Impact of down-regulation of starch branching enzyme IIb in rice by artificial microRNA- and hairpin RNA-mediated RNA silencing

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Abstract

The inactivation of starch branching IIb (SBEIIb) in rice is traditionally associated with elevated apparent amylose content, increased peak gelatinization temperature, and a decreased proportion of short amyllopectin branches. To elucidate further the structural and functional role of this enzyme, the phenotypic effects of down-regulating SBEIIb expression in rice endosperm were characterized by artificial microRNA (amiRNA) and hairpin RNA (hp-RNA) gene silencing. The results showed that RNA silencing of SBEIIb expression in rice grains did not affect the expression of other major isoforms of starch branching enzymes or starch synthases. Structural analyses of debranched starch showed that the doubling of apparent amylose content was not due to an increase in the relative proportion of amylose chains but instead was due to significantly elevated levels of long amyllopectin and intermediate chains. Rices altered by the amiRNA technique produced a more extreme starch phenotype than those modified using the hp-RNA technique, with a greater increase in the proportion of long amyllopectin and intermediate chains. The more pronounced starch structural modifications produced in the amiRNA lines led to more severe alterations in starch granule morphology and crystallinity as well as digestibility of freshly cooked grains. The potential role of attenuating SBEIIb expression in generating starch with elevated levels of resistant starch and lower glycaemic index is discussed.

Key words: CP/MAS NMR, crystalline polymorph, RNA interference, starch granules, XRD.

Introduction

High amylose cereals are attracting considerable attention because of their potential health benefits, along with their industrial uses (Jobling, 2004; Morell and Myers, 2005; Rahman et al., 2007). High amylose maize (Shannon et al., 2009), wheat (Regina et al., 2006; Sestili et al., 2010), and barley (Morell et al., 2003; Regina et al., 2010) have so far been developed. The apparent amylose content (AAC) of these high amylose cereals ranges from 50% to 90% but, in rice, the highest reported AAC is only ~30% for wild types (Juliano, 2003) and between 25% and 40% for chemical- and irradiation-induced mutants (Yano et al., 1985; Nishi et al., 2001; Kang et al., 2003; Yang et al., 2006). Recently, Wei et al. (2010a) have reported higher amylose contents, but the detailed characterization of the rice plants has yet to be described.
Amylose is the predominantly linear component of native cereal starches with a degree of polymerization (DP) <5000, whereas amyllopectin is a very large (DP 5000–50 000) and highly branched biopolymer (Ball et al., 1998). When rice starch is debranched by isoamylase, amyllopectin chains are in the range of DP 6–120, while amylose is in the range of DP 230–10 000 (Takeda et al., 2003; Ward et al., 2006; Fitzgerald et al., 2009). Granule-bound starch synthase I (GBSSI), encoded by the Waxy (Wx) gene, is essential for amylose biosynthesis, while amyllopectin is synthesized by the combined action of several isoforms of starch synthases, starch branching enzymes, and starch debranching enzymes (Smith, 2001; Nakamura, 2002; Ball and Morell, 2003; Tetlow et al., 2004; Jeon et al., 2010).

In most wild-type cereal starches, amylose is usually 15–25% by weight and amyllopectin is in the order of 75–85% (Ball et al., 1998; Fitzgerald, 2004). Screening a subset of the International Rice Research Institute’s germplasm collection revealed that the range of amylose in wild and cultivated rice ranges from 0% to 30% (Butardo et al., 2008). To increase the levels of amylose further, one can overexpress a suitable Wx allele (Itoh et al., 2003; Hanashiro et al., 2008), but the more common method is to down-regulate the expression of enzymes involved in amyllopectin biosynthesis to direct starch synthesis towards amylose production (Morell and Myers, 2005; Regina et al., 2006, 2010; Rahman et al., 2007).

The prime target for down-regulation to achieve high amylose is starch branching enzyme II (SBEII) based on elevated amylose mutants in maize, wheat, barley, and rice (Boyer and Preiss, 1978; Nishi et al., 2003; Regina et al., 2006, 2010; Wei et al., 2010a). The two isoforms of SBEII, SBEIIa and SBEIIb (Vandeputte and Delcour, 2004), share ~80% sequence identity, but their expression patterns differ. In rice, SBEIIa (RBE4) is primarily expressed in the leaves while SBEIIb (RBE3) is primarily expressed in the grains (Yamanouchi and Nakamura, 1992; Ohdan et al., 2005; Yamakawa et al., 2007), although massively parallel sequencing data indicate that it is also expressed weakly in roots and seedlings (http://mpss.udel.edu/rice/mpss_index.php). In vitro studies suggest that rice SBEIIb acts preferentially on DP 6 and 7, while SBEIIa acts on a wider range of chain lengths of DP 6–15 from the outer chains of amyllopectin and possibly amylose (Nakamura et al., 2010). High amylose rice and maize, which exhibit the amylose extender (ae) phenotype, result from the inactivation of SBEIIb (Boyer et al., 1980; Hedman and Boyer, 1982; Yano et al., 1985; Kim et al., 1998; Nishi et al., 2001). The ae mutants in rice have higher AAC than their wild-type parents, but only 35% AAC is found, in contrast to 50–75% in ae maize (Shannon et al., 2009).

Most high amylose mutants in rice have been obtained by chemical mutagenesis or by exposure to sublethal doses of radiation (Yano et al., 1985; Kim et al., 2005; Shu et al., 2006). Mutation of SBEIIb in rice (Yano et al., 1985; Asaoka et al., 1986) and in maize (Moore and Creech, 1972; Boyer et al., 1976; Garwood et al., 1976) was accomplished by directly mutating the genome, hence the expression of active SBEIIb in the entire plant is affected. Furthermore, genetically tightly linked but uncharacterized mutations may also be present in the genome. Possible effects on the whole plant from such genomic alterations can be avoided by RNA silencing using seed-specific promoters (Wang et al., 1998; Kawakatsu et al., 2008; Qu et al., 2008). RNA interference using hairpin RNA (hp-RNA) has been successfully demonstrated in wheat (Regina et al., 2006; Sestili et al., 2010) and in barley (Regina et al., 2010) endosperm. Another method that has been recently developed is RNA silencing by artificial microRNA (amiRNA) (Ossowski et al., 2008). Although this has not yet been successfully demonstrated in cereal endosperm, one study was able to silence the expression of three rice genes systemically in japonica (Nipponbare) and indica (IR64) backgrounds using an amiRNA driven by a ubiquitin promoter (Warthmann et al., 2008).

In the present study, the expression of SBEIIb in the endosperm has been reduced using both hp-RNA and amiRNA approaches. The amiRNA approach reduces yet further the possibility of non-specific targets, and this paper reports the first, highly effective, use of this technique in the grain endosperm. It is shown here that the ae phenotype in rice can be obtained by down-regulating the expression of SBEIIb alone, thereby further corroborating previous findings that this mutation is due to a defective SBEIIb. It is further demonstrated that the phenotype in a japonica background is due solely to the increased proportion of long amyllopectin chains, not to an increase in ‘true’ amylose. Rice grains with different crystalline polymorphs and digestibility were obtained using the two different techniques although they only differed slightly in starch branch length distribution, and these starches are comprehensively characterized herein.

Materials and methods

Construction of RNA silencing expression vectors

The construction of SBEIIb hairpin RNA (hp-BEIIB) was based on previous methods (Regina et al., 2006, 2010). Briefly, a 397 bp SBEIIb fragment located at the 5’ end of the SBEIIb gene (254–650 bp of LOC_Os02g32660 based on Oryza sativa MSU Osa1 Release 6.1 Annotation) was PCR amplified (Supplementary Table S1 available at JXB online) from Nipponbare cDNA and cloned into pGEM-T Easy (Promega) using Escherichia coli DH5α. The cloned SBEIIb fragment was inserted in forward and reverse orientations in an intermediate cloning vector containing a wheat high molecular weight glutenin (wHMWG) promoter and a nopaline synthase (NOS) 3’ terminator (pBx17). The hairpin construct was then transferred into an Agrobacterium Ti binary expression vector (pVec8) containing a hygromycin resistance gene driven by a cauliflower mosaic virus (CaMV) 35S promoter (Wang et al., 1998). The binary vector containing the hp-BEIIb sequence was electroporated and maintained in Agrobacterium tumefaciens AGL1 using LB broth supplemented with 50 μg ml⁻¹ rifampicin and spectinomycin.

The construction of SBEIIb artificial microRNA (ami-BEIIB) was based on a previous protocol (Warthmann et al., 2008), with modifications to express the amiRNA in the endosperm using pVec8. A 21 nucleotide microRNA (miRNA) target located at the
middle of the \textit{SBEIIb} gene (1258–1278 bp) was identified using Web MicroRNA Designer 2 (WMD2) (Ossowski et al., 2008). An amiRNA was selected from the list of potential amiRNAs based on its binding energy and specificity with the target gene. The secondary structure of ami-\textit{BEIIb} in the \textit{osa-mir528} backbone was predicted using RNAfold (Hofacker et al., 1994).

The selected amiRNA (TTAATCGGTATCTGTACCAG) was synthesized by fusion PCR (Supplementary Table S1) using Expand Taq (Roche) and \textit{osa-mir528} (Liu et al., 2005) endogenous miRNA precursor as stem-loop backbone (Warthmann et al., 2008). After PCR purification using Wizard SV PCR Clean-up System (Promega), the amiRNA precursor (254 bp) was cloned into pGEM-T Easy (Promega) using \textit{E. coli} DH5a. The resulting amiRNA (ami-\textit{BEIIb}) was cloned in the forward orientation as described above.

\textit{Nipponbare} transformation

Rice transformation was undertaken by standard procedures as previously described (Upadhyaya et al., 2000) but using 50 mg l\(^{-1}\) hygromycin to select for transformed calli. Regenerated hygromycin-resistant plants were acclimatized for 1 week inside a moist growth chamber before they were individually planted in pots (8 cm diameter) with soil supplemented with 1 g kg\(^{-1}\) Osmocote (Scots Australia). The pots were maintained in submerged tanks inside a biosafety glasshouse with temperature maintained at 26.5\(\pm\)3.5 \textdegree C. The succeeding generations of transgenic and control plants were grown under similar conditions.

Genomic DNA analyses

Genomic DNA was extracted from 1-month-old leaves using a FastDNA Kit (Q-BIOgene). Initial screening for putative transformants was done using the hygromycin resistance gene (Supplementary Table S1 at JXB online). The putative transformants were verified using gene-specific primers that amplify a fragment containing a portion of the \textit{wHMWG} promoter and a portion of the forward \textit{hp-SBEIIb} or ami-\textit{SBEIIb} fragment (Supplementary Table S1). PCR amplification was carried out using HotStar Taq (Qiagen) and \textit{products were resolved in 13\% agarose, using the wHMWG reporter dye. Comparative quantitation was conducted using Taq DNA polymerase (Invitrogen) and Sybr Green I (Invitrogen) reporter dye. Comparative quantitation was conducted using tubulin as a reference gene (Toyota et al., 2006), with data validation and melt curve analysis done using Rotor-Gene 6000 Series Real Time Rotary Analyzer Software (Corbett).

Protein expression analyses

Anti-\textit{rice SBEIIa} was developed by conjugating the sequence IPAYAESISIKVVAED or AGAPGKVLVPG (GC was added to the C-terminal end of both peptides) to either keyhole limpet haemocyanin or ovalbumin. The antiserum raised in rabbits against AGAPGKVLVPG conjugated to ovalbumin was validated to be the most satisfactory and was used for the experiments described herein. On the other hand, anti-\textit{wheat SBEIIb} rabbit polyclonal antibodies (Regina et al., 2005) which were shown by mass spectrometry to recognize SBEIIb in rice (unpublished data) were used for the western blot detection of \textit{SBEIIb}.

Native soluble proteins were extracted as previously described (Regina et al., 2006). A total of 100 \textmu g of protein quantified using \textit{C. E. coli Protein Assay Reagent} (Bio-Rad) was loaded into each lane. Two gels blotted separately were prepared to detect \textit{SBEIIa} and \textit{SBEIIb} individually using anti-\textit{SBEIIa} and anti-\textit{SBEIIb} antisera (1:2000 dilution). The immunoreactive proteins were probed by goat anti-rabbit immunoglobulins conjugated to horseradish peroxidase (Bio-Rad). Detection was carried out using ECL Western Blotting Detection Reagents (GE Healthcare) and Hyperfilm ECL chemiluminescence film (Amersham Biosciences). The film was developed using a CP 1000 automatic film processor (Agfa).

Branching enzyme zymograms were carried out as described (Nishi et al., 2001), with slight modifications. For each lane, 100 \textmu g of total protein was loaded and resolved using native PAGE with 3.3\% stacking and 5\% separating layers. The gel was incubated overnight with gentle shaking at ambient temperature using a branching enzyme buffer (Nishi et al., 2003) containing 1 mM dithiothreitol (DTT) and 1.8 mM maliotrose. The addition of maltotriose was found to enhance the detection of branching enzyme activity (JP Ral, personal communication). The gel was stained with iodine solution (0.1\% I\(_2\) and 1\% KI) the following day. The reduction in branching frequency of debranched starch was determined based on a reducing end assay (Bernfeld, 1955) as modified by Regina et al. (2010).

Grain and starch granule analyses

Mature panicles were harvested and dried at 37 \textdegree C for at least 3 d. The seeds were then manually threshed and machine dehulled (Satake). Ten brown grains from selected lines were chosen and weighed in triplicate. Grain appearance and dimensions were determined using a SeedCount (SeedCount Australasia Pty Ltd), with the digital image analysis software module for medium grain rice. Opacity was measured using the chalkiness index for the Australian rice industry standard. Photomicrographs of whole rice grain samples were obtained using a Leitz M8 stereomicroscope.

Cross-sections of rice grains were observed uncoated with an environmental scanning electron microscope (Zeiss EVO LS15) under variable-pressure mode. Images of starch granules were taken with a back-scattered electron detector. Starch granules were isolated and viewed under a polarized light microscope to check for birefringence. The isolated starch granules were also stained with APTN (8-amino-1,3,6-pyrenetrisulphonic acid) and viewed under a fluorescence microscope as previously described (Wei et al., 2010).

Characterization of starch crystallinity by X-ray diffraction (XRD) was carried out on a Panalytical XPert Pro diffractometer. The instrument was equipped with a Cu long fine focus tube, programmable incident beam divergence slit and diffracted beam scatter slit (both fixed at 0.125 \textdegree), and a Xcelerator high speed detector. The samples were examined over the angular range of 4–40 \textdegree with a step size of 0.0332 \textdegree and a count time of 220 s per point. Crystallinity was determined using the crystal defect method as previously described (Lopez-Rubio et al., 2008).

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Solid-state $^{13}$C cross-polarization/magic angle spinning (CP/MAS) nuclear magnetic resonance (NMR) experiments were performed at a $^{13}$C frequency of 75.46 MHz on a Bruker MSL-300 spectrometer. A standard of amorphous, regular maize starch was prepared by heating a starch suspension (1% w/v) for 30 min at 95°C. The suspension was then lyophilized. Approximately 200 mg of rice flour was packed in a 4 mm diameter, cylindrical, partially stabilized zirconium oxide (PSZ) rotor with a KelF end cap. The rotor was spun at 5 kHz at the magic angle (54.7°). The 90° pulse width was 5 μs and a contact time of 1 ms was used for all starches with a recycle delay of 3 s. The spectral width was 38 kHz, acquisition time 50 ms, time domain points 2 k, transform size 4 k, and line broadening 50 Hz. At least 1000 scans were accumulated for each spectrum. Spectra were referenced to external adamantane. Data fitting was carried out as previously described (Tan et al., 2007).

Carbohydrate analyses
Brown rice grains from selected lines were polished for 1 min using a fabricated machine with a circulating abrasive. Opaque or chalky seeds from each line were ground with a metal ball bearing for 30 s using an ESPE Capmix (3M). The amylose content of flour samples was determined by iodine colorimetry against a standard curve from five rice varieties whose actual amylose contents were determined by debranched size-exclusion chromatography (SEC; described below). Amylose estimations were done at two wave-lengths: the traditional method using 620 nm (Juliano et al., 1981) and using 720 nm which was found to reduce the effect of long chain amyllopectin on absorbance values (Fitzgerald et al., 2009). Peak gelatinization temperature (GT) was measured by differential scanning calorimetry (Cuevas et al., 2010).

The total starch content of rice flour (dry weight basis) was determined using a Megazyme total starch assay procedure (AACC Method 76.13) but adapted to a 96-microwell plate format. For samples which showed a reduction in total starch content compared with Nipponbare, resistant starch content was determined (AACC Method 32-40) using the 96-well plate format (Megazyme, Wicklow, Ireland). The resistant starch content was added to the amount of solubilized (non-resistant) starch to determine the actual total starch content.

Analysis of mixed-linkage β-glucan was conducted using a scalable method (McCleary et al., 1994) with modifications, including using 50 mg of flour that was pre-washed in 1 ml of 70% ethanol (80°C, 20 min) to remove free sugars before washing in 50% ethanol (80°C, 5 min) and resuspending in 1 ml of sodium phosphate buffer (90°C, 30 min). Subsequent incubations were performed at 42°C, including with lichenase (2 U, 90 min) before a 20 μl aliquot of supernatant was taken (after 5 min centrifugation, 14 000 rpm) and addition of β-glucosidase (0.02 U ml$^{-1}$, 30 min). After incubation with 200 μl of glucose oxidase/peroxidase reagent, the absorbance (510 nm) was used to calculate released glucose by blank subtraction. All flour incubations were shaken (1200 rpm) on a Thermomixer (Eppendorf).

Total pentosan content was determined colorimetrically as previously described by Bell (1985) with modifications. Following hydrolysis of samples with sulphuric acid (0.5 M, 30 min, 100°C), sugars were measured by adding 500 μl of freshly prepared reagent (55 ml of acetic acid, 1.1 ml of HCl, 0.6 ml of 0.7% glucose, and 2.4 ml of 25% phloroglucinol in ethanol) to 100 μl of sample containing 0–20 μg of pentose sugars (25 min, 100°C); the mixture was then cooled for 5 min. The difference in absorbance ($A_{540}$–$A_{425}$) was used to calculate the pentosan content using a xylose standard and a conversion factor of 0.88 to express the pentose sugars in a polysaccharide form.

Determination of chain length distribution of amyllopectin by fluorescence-activated capillary electrophoresis (FACE) was performed as previously described (O’Shea and Morell, 1996) with debranching as described by Ward et al. (2006). Molecular size distribution of debranched starch was determined by SEC as previously described (Ward et al., 2006; Cuevas et al., 2010). Two SEC columns were used: Ultrahydrogel 250 (Waters, Milford, MA, USA) which starts separating debranched starch at a DP of ~2750, and Proteoma (Polymer Standards Service GmbH, Mainz, Germany) which starts separating at DP ~650. The molecular weight was estimated from the elution time using pullulan standards (Shodex P-82) calibrated with the Mark–Houwink–Sakurada equation and universal calibration (Castro et al., 2005; Ward et al., 2006). A waxy rice was used to delineate the debranched amyllose and amyllopectin regions with a cut-off at DP 120 (Fitzgerald et al., 2009).

Resistant starch and glycaemic index predictions
The total resistant starch (RS) content and glycaemic index (GI) of freshly cooked polished rice grains were predicted using an in vitro incubation system which models the buccal, gastric, and pancreatic phases of food digestion as occurs in the human upper gastrointestinal tract. The methods have been extensively validated to have a high correlation with in vivo RS levels and GI values (AR Bird, S Usher, B Klingler, DL Topping, and MK Morell, unpublished data). Rice samples were prepared using the absorption method using a ratio of water to rice depending on amylose content (Supplementary Table S2 at JXB online). A total of 50 mg and 500 mg of available carbohydrates were used to predict GI and RS, respectively. For GI prediction, aliquots of supernatant were sampled at the designated time points for up to 5 h and the glucose concentration determined using an automated electrochemical procedure.

The total starch content of freshly cooked rice was determined based on a previously published protocol (McCleary et al., 1994) after freeze drying and milling into a fine powder. The predicted GI of the sample was calculated as the percentage of available carbohydrate converted to glucose and released during the time course of the incubation. For RS, the incubation period was extended to 16 h and the amount of starch remaining in the sample at that time was determined using conventional enzymatic and spectrophotometric techniques. The predicted RS content of the sample was calculated as the amount of starch remaining in the digest as a percentage of sample weight.

Neutral non-starch polysaccharides (NNSPs) were measured by a gas chromatographic technique using a slightly modified version of Theander et al. (1995) (AOAC 994.13). The insoluble and soluble NNSPs were separated by selective precipitation. The insoluble NNSPs were hydrolysed with 1 M sulphuric acid, while the soluble NNSPs were hydrolysed with 2 M trifluoroacetic acid.

Sampling and statistical analyses
Four generations of transformed lines (T$_0$–T$_3$) were selected for analysis. Negative segregants and empty vector Nipponbare callus regenerated by tissue culture were used as a negative controls for every generation. One-month-old leaves (T$_0$ and T$_1$) and mid-milk stage (10 dpa) grains (T$_2$ and T$_3$) were used for DNA and protein analyses, respectively. Homozygous T$_3$ grains (10 dpa) were used for gene expression analyses. Developing T$_4$ grains (5, 10, 15, and 20 dpa) were analysed by western blot and zymogram to compare the extent of protein down-regulation between ami-BEIIBs and hp-BEIIB. Mature chalky to opaque grains were used for whole grain and starch analyses for three generations (T$_1$, T$_2$, and T$_3$) planted at different seasons to assess stability of traits. At least three biological replicates from three independent transformed lines were used for every analysis, each with at least two technical replicates. IR36 and IR36ae were obtained from Yanco Agricultural Institute (NSW, Australia) and grown at the CSIRO Plant Industry (ACT, Australia).

Statistical analyses [one-way analysis of variance (ANOVA) with Tukey post-test, two-way ANOVA with Bonferroni post-test, and unpaired t-test] were done using GraphPad Prism.
Results

Constructs and genomic DNA screening

Two RNA silencing constructs were cloned into pVec8 (Wang et al., 1998): an artificial microRNA (ami-BEIIb, Fig. 1A, B) and a hairpin RNA (hp-BEIIb, Fig. 1C), which were expressed in the rice endosperm using a wheat high molecular weight glutenin promoter. The selected amiRNA fragment has a predicted hybridization energy of \(-38.39\) kcal mol\(^{-1}\), with a putative target cleavage site at positions 10 and 11, and mismatches at positions 1 and 21 (Fig. 1B).

PCR screening of T\(_0\) plants revealed that \(~75\%\) of the putative transformants contained the constructs (Supplementary Fig. S1 at JXB online). The transgenic plants stably retained the hp-BEIIb and ami-BEIIb transgene in subsequent generations (T\(_1\)–T\(_4\)). Southern blot analyses of selected T\(_1\) plants revealed an insertion of one copy of each silencing construct for most of the hp-BEIIb and amiRNA plants tested (data not shown). Segregating PCR-positive T\(_1\) seeds (\(~3:1\) ratio based on grain appearance) were planted to obtain homozygous seeds. Four lines of hp-BEIIb and five lines of ami-BEIIb were homozygous in the T\(_2\) generation and this was verified up to T\(_4\). These lines were used in the following experiments.

Gene expression analyses

Gene expression analyses of homozygous T\(_3\) lines by real-time PCR revealed a \(>5\)-fold decrease in the expression of SBEIIb in ami-BEIIb lines, while only a 2-fold decrease was observed in transgenic lines harbouring hp-BEIIb (Fig. 2). The expression of transcripts for other starch branching isoforms (SBEI and SBEIIa) and major starch synthase isoforms (SSI, SSIIa, and SSIIIa) was unaffected (Fig. 2).

Enzyme expression and activity detection

Western blot analyses showed trace levels of SBEIIb protein in the hp-BEIIb lines at 10 dpa, which was undetectable in the amiRNA lines (Fig. 3A), while the wild type had considerably more SBEIIb. The levels of SBEIIa remained similar to those of the wild-type Nipponbare for both the ami-BEIIb and the hp-BEIIb lines (Fig. 3B). This was verified by in-gel activity staining of branching enzymes, which showed that SBEIIb activity was only detectable in Nipponbare but not in the transgenic lines, while the

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**Fig. 1.** Diagrammatic representation of the RNA silencing constructs (not drawn to scale). (A) A 21 nucleotide artificial microRNA (ami-BEIIb)-based osa-miR528 was synthesized by fusion PCR and cloned into Vec8, a Ti binary vector with a wheat high molecular weight glutenin promoter (wHMGPro) and nopaline synthase terminator (NOS). (B) The secondary structure of the osa-miR528 backbone as predicted by RNAfold, including information on ami-BEIIb (reverse complement). The predicted target cleavage site (arrow with sequence bold and highlighted) is located between positions 10 and 11 of the amiRNA, while the two mismatches (grey highlight) are located at positions 1 and 21. (C) The hairpin RNA (hp-BEIIb) was cloned in Vec8 by inserting a 397 bp BEIIb fragment in the sense (BEIIb→) and antisense (BEIIb←) orientations. The two fragments are flanked by two rice introns, Rint4 and Rint9, which form a hairpin loop. The amiRNA and hp-RNA fragments were directionally cloned using several restriction sites (H, HindIII; B, BamHI; K, KpnI; N, NotI; E, EcoRI; S, SpeI).
SBEIIa activity remained unaffected (Fig. 3C). The activity of SBEI was unaffected (data not shown).

Western blot detection of developing T4 endosperm revealed that one homozygous line of ami-BEIIb had stably down-regulated and almost undetectable amounts of SBEIIb at 5, 10, 15, and 20 dpa, while one homozygous hp-BEIIb line had a down-regulated but higher concentration of SBEIIb (Supplementary Fig. S2 at JXB online). In contrast, the levels of SBEIIa in both lines were similar to those of the Nipponbare control at each stage (Supplementary Fig. S2). The decrease in branching enzyme activity due to SBEIIb down-regulation led to reduced branching frequency, with a decrease in the number of reducing ends per milligram of starch from ~19 in Nipponbare wild type to 14 in the ami-BEIIb lines (Fig. 3D).

**Grain and starch granule analyses**

Polished grains of ami-BEIIb lines were opaque throughout (Fig. 4A), while polished grains of the hp-BEIIb lines were chalky, showing streaks of white along the translucent endosperm (Fig. 4B). In comparison, Nipponbare grain appeared uniformly translucent (Fig. 4C). Grain weight, width, and thickness of the transgenic lines were lower than those of Nipponbare (Table 1). Grain yield of the transgenic lines was also lower than for Nipponbare (data not shown).

Scanning electron micrographs of ami-BEIIb (Fig. 4D) and hp-BEIIb (Fig. 4E) lines revealed big and small rounded starch granules with large spaces in between, exhibiting a loss of compound granular organization. In
contrast, the starch granules of Nipponbare appeared compact, compound, and angular, with fewer spaces in between (Fig. 4F). Changes in starch granule morphology were more pronounced in ami-BEIIB (Fig. 4D) than in hp-BEIIB (Fig. 4E). Isolated starch granules of hp-BEIIB and ami-BEIIB stained with APTS did not show any distinct difference from the wild type, and all showed normal birefringence under polarized light (data not shown).

XRD analyses showed that some hp-BEIIB lines retained the A-type starch crystalline polymorph (similar to the Nipponbare as well as another wild type, IR36) while others showed a mixture of A- and B-type crystallinity, also known as C-type starch (Fig. 5). In contrast, all ami-BEIIB lines fully shifted to B-type crystallinity, as also found in the SBEIIb mutant IR36ae (Fig. 5). These differences were also consistent with solid-state $^{13}$C CP/MAS NMR analyses (Supplementary Fig. S3 at JXB online).

**Starch structural analyses**

The total starch content of hp-BEIIB and ami-BEIIB lines was comparable with that of the control at ~90%, although the ami-BEIIB lines contain a greater proportion of starch resistant to enzymatic breakdown (Tables 1, 2). When measured at 620 nm, a >2-fold increase in AAC was observed in ami-BEIIB compared with Nipponbare (Table 1). The AAC levels of the hp-BEIIB lines were also significantly elevated compared with the control, but not as high as those of the ami-BEIIB lines (Table 1). However, when measured at 720 nm, the amylose content of ami-BEIIB lines was comparable with that of Nipponbare (Table 1). The hp-BEIIB lines showed a slight reduction, though this was not significant (Table 1).

Chain length distributions (CLDs) of debranched starches revealed a decrease in the proportion of amylopectin short chains (DP 6–12) with a concomitant increase in the longer chains (DP >14) in both the amiRNA (Fig. 6A) and hp-RNA (Fig. 6B) lines. The decrease in the ratio of short chains was more pronounced in the amiRNA lines to DP 36 but <1000 in the transgenic lines compared with the wild type was observed and this was more pronounced for the ami-BEIIB lines (Fig. 7, Supplementary Table S3). There was also a reduction in debranched short amylopectin chains (DP 6–36) in the transgenic lines, which was again more pronounced in ami-BEIIB lines (Fig. 7, Supplementary Table S3). These results are consistent with the increase in the average chain length in the ami-BEIIB samples to DP ~400 compared with DP ~300 in the Nipponbare samples as deduced from the number of reducing ends per milligram of starch (Fig. 5D).

Peak GT was significantly increased in ami-BEIIB compared with the wild type (Table 1). The mean GT of hp-BEIIB was slightly elevated but the difference was not significant (Table 1).

**Nutritional properties**

The in vitro RS content of freshly cooked ami-BEIIB grains was 10-fold higher than that of Nipponbare (Table 2). In comparison, a 4-fold increase was observed for IR36ae compared with its parent IR36 (Table 2). Moreover, the RS content of ami-BEIIB grains was also considerably higher than that of IR36ae. On the other hand, the RS values

<table>
<thead>
<tr>
<th>Properties</th>
<th>ami-BEIIB lines</th>
<th>hp-BEIIB lines</th>
<th>Nipponbare</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ten brown grain weight (mg)</td>
<td>140.3±8.5 a</td>
<td>144.2±5.7 a</td>
<td>205.2±2.3 b</td>
</tr>
<tr>
<td>Length (mm)</td>
<td>5.2±0.1</td>
<td>5.1±0.0</td>
<td>5.2±0.0</td>
</tr>
<tr>
<td>Width (mm)</td>
<td>2.7±0.1 a</td>
<td>2.7±0.1 a</td>
<td>2.9±0.0 b</td>
</tr>
<tr>
<td>Thickness (mm)</td>
<td>2.0±0.0 a</td>
<td>2.0±0.0 a</td>
<td>2.2±0.0 b</td>
</tr>
<tr>
<td>Chalkiness (% per grain)</td>
<td>75–100</td>
<td>25–50</td>
<td>0–10</td>
</tr>
<tr>
<td>Total starch, flour samples (g 100 g$^{-1}$)</td>
<td>91.4±1.7*</td>
<td>91.0±0.4</td>
<td>90.4±0.9</td>
</tr>
<tr>
<td>Total starch, freshly cooked grains (g 100 g$^{-1}$)</td>
<td>86.9±0.6</td>
<td>86.0±0.4</td>
<td>85.5±0.0</td>
</tr>
<tr>
<td>Apparent amylose content (%) at 620 nm</td>
<td>41.2±0.5 a</td>
<td>34.0±1.6 b</td>
<td>19.6±0.7 c</td>
</tr>
<tr>
<td>Apparent amylose content (%) at 720 nm</td>
<td>16.9±0.4 a</td>
<td>13.7±0.8 b</td>
<td>15.2±0.2 a,b</td>
</tr>
<tr>
<td>Pentosans (%)</td>
<td>0.31±0.02 a</td>
<td>0.25±0.01 a</td>
<td>0.05±0.01 b</td>
</tr>
<tr>
<td>β-Glucan (%)</td>
<td>0.07±0.01</td>
<td>0.05±0.01</td>
<td>0.05±0.01 b</td>
</tr>
<tr>
<td>Neutral non-starch polysaccharides (g 100 g$^{-1}$)</td>
<td>1.5±0.2 a</td>
<td>0.8±0.1 b</td>
<td>0.8±0.0 b</td>
</tr>
<tr>
<td>Peak gelatinization temperature (°C)</td>
<td>80.7±0.0 a</td>
<td>75.0±1.2 b</td>
<td>71.2±0.5 b</td>
</tr>
</tbody>
</table>

*Of which, 9.5±0.8 g 100 g$^{-1}$ are resistant starch.
obtained for hp-BEIIb were not significantly different from the control (Table 2).

The predicted GI for ami-BEIIb was reduced compared with Nipponbare and the SBEIIb mutant, IR36ae (Table 2). The predicted GI of IR36ae was also decreased compared with its parent IR36. The GI estimate for hp-BEIIb was also reduced, but this was not significantly different from the control (Table 2).

The NNSP content of ami-BEIIb lines was approximately twice that of hp-BEIIb and Nipponbare (Table 1), while the level of pentosans was significantly greater in ami-BEIIb and in hp-BEIIb lines compared with the control. On the other hand, the levels of β-glucans remained unchanged (Table 1).

Discussion

In the present study, the down-regulation of SBEIIb gene expression (Fig. 2) in the rice endosperm was achieved using an amiRNA (Fig. 1A, B) and a hp-RNA (Fig. 1C). This led to the reduction of SBEIIb protein, SBEIIb enzymatic activity, and the abundance of reducing ends present in debranched starch (Fig. 3). SBEIIa expression was unaffected (Fig. 3). The down-regulation of SBEIIb was further confirmed using endosperm at different stages of development, which also showed that SBEIIa expression was unaffected (Supplementary Fig. S2 at JXB online). The SBEIIa and SBEIIb isoforms are encoded by separate genes in maize, wheat, and rice (Fisher et al., 1996). In the current work, the mRNA and protein levels as well as the enzymatic activity of SBEIIa were not affected (Figs 3, 4), similar to what is observed in an ae maize (Fisher et al., 1996; Gao et al., 1997) and transgenic barley with down-regulated BEIIb (Regina et al., 2010). Furthermore, the expression of BEI and other major starch synthase isoforms were also unaffected (Fig. 3). Even the expression of SSI, which is slightly down-regulated in an ae mutant of rice (Nishi et al., 2003), and corn (Boyer and Preiss, 1978), was not altered (Fig. 2), in either the hairpin or amiRNA lines. These results indicate that RNA silencing (Fig. 1) can be gene- and isoform-specific, given a unique target sequence and an accurate target-finding algorithm such as Web microRNA Designer (Ossowski et al., 2008).

Comparison of artificial microRNA and hairpin RNA techniques

RNA interference using hp-RNA was found to be effective in down-regulating branching enzymes in wheat (Regina et al., 2006; Sestili et al., 2010) and in barley (Regina et al., 2010), as also demonstrated in this study (Figs 1–4). This study also established that amiRNA (Fig. 1A, B) is very efficient in attenuating SBEIIb expression in rice grains (Fig. 2), demonstrating the isoform specificity and efficacy of this technique in regulating gene expression in the rice endosperm.

The amiRNA technique (Fig. 1A, B) was more effective in reducing SBEIIb gene expression (Fig. 2) and in producing more extreme starch properties (Figs 5–7, Tables 1, 2) than the hp-RNA technique for the traits assessed here. The hp-BEIIb lines, especially those that retained the A-type polymorph (Fig. 5), might not have exceeded an RNA inhibition threshold necessary to achieve the phenotypes produced by the ami-BEIIb lines. This was supported by the observation that the amount of SBEIIb was more stably reduced in developing endosperm of an ami-BEIIb line compared with an hp-BEIIb line (Supplementary Fig. S2 at JXB online). Very high amylose was not achieved in potato because the necessary RNA inhibition threshold was also not exceeded (Safford et al., 1998).

One advantage of the amiRNA technique is that mismatches can be deliberately introduced in its 21 nucleotide sequence (Fig. 2) to facilitate not only mRNA cleavage (Kasschau et al., 2003; Bagga et al., 2005; Schwab et al., 2005) but also translational repression (Aukerman and Sakai, 2003; Chen, 2004). Such mRNA cleavage and

### Table 2. Nutritional properties of freshly cooked grains

<table>
<thead>
<tr>
<th>Lines</th>
<th>Resistant starch cooked grains (g 100 g⁻¹)</th>
<th>Predicted glycaemic index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Down-regulated BEIIb lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ami-BEIIb</td>
<td>4.8±0.2 a</td>
<td>44±1 a</td>
</tr>
<tr>
<td>hp-BEIIb</td>
<td>0.4±0.2 b</td>
<td>70±8 b</td>
</tr>
<tr>
<td>Nipponbare</td>
<td>0.2±0.0 b</td>
<td>85±2 b</td>
</tr>
<tr>
<td>amylose extender mutant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IR36ae</td>
<td>3.1±0.0 c</td>
<td>54±2 a</td>
</tr>
<tr>
<td>IR36</td>
<td>0.7±0.4 d</td>
<td>68±1 b</td>
</tr>
</tbody>
</table>

Fig. 5. XRD patterns of starches with down-regulated SBEIIb and its comparison with the wild type and the amylose extender mutant. Two hp-BEIIb lines (hp-BEIIb A-type) share the same A-type crystalline polymorph (peak at 18°, but not 5°, 2θ) with the wild-type rices (Nipponbare and IR36) while all four ami-BEIIb shifted to a B-type polymorph (peak at 5°, but not 18°, 2θ) similar to IR36ae. Two hp-BEIIb showed a crystalline structure that is intermediate between A- and B-type (hp-BEIIb C-type). Data are off set for clarity.
translational repression is consistent with the observation of the 4-fold reduction in the gene expression of SBEIIb (Fig. 2) and the undetectable levels of SBEIIb protein and enzymatic activity (Fig. 3, and Supplementary Fig. S2 at JXB online).

Effects of down-regulating SBEIIb expression on starch structure

The ‘amylose’ content of both ami-BEIIb and hp-BEIIb lines was double that of the control when measured at 620 nm (Table 1), the wavelength routinely used for

Fig. 6. Chain length distribution (CLD) profile of debranched starch (A and B) and mol% difference of ami-BEIIb and hp-BEIIb compared with Nipponbare (C). Compared with Nipponbare, ami-BEIIb (A) and hp-BEIIb (B) have reductions in DP 6–12 and an increase in DP 13 onwards. A difference plot (C) revealed that the reduction in short DP is more pronounced in ami-BEIIb (up to 4%) than in hp-BEIIb (up to 3%). Likewise, the increase in longer DPs is more pronounced in ami-BEIIb (up to 0.9%) than in hp-BEIIb (up to 0.7%). Error bars indicate the SEM.
apparent amylose content estimation (Juliano et al., 1981). However, the molecular size distribution of debranched starch revealed that down-regulating BEIIb, at least in a japonica background like Nipponbare where GBSSI is less active, does not increase the amount of the very long amylose chains (Fig. 7, and Supplementary Table S3 at JXB online). Instead, the reduction in the amount of BEIIb alters starch properties by increasing the proportion of long amylopectin chains (DP 37–120) and decreasing the proportion of short amylopectin chains (DP 6–36) (Fig. 7, and Supplementary Table S3). ‘Long amylopectin’ chains here refer to the maximum DP spectrum observed in debranched starch in the absence of a functional GBSSI. Based on the result of Fitzgerald et al. (2009), debranched chains observed in the absence of GBSSI, as in waxy rice lines, have a maximum DP of ~120. In the same study, much higher DPs are found in debranched starch of rice lines where GBSSI is catalytically active, which can reach beyond DP 7000. The general shift to longer branch lengths for ami-BEIIb compared with hp-BEIIb seen in the SEC data (Fig. 7) can also be observed in the FACE data (Fig. 6).

Moreover, the proportion of intermediate chains from DP 120–1000 was also elevated (Fig. 7, and Supplementary Table S3), consistent with either significantly elevated levels of amylopectin B3 and B4 chains (Hizukuri, 1986) or the presence of short amylose branches. Longer average chain lengths and increased proportions of intermediate fractions were also observed in ae mutants of maize (Ikawa et al., 1978; Yeh et al., 1981).

The total proportion of long amylopectin and intermediate chains was 13% higher than Nipponbare for hp-BEIIb and 18% for ami-BEIIb (Supplementary Table S3 at JXB online). Whether these debranched intermediate chains—longer than traditionally found in amylopectin and shorter than classic long chain amylose—are attached to amylopectin or amylose, it is likely that they are the origin of the doubling of apparent amylose content when measured using 620 nm (Table 1). No difference in amylose levels was observed in the transgenic lines compared with Nipponbare when the estimation was done at 720 nm (Table 1). It is possible that these intermediate chains complex with iodine in a similar fashion to amylose, as they represent the main structural difference between the transgenic starches compared with the wild type (Figs 6, 7). If these intermediate chains connect clusters of branching density similar to those of the wild type, then little change in iodine binding would be expected due to the disruptive influence of branch points on the formation of the iodine complex. However, if these intermediate chains are lightly branched, they could complex with iodine, explaining the large increase in amylose content in the transgenic lines (Table 1). It is therefore likely that the increased proportion of intermediate chains is associated with lightly branched structures so that that iodine binding occurs similar to linear amylose.

Effects of amylopectin structure on starch granule morphology and crystallinity

The ae mutants in maize are often associated with slight reductions in grain dimensions and weight (Shannon et al., 2009), as also observed in this study (Table 1). Additionally, the opacity or chalkiness in grains (Fig. 4A, B, Table 1) is consistent with previous observations (Nishi et al., 2001; Sawada et al., 2009). The starch granules of the recombinant lines (Fig. 4D, E) appeared loose and spherical and seemed to have lost the higher level of organization into compound granules that is seen in the control (Fig. 5F). The increase in apparent amylose content also associates with decreases in the proportion of angular starch granules and increases in the proportion of elongated forms in maize (Banks et al., 1974). Furthermore, chains intermediate between amylose and amylopectin can distort starch granule morphology (Blennow et al., 2003). The differences in granular packing could therefore be due to the altered molecular structure of the transgenic starches (Figs 6, 7).

A-type crystallinity is commonly found in cereal starches, but mutations in SBEIIb often lead to B-type crystallinity (Gerard et al., 1999; Tanaka et al., 2004; Kubo et al., 2008). Interestingly, in the present study, the ami-BEIIb starches

![Fig. 7. Debranched HP-SEC of transformed lines compared with Nipponbare using (A) Ultrahydrogel 250 and (B) Proteema columns. Intermediate length chains (apparent DP ≥37) were increased while the short amylopectin chains (DP ≤36) were decreased in both hp-SBEIIb and ami-BEIIb, with the changes more pronounced in the latter. No change in the proportion of long chain amylose peak (apparent DP ≥1000) was observed, while the mutants have elevated amounts of intermediate material—longer than traditionally found in amylopectin (DP 6–120) and shorter than classic long chain amylose (inset).](image-url)
completely shifted to a B-type, while hp-BEIib starches retained the A-type, or exhibited the intermediate C-type (Fig. 5, and Supplementary Fig. S3 at JXB online). The results obtained by Tanaka et al. (2004) are illuminating in this context. They reported that the absence of BEIib in the mutant EM10 resulted in B-type crystallinity but when the mutation was complemented by the introduction of a BEIib transgene, the starch crystallinity changed from B to C to A, depending on the strength of transgene expression. In the results of this present study, it is clear that the ami-BEIib lines have more reduced SBEIib expression than the hp-BEIib lines (Fig. 3, and Supplementary Fig. S2). The reduction in the ami-BEIib is sufficient to phenocopy the EM10 and 7.8 lines reported by Tanaka et al. (2004), whereas the reduction in the hp-BEIib lines phenocopies lines 9.8 (C type) and 106-1 (A type). Furthermore, the difference in crystalline polymorphic form between ami-BEIib and hp-BEIib is consistent with the slight increase in longer amylopectin branches (Hizukuri et al., 1983) in the ami-BEIib lines compared with hp-BEIib lines, as this could cause a shift from the thermodynamically more stable A form to the kinetically trapped B form (Gidley, 1987). A difference in chains of DP 6–12 has also been associated with a shift from the A-type to B-type polymorph in several cereals (Hanashiro et al., 1996). Although it is not possible in this study to separate the effects of the changes in long amylopectin chains from the changes in the short amylopectin chains, it appears that rice starch exists at the boundary of the structural requirements for A- versus B-type crystallinity, and that only a relatively small change in amylopectin branch lengths of rice starch is sufficient for this transition.

Wei et al. (2010a) reported that simultaneous antisense inhibition of SBEI and SBEIib (TRS line) only led to the intermediate C-type crystalline polymorph, which may indicate insufficient down-regulation of branching activity as also observed in hp-BEIib lines (Figs 2, 3). However, alterations in starch granule morphology produced in the TRS line are more extreme that what was observed in this study (Wei et al., 2010a, b, c, d), maybe because the antisense inhibition was done in a high amylose background (Teqing). Elevated proportions of amylose chains might introduce additional perturbations in granule packing in this variety.

Effects of amylopectin structure on functional and predicted nutritional properties

It is known that ae starch granules are more resistant to thermal gelatinization than the wild type, at least in part due to alterations in amylopectin fine structure (Tanaka et al., 2004; Shannon et al., 2009). The decrease in short chains and the increase in longer chains (Figs 6, 7, and Supplementary Table S3 at JXB online) observed in the ami- and the hp-BEIib lines is consistent with previous observations (Nishi et al., 2001) and with the proposed branching role of SBEIib in the crystalline lamellae of the amylopectin cluster in the rice endosperm (Nakamura, 2002; Nakamura et al., 2010). Although B-type crystallities melt at lower temperature than A-type crystallites of the same chain length (Whittam et al., 1990), it is probable that the higher peak GT in ami-BEIib is due to the elevated proportions of long amylopectin and intermediate chains as compared with hp-BEIib (Fig. 7, and Supplementary Table S3). It was found that an ~6% decrease in the proportion of chains of DP 6–12 relative to chains of DP 12–24 can lead to an increase in GT of at least 10 °C in waxy rices (Cuevas et al., 2010).

High amylose rice mutants, including the lines developed in this study, are interesting from a nutritional perspective because they are typically shown to contain higher levels of dietary fibre and/or resistant starch (Lee et al., 2006; Shu et al., 2006; Yang et al., 2006). The alterations in starch digestibility are consistent with the observed alterations in starch structure as it is known that linear amylose chains of DP ~100 have the fastest rate of double helix formation and retrogradation in water of any starch polymer (Gidley and Bulpin, 1989). Increases in the proportion of DP 100 chains were observed (Fig. 7, and Supplementary Table S3 at JXB online).

In the lines described here, there is a 20-fold increase in the proportion of resistant starch in the amiRNA lines compared with hp-BEIib and wild-type lines (Table 2). In fact the ami-BEIib lines have higher RS content than the traditional amylose extender mutant IR36ae (Table 2). This is despite the fact that ami-BEIib is in a japonica background with intermediate to low amylose content (Nipponbわれ), while IR36ae is in an indica background with a high amylose content (IR36). Likewise, the ami-BEIib lines have lower GI estimates compared with IR36ae (Table 2), despite the former having a lower proportion of true amylose chains. This strongly suggests that aside from amylose, intermediate chains and long chain amylopectin molecules (Fig. 7) may play an important structural role in rendering the starch molecule less digestible. In particular, it appears that elevations in the proportion of intermediate chains may be associated with increase in the levels of resistant starch in rice.

Implications for starch biosynthetic pathways in cereals

While the rice mutants obtained by traditional mutagenesis techniques in previous studies as well as the recombinant lines obtained in this study have high apparent amylose contents compared with common cultivated varieties, these are significantly lower than apparent amylose contents that have been reported for analogous mutations in maize, wheat, and barley. In ae maize, lack of BEIib is thought to be partially complemented by BEIIa and BEI (Liu et al., 2009). The same mechanism might also exist in rice. However, maize can produce up to 50–80% amylose when SBEIib is altered, with levels increasing due to the presence of some modifier genes (Shannon et al., 2009). Another reason for this may be that in maize, the proportion of SBEIib to SBEIIa in the endosperm is 50:1 (Gao et al., 1997), whereas in rice the ratio is closer to 5:1 (Regina et al., 2006).
In contrast to maize, down-regulation of SBEIIb alone did not significantly increase the levels of amylose in either wheat or barley (Regina et al., 2006, 2010). This is because SBEIIa is expressed at much higher levels in the grains of both wheat and barley (Regina et al., 2005, 2010), than in maize and even rice (Gao et al., 1997; Mizuno et al., 2001; Ohdan et al., 2005). The differences in expression levels of the SBEIIa and SBEIIb enzymes in cereals therefore appear to define which of these two enzymes should be down-regulated to generate high amylose starches. Furthermore, since down-regulating BEIIa in wheat produces high amylose (Regina et al., 2006) and down-regulating both BEIIa and BEIIb (Regina et al., 2010) or SSIIa alone (Morell et al., 2003) is necessary to produce high amylose in barley, it appears that producing very high amylose in rice might require modification of the expression of a different combination of target isoforms. Even the simultaneous down-regulation of SBEI and SBEIIb only resulted in a doubling of the apparent amylose content despite significant alterations in starch granule morphology (Wei et al., 2010a, b, c, d). The use of amiRNAs, which has been successfully demonstrated in this study to be effective in producing a more extreme starch phenotype than the conventional hp-RNA constructs, may be helpful in silencing other genes to produce very high amylose rice grains. Comparison of the efficacy of these techniques in down-regulating other genes in the cereal endosperm will be important to better understand the relative efficacy of the amiRNA and hp-RNA approaches.

Conclusion
The ae phenotype can be obtained by down-regulating the expression of SBEIIb in rice using RNA silencing. For the first time, the amiRNA technique was demonstrated to facilitate RNA silencing in rice endosperm and was better at producing a more extreme starch and nutritional phenotype than the hp-RNA technique in these experiments. For rice SBEIIb, the greater effectiveness of the amiRNA technique resulted in distinct differences in rice starch chemistry, crystallinity, and digestibility compared with the hp-RNA technique. However, in order to obtain very high amylose rice grains, the expression of multiple starch-synthesizing enzymes may need to be down-regulated simultaneously.

Supplementary data
Supplementary data are available at JXB online.

- Figure S1. PCR screening of transgenic lines.
- Figure S2. Western blot detection of SBEIIa and SBEIIb in developing endosperms of ami-BEIIb and hp-BEIIb compared with Nipponbare at four development stages.
- Figure S3. 13C NMR CP/MAS spectrum and deconvolution of an ordered subspectrum of rice flour samples.
- Figure S4. Chain length distribution difference (mol%) of generated transgenic lines as compared with IR36ae.

Table S1. PCR primers used.
Table S2. Amylose classification and water to raw rice ratio used during the absorption method of resistant starch and glycaemic index estimations.
Table S3. Molecular size distribution of debranched amylose and amylopectin chains of transgenic lines compared with Nipponbare.

Acknowledgements
The authors thank Norman Warthmann and Detlef Weigt for providing the osa-miR528 amiRNA backbone. Ivan Moore produced the rice anti-BEIIa polyclonal antibodies. Robin Chapple is acknowledged for carrying out the β-glucan assay, Bradley Klingner for performing the in vitro RS and GI tests, and Rosa Paula Cuevas for running the DSC measurements. Mark Talbot is also acknowledged for his assistance during the scanning electron microscopy experiments. VMB is a recipient of an Australian Leadership Award Scholarship.

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