: maximum grain weight (g). T_e: end of growth period (day). T_m: time when maximum growth rate was achieved (day). C_m: maximum growth rate (g day^{-2}). [a]Calculated by Eq. (2). "Upper A"and "Lower B" mean position of grains in a panicle and see the Materials and Methods for the details. ***$P < .001$, **$P < .01$, *$P < .05$.

insufficient to exploit its high source potential. On the other hand, source shortage relative to a large sink capacity may be responsible for the low ratio of grain filling in Teqing. Unutilized source seems to be low in Teqing because very little NSC remained in the stem, and the harvest index was higher in Teqing than in H193 (Tables 1 and 4), suggesting higher efficiency of translocation. However, the ratio of unfilled but fertile grains was higher in Teqing than in H193 (Table 5), indicating that the efficiency of carbon distribution among spikelets was higher in H193 than in Teqing. Therefore, improving this distribution efficiency also could increase grain yield of Teqing.

5. Conclusion

We compared the grain-filling-related traits of Momiroman, which has high sink capacity but low grain-filling ratio, with those of H193 and Teqing. Examination of the sink–source balance revealed essential differences in grain-filling properties and led to the conclusion that low sink strength is likely the major cause of low grain filling in Momiroman. Although further studies to investigate the rate-limiting steps in sucrose breakdown and starch synthesis in the endosperm of Momiroman are needed to prove this hypothesis, this study provides basic knowledge for improving the grain filling of high-yielding rice cultivars.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.fcr.2018.01.035.

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