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Identification of a Major Genetic Determinant of Glycaemic Index in Rice

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Abstract Type II diabetes is a major chronic disease. In developing countries, the prevalence of type II diabetes is increasing enormously. Much research indicates that choice of carbohydrates, particularly those with low glycaemic index (GI) is able to assist in the management or prevention of type II diabetes. Most developing countries consume rice as the staple. The objectives of this study were to determine the variability in the GI of popular improved and traditional varieties of rice and to find the genetic basis of GI. A method to predict GI using an in vitro system was compared to the in vivo system using a range of rice varieties differing in GI. Large variability in GI, ranging from low to high GI, was found using a set of 235 varieties. The major gene that associated with GI in the 235 varieties was the *Waxy* gene. This paper reports the first large-scale phenotyping of this trait, provides important information for nutritionists to identify and quantify the impact of low GI rices on blood sugar status and offers a mechanism for breeding programmes to select for GI based on amylose content. Furthermore, it allows rice consumers to select particular varieties of rice as their choice of carbohydrate.

Keywords Glycaemic index · Rice · Type II diabetes · Amylose

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Introduction

Type II diabetes is a major global health problem and its prevalence is increasing dramatically throughout the world, especially in Asia (Chan et al. 2009; Danaei et al. 2011). By 2030, almost 330 million people will be affected by diabetes and the greatest burden of this disease will be borne primarily by the socioeconomically disadvantaged in low and middle income societies (Misra et al. 2010; Walgate 2008). In those communities, the populace has limited access to basic health services and the presence of diabetes is often only discovered when serious complications arise, such as cardiac and kidney failure and peripheral vascular damage leading to strokes, blindness and amputations of toes, feet and limbs (Walgate 2008).

The escalating diabetes pandemic is largely a consequence of the shift away from traditional lifestyles and dietary patterns to increasingly sedentary behaviours coupled with excess intake of energy dense foods and rising rates of obesity. A growing body of evidence from epidemiological and clinical studies points to the adverse health

consequences of foods and diets rich in carbohydrates which are readily and extensively digested (Brand-Miller et al. 2009; Hu et al. 2001; Sluijs et al. 2010). Mechanistic studies demonstrate that chronically elevated blood glucose levels induce deleterious structural changes in many tissues of the body, in particular the macro- and microvasculature (Kaushik et al. 2009). Postprandial glycaemia is emerging as a clinically useful independent risk factor for cardiovascular disease in non-diabetics and those with established diabetes (Sheu et al. 2011). Carbohydrate-based foods which elicit a modest metabolic response, namely slowed or delayed postprandial intestinal glucose absorption and consequent insulin secretion are likely to be of benefit for reducing risk of chronic diseases, such as type II diabetes. There is also a growing body of data suggesting that certain populations are inherently more susceptible to developing type II diabetes (Kooner et al. 2011); for such people, dietary options for managing blood glucose levels are important.

The glycaemic index (GI) ranks foods (and diets) on the basis of their propensity to raise blood glucose, thereby providing a relative measure of dietary carbohydrate quality. A prospective cohort study showed that dietary GI and glycaemic load (GL=0.01GI × grams of carbohydrate consumed) were positively associated with diabetes risk (Barclay et al. 2008; Halton et al. 2008). Subsequent research has confirmed those findings and generated strong evidence from meta-analysis and meta-regression studies demonstrating that low GI diets are linked to improved risk markers for prevention of type II diabetes and its co-morbidities (Barclay et al. 2008; Brand-Miller et al. 2003; Halton et al. 2008; Livesey et al. 2008; Marsh and Brand-Miller 2008; Opperman et al. 2004; Wolever and Mehling 2002). Lower GI foods and diets provoke only transient, moderate postprandial glycaemia and improve insulin sensitivity along with other endpoints of cardio-metabolic health in obese and overweight subjects as well as those with type II diabetes (Brand-Miller et al. 2003; Dickinson and Brand-Miller 2005; Livesey et al. 2008; Marsh and Brand-Miller 2008; Opperman et al. 2004; Wolever and Mehling 2002). Furthermore, low GI diets improve metabolic health indices independent of the amount of carbohydrate consumed (Psaltopoulou et al. 2010). Accordingly, lowering the GI of the diet could help in preventing the development and slowing the progression of type II diabetes and thereby lead to an improvement in public health. It also may offer a practical means for diabetes sufferers in low income countries to better manage their condition without expensive medication.

For the majority of the world's population, polished rice is a dietary staple and has been since its domestication many thousands of years ago (Sweeney and McCouch 2007). It serves as the primary source of dietary energy and carbohydrates for most Asians, and increasingly for Africans, especially those in poorer urban and rural communities (www.irri.org). However, prospective cohort studies in genetically divergent populations show that white rice consumption is associated with increased risk of developing type II diabetes independent of ethnicity (Murakami et al. 2006; Sun et al. 2010). In a study of middle-aged Chinese women, type II diabetes risk was 78% greater in those consuming more than 300 g rice/day relative to those eating <200 g/day (Villegas et al. 2007). Whereas white rice has been shown to adversely affect metabolic health, brown rice may be protective. In a study of US men and women, a moderate inverse association between diabetes risk and brown rice consumption was observed (Sun et al. 2010); however, varietal differences were not taken into account. Furthermore, brown rice intakes were overall very low and the results may have been confounded, in that certain populations of rice eaters often have healthier diets and lifestyles (Batres-Marquez and Jesen 2009; Fulgoni et al. 2010). Further, two studies that tested the GI of brown and white rice of the same variety, each using different varieties, reached opposing conclusions (Brand-Miller et al. 1992; Panlasigui and Thompson 2006).

Replacing white with brown rice or other whole grains has been recommended to mitigate the adverse metabolic consequences associated with refined rice consumption (Dixit et al. 2011; Kumar et al. 2011; Sun et al. 2010). However, consumption of brown rice is very low relative to white rice. Most Asians, for instance, consider it inferior to white rice because of its shorter shelf-life, longer cooking time and unappealing taste and texture (Zhang et al. 2010). Accordingly, strategies to encourage brown rice consumption over that of polished rice so as to improve consumer health are unlikely to be successful at the population level, whereas a more effective approach may be to reduce the glycaemic impact of polished rice through the development and introduction of suitable low GI rice varieties. Furthermore, most commonly consumed rices have a high GI irrespective of whether they are unpolished or refined (Brand-Miller et al. 2003; Lin et al. 2010).

While primary prevention of type II diabetes through more judicious food choices may be the frontline strategy, behavioural change that is sustained and of meaningful magnitude is difficult to achieve in practice, especially in the short term. Lowering the GI of staple foods such as rice is likely to be more effective in promoting public health, especially in communities in which rice accounts for a large share of dietary glycaemic load and where there are entrenched cultural preferences for consumption of white rice. Some Australian, Indonesian, Indian and Bangladeshi varieties have been reported to have lower GI than other rices, but the genetic basis of GI has not been determined. In barley, a mutation in starch synthase IIa (*SSIIa*) led to a significant lowering of GI (King et al. 2008). Four

haplotypes of SSIIa are known in rice, two of which are inactive (Cuevas et al. 2010b; Waters et al. 2006), and if the enzymes operate the same way in barley and rice, this could lead to greater understanding of differences in GI in rice. Understanding this could enable breeding programmes to target this trait, leading to new varieties with even lower GI values than are currently known for rice.

The purpose of the present study was to establish the major genetic determinants of GI in rice for informing future varietal development. The GIs of a diverse set of Asian rices, both improved and traditional varieties, were predicted using a newly developed, high throughput instrument designed to simulate carbohydrate assimilation in the human gut. The relationship between the predictive method and in vivo measurements of GI was confirmed on a subset of lines. A secondary objective of the study was to offer an insight as to whether rice improvement programmes have changed the glycaemic properties of rices commonly eaten in Asian countries.

Results

Method validation

In vivo values of GI were determined for both the International Rice Research Institute (IRRI) and Australian sets of rice at the International Diabetes Institute and the University of Sydney, respectively. The available carbohydrate for both sets was calculated by the direct method, and GI was predicted for both sets. Figure 1 shows that the in vivo values of GI associate well with the in vitro values (Fig. 1).

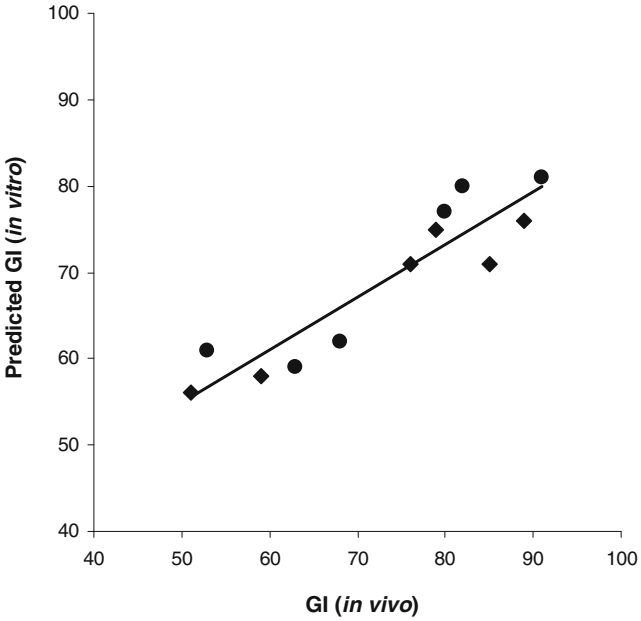

Fig. 1 Correlation (r^2=0.85) between in vitro and in vivo measures of GI in 12 varieties of rice. *Diamonds* are in vivo values from the literature (Williams et al. 2005) with in vitro values tested in this study, and *circles* represent varieties for which in vitro and in vivo values are from the present study.

Association between GI and grain properties

Using a diverse set of rices from different countries and germplasm classes, the predicted GI values range from 48 to 92 (Fig. 2), spanning low, intermediate and high GI categories, with an average reading of 64. Predicted GI also associates with amylose content (Fig. 3a) for the 235 varieties, with increasing amylose content leading to decreased values of GI. In Fig. 3a, clusters can be seen for high, intermediate, low and waxy rices. Table 1 shows that for most alleles of the *Wx* gene, the average GI values are significantly different, with the *wx* allele showing the highest GI and the Wx^a allele showing the lowest values of GI. Figure 3b shows that one waxy, IRIS 6-59997, one low, IRIS 298-52752 and four high amylose, IRIS 266-4060, IRIS 249-1353606, IRIS 249-1353606 and IRIS 109–8916 (Table S1) varieties lie beyond the lower boundary of the interquartile range, and four high amylose varieties lie above the upper boundary. The activity of SSIIa in each sample was determined by genotyping for the four haplotypes (Cuevas et al. 2010a). No significant difference was found between *SSIIa* haplotype and GI, nor was there any interaction or modifying effect of *SSIIa* haplotype on the association between amylose alleles and GI (data not shown).

High amylose rices, those carrying the Wx^a allele, differ in the texture of the cooked rice in that some are soft and some are firm. Using progeny from a mapping population derived from parents both with the Wx^a allele and segregating for cooked rice texture, the predicted GI was 60±6 for the soft textured progeny and 59±4 for the firm-textured progeny.

Figure 4 shows that traditional varieties, selected by ancient farmers, are not of significantly different GI than improved varieties, with the average GI of improved varieties being 64.9 and of traditional, 64.0. However, traditional varieties do not show so many high GI samples as improved varieties, so the weighted average GI of these is 63, whereas it is 68 for the improved varieties.

Discussion

The prevalence of lifestyle-related chronic diseases and conditions, such as obesity, cardiovascular disease, certain cancers and type II diabetes is continuing to grow at an alarming pace throughout the world, the Asian region

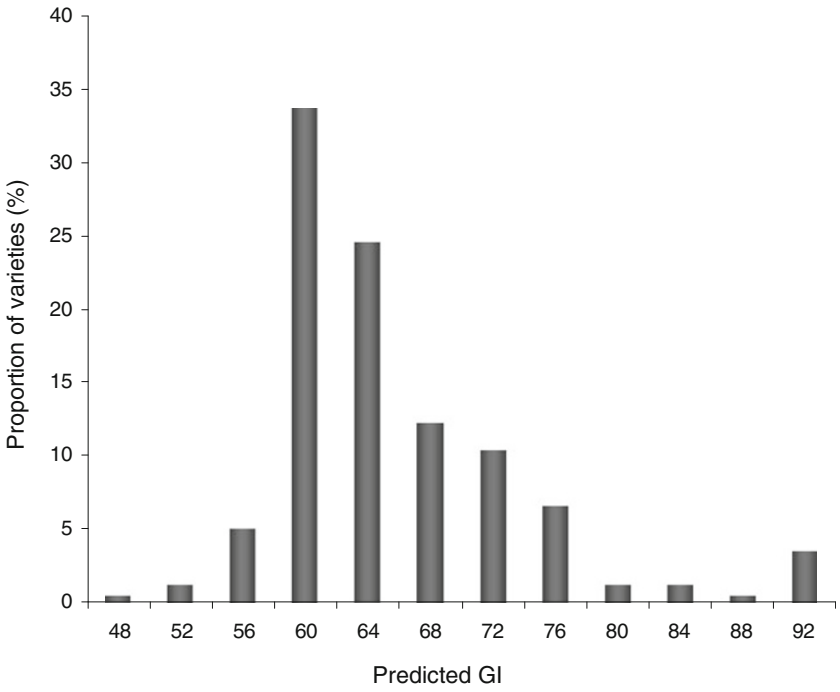

Fig. 2 Range in predicted GI values of 235 varieties of cooked, polished rice.

especially (Shaw et al. 2010). Diet is implicated in the onset and progression of these health problems and carbohydrate quality is a strong predictor of disease risk. Choosing to eat foods with predominately slowly digestible carbohydrates has been shown to be linked to favourable health outcomes including reduced risk of type II diabetes and related conditions (Barclay et al. 2008; Halton et al. 2008; Livesey et al. 2008; Marsh and Brand-Miller 2008).

Rice is a traditional staple food and primary dietary source of carbohydrates for most Asians and is increasingly playing the same role in African diets. Improving the carbohydrate quality of this popular commodity offers potential as a dietary strategy for preventing and managing type II diabetes and its co-morbidities, thereby promoting population health and alleviating the public health burden of chronic diseases. In countries with very high incidences of type II diabetes, such as Sri Lanka, Bangladesh, Indonesia, Malaysia and India, there is a belief that specific varieties of rice can elicit lower glycaemic responses and these are sold and marketed to type II patients.

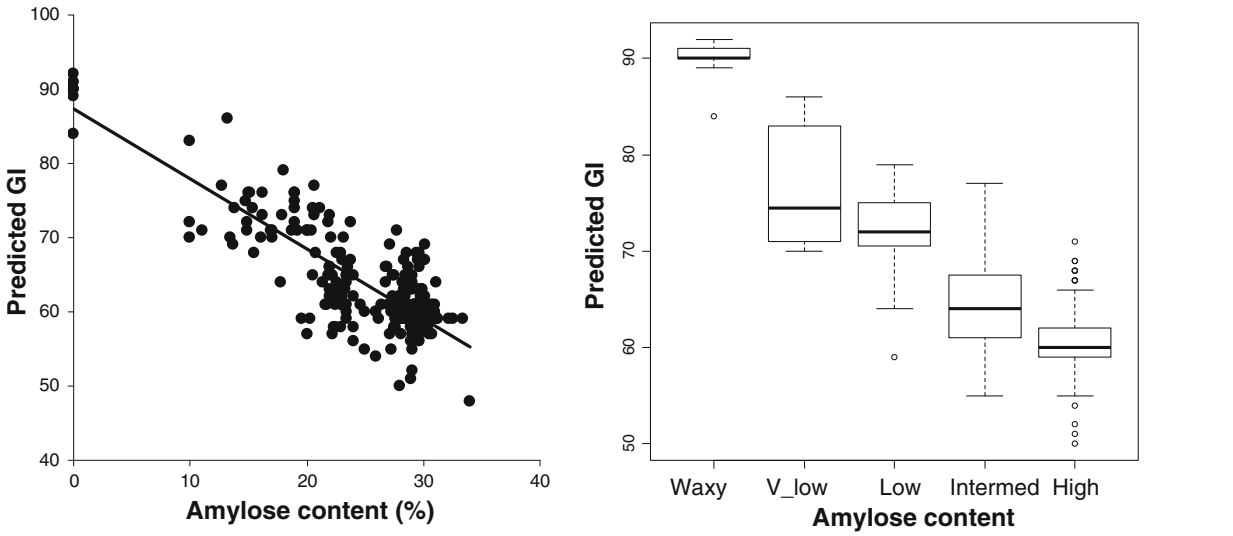

Fig. 3 **a** Correlation (r^2=0.73) between amylose content and predicted GI using 235 diverse samples and **b** box and whiskers plot of GI vs. amylose content showing that most samples associate with amylose content, but for those with high amylose, a number of outliers extend in both directions beyond the interquartile range.

Table 1 Average GI is significantly different between each known allele of the *Waxy* gene using the Welch's *T* test and pairwise comparisons ($p<0.05$). Different letters indicate signficant differences in GI values

Mutation	Predicted GI
wx	89.70[a]
Wx^{op}	76.50[b]
Wx^{b}	72.04[b]
Wx^{in}	64.33[c]
Wx^{a}	60.53[d]

GI glycaemic index

GI provides a measure of the glycaemic potency of foods and is widely used as a guide for choosing healthier foods (Chiu et al. 2011; Mitchell 2008). Low GI diets are effective in the prevention and treatment of type II diabetes (Barclay et al. 2008; Gnagnarella et al. 2008; Jenkins et al. 2002). However, rice improvement programmes have not been able to focus on the development of varieties with potential for reducing the incidence and severity of type II diabetes because variability for GI in rice is unknown, the genetics of GI are unknown and phenotyping tools for nutritional traits, such as GI, are not yet available.

Although a number of studies have attempted to draw associations between components of the grain and the GI of the rice (Babu et al. 2007; Brand-Miller et al. 1992; Frei et al. 2003; Hettiarachchi et al. 2001; Hu et al. 2004; Matsuo et al. 1999; Panlasigui et al. 1991), establishing relationships between composition and genotypes of rices and GI has been hampered by the low throughput, poor precision and considerable expense of in vivo determination of GI (De Vries 2007; Muller and Bird submitted). Consequently, previous studies have measured GI on only a small subset of rice varieties and so variability in the diversity of rice is not captured and definitive conclusions about possible relationships between GI and other grain traits cannot be drawn.

GI is a numerical measure of the extent to which carbohydrates in foods affect postprandial blood glucose levels. Conventional GI determination therefore involves testing in humans. For most applications, this is impractical for the reasons stated earlier. Indeed, stringent testing conditions are essential to achieve satisfactory levels of precision (De Vries 2007; Muller and Bird submitted; Pi-Sunyer 2002; Venn and Green 2007). In the present study, we demonstrate that the use of an automated laboratory-based assay for predicting GI overcomes the methodological and ethical constraints of in vivo testing and provides a practical solution to screening large volumes of samples. The validity of the assay was established by testing a diverse set of rice varieties and comparing the results against those obtained using standardised in vivo procedures performed by two highly experienced testing agencies. Correlation analysis of the resultant data demonstrated a strong relationship between predicted and actual GI values confirming that the in vitro method has high predictive power, thereby justifying its use in the current study to explore, for the first time, the range in GI values across a large and diverse collection of improved and traditional rice varieties. The information we have generated on the relative glycaemic properties of

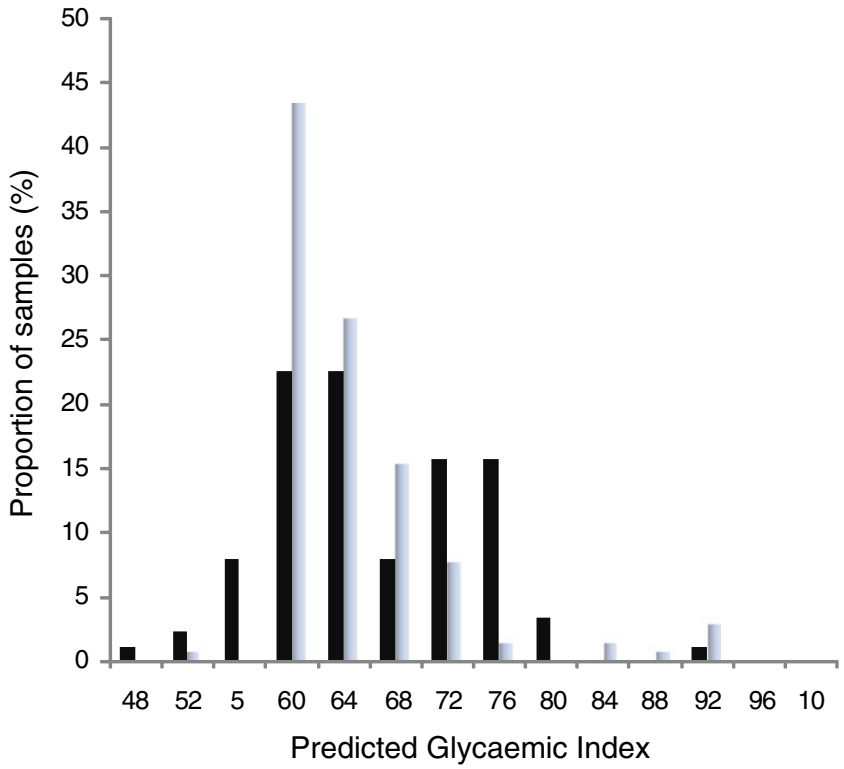

Fig. 4 Frequency histogram showing that the distribution in GI of improved varieties of rice (*black bars*) contains more high GI rices than the distribution of the traditional varieties (*grey bars*).

different types of rice has a wide scope of applicability. In vivo GI values for a given food are essentially the same regardless of ethnicity or physiological status of the volunteers on which the data are based (Chiu et al. 2011). Furthermore, the relationship between GI and adverse health impacts is just as pertinent to Asians as it is to Caucasians and other racial and ethnic groups (Murakami et al. 2006; Villegas et al. 2007).

The varieties measured included both landraces (143) and improved (92) varieties developed by leading rice breeding programmes in many different countries. The collection included indica and tropical and temperate japonica rices. GI ranged from 48–98 in the set of rices, with no association between germplasm class. A similar range in the GI of rices, including low GI varieties as determined by acceptable in vivo methods, has been reported previously (Brand-Miller et al. 1992; Larsen et al. 1996). While there are in vitro GI data on different rice lines (Frei et al. 2003), caution should be exercised in interpreting these findings because their physiological relevance has not been satisfactorily substantiated. Our data demonstrate that improved varieties have on average a slightly higher GI than traditional ones (Fig. 4), suggesting that breeding programmes have produced a slow, passive drift towards higher GI rices. Until recently, the nutritional potential of rice has not been a target of rice improvement programmes, and while various countries would like to develop low GI rices, the limitation lies in selecting for the trait.

Strong correlations between amylose content, the *Waxy* locus and GI were observed across all samples (Fig. 3a, Table 1), indicating that amylose is the major grain constituent that affects GI. However, the size of the interquartile ranges and the presence of outliers in Fig. 3b suggest that (a) within each class of amylose content, and for each allele of the *Waxy* gene, variability can be found for GI and (b) there must be other loci that interact with the *Waxy* gene to produce variability within each class. The absence of starch synthase IIa (SSlla) activity lowers GI in barley (King et al. 2008) but no association was found between varieties with active and inactive haplotypes of *SSlla* in this study. This suggests that SSlla plays different roles in starch synthesis in barley and rice.

In the high amylose classification of rice, the texture of freshly cooked rice can be either soft or firm (Cagampang et al. 1973). This difference is due to a single-nucleotide polymorphism in the *Waxy* gene that leads to different structures of amylose within the grain and different retrogradation rates (Tran et al. 2011). Intuitively, it might be assumed that amylose structure would affect the GI and that the firm-textured varieties might have a lower GI. However, the difference in GI between progeny of a mapping population with soft and firm texture after cooking was not significantly different, suggesting that the amount of amylose is more important than the structure of the amylose in determining GI.

Intermediate amylose rices are preferred in many countries in which over 5% of the population have type II diabetes. There is also a strong positive association between amylose content and texture of the rice (Bhattacharya 2009; Juliano 1979). The high amylose class contains both soft- and firm-textured rice, and given that there is no effect of the single-nucleotide polymorphism (SNP) on exon 10 of the *Wx* gene on GI within the high amylose class, it should be possible for breeding programmes concerned with the GI of rice to develop soft-textured high amylose rices to replace the intermediate amylose rices, especially for countries and regions with high incidences of type II diabetes, such as the Philippines, South Asia and the Middle East.

While this study has identified a major determinant of GI in rice, the data do not preclude the possibility that other genes have a modifying effect on GI. Additional studies in genetic populations will be required to identify such genes and quantify their contribution to determining GI.

The identification of low GI rice varieties offers the possibility of conducting well-designed, randomised controlled trials and epidemiological studies (long-term prospective investigations that take into account the various confounding factors operating across different populations, regional locations, culinary customs and ethnic groups) to examine the relationship between sustained consumption of low GI rice, metabolic control and health outcomes. Such information will be useful for informing the development of long-term public health strategies and clinical management plans for people with metabolic diseases.

Materials and methods

Method validation

In order to predict the GI of a large and diverse set of rice varieties with an in vitro method, it is necessary to determine the association between the in vivo method of measuring GI and the in vitro method. A set of six varieties of rice, *Oryza sativa* L., that have been widely researched in previous studies were selected to validate the method. These were IR65, IR24, IR64, IR8, BD192 (from Bangladesh) and Samba Mahsuri (from India). All varieties, other than BD192, were grown in the dry season of 2008 at the International Rice Research Institute in the Philippines. Grain was harvested at maturity, stored for 6 weeks to equilibrate for moisture content, then 150 g was dehulled (THU35A Test Husker, Satake) and polished (Grainman 60-230-60-2AT, Grain Machinery Mfg. Corp.). BD192 was obtained from the Genetic Resources Centre of the Bangladesh Rice Research Institute (BRRI) and grown in

both the Aman and Boro seasons at BRRI. Grain was harvested at maturity, dehulled (THU35A Test Husker, Satake) and polished using a home-made polisher.

Polished grain from each variety was cooked in excess water. Once the water reached a light boil, the rice was added, and after 17 min, the rice was drained and allowed to cool for 5 min. Total starch of the cooked rice was determined as described using the Megazyme total starch kit (Megazyme, Wicklow, Ireland) (AACC Standard 76 13.01).

In vivo testing for GI

GI of the six rices was tested according to the Australian Standard AS 4694-2007: Glycaemic Index of Foods. The tests were performed by the International Diabetes Institute testing facility, Caulfield, Victoria, Australia. Blood glucose was determined in 10 to 12 volunteers who had fasted for 10 h prior to the test. The volunteers then consumed the test (glucose drink) or reference food (rice) over 12 min, and then, changes in circulating levels of blood glucose were measured over the following 2 h. The test rices contained 50 g of glycaemic (available) carbohydrate, based on total starch of the cooked rice, and the reference food (glucose drink) had been tested in each volunteer on three previous occasions. The incremental area under the blood glucose curve (IAUC) was calculated and indexed to that of the mean IAUC for the reference food and then GI of each of the six rices was calculated according to the trapezoid rule. The area beneath the fasting concentration is ignored in the calculation of GI. Glucose is used as the reference food and by definition has a GI of 100.

A second set of values of in vivo GI was previously obtained for another six Australian varieties of rice (Williams et al. 2005). These were Amaroo, Doongara, Opus, Langi, Kyeema and Basmati. Samples of each were supplied by Sunrice Cooperative Ltd to the University of Sydney and to IRRI as polished rice. Each sample was analysed for proximate analysis and amylose content to determine available carbohydrate, and GI values were obtained by the University of Sydney (Williams et al. 2005).

In vitro testing for GI

Both sets of samples that were tested for in vivo GI at the International Diabetes Institute and the University of Sydney were sent to Commonwealth Scientific and Industrial Research Organisation (CSIRO) Food Nutrition for determining predicted GI. Samples of the test rices IR65, IR24, IR64, IR8, BD192 and Samba Mahsuri were cooked using a scaled-down version of the rapid boil procedure that was used in the in vivo GI studies described previously.

Quantities of raw test rices equating to 5 g of glycaemic (available) carbohydrate were added to an excess of boiling water (approximately 60 mL) and cooked for 16 min. The rices were then drained using a domestic sieve and allowed to cool for 5 min at room temperature before intact rice grains were assayed immediately for their predicted GI using an in vitro system which models the buccal, gastric and pancreatic phases of food digestion as it occurs in the human upper gastrointestinal tract (Bird, Usher, Klingner, Topping and Morrell, unpublished data). Briefly, cooked rices were added to a conical flask and mixed with artificial saliva (250 U/mL of α-amylase) at pH 7.0. After approximately 20 s, acidified (0.02 M HCl) pepsin (1 mg/mL) was added and the flask incubated at 37°C for 30 min in a shaking water bath. The digest was adjusted to pH 6.0 (0.2 M acetate buffer pH 6.0) followed by the addition of pancreatin (2 mg/mL) and amyloglucosidase (28 U/mL) and the digest incubated for a further 5 h. Aliquots of supernatant were sampled at preset intervals and the glucose concentration determined using an automated electrochemical technique (YSI 2700 Select Bioanalyser) (Yellow Springs, OH). The predicted GIs of the rices were calculated as a percentage of available CHO converted to glucose over the duration of the incubation.

Range in predicted GI using diverse varieties of rice

A set of 111 varieties was randomly selected from the Genetic Resources Centre of (BRRI). Of these, 72 were traditional varieties and 39 were improved. A second set was selected at IRRI to represent the popular and traditional varieties of many other Asian countries. All samples (235) were shipped to CSIRO as polished grain for the prediction of GI by the in vitro method described above.

Association between grain properties and GI

A set of 40 samples from a population (F_7) of recombinant inbred lines derived from a cross between two high amylose varieties, IR5 and IR8, were selected to determine whether predicted GI associates with the texture of the grain after cooking. Twenty soft-textured and 20 firm-textured progeny were selected for GI testing. Cooked rice texture of the 40 samples was measured by the gel consistency test and confirmed by texture profiling using a TaXt-plus as described previously (Tran et al. 2011). Amylose content was measured on the 235 varieties of rice, including those used to validate the method, by the standard method (AACC-6647), except that an additional standard was included in the standard curve, IR65, which is a waxy variety and contains no amylose.

Three SNPs at the Waxy locus, on the splice site of exon 1, exons 4 and 6, define the haplotypes that associate with amylose class. DNA was extracted from each sample (Fitzgerald et al. 2008). The SNP status (G/T) at exon 1 was determined by amplifying a region containing the SNP using primer pair RM190, and then, a restriction enzyme, Acc1 (New England BioLabs), which splices when a G is present at the site (Ayres et al. 1997). The presence of T at the site signifies low amylose. Intermediate and high amylose varieties were genotyped for SNP status at exon 6 (A/C) using allele-specific primers (5′-CCC ATA CTT CAA AGG AAC ATA-3′, 5′ - GGT TGG AAG CAT CAC GAG TT – 3′ and 5′ - TCT TCA GGT AGC TCG CCA GT – 3′), where a product size of 292 bp indicates C (intermediate amylose) and products of 200 and 292 bp identify an A (high amylose). Very low amylose varieties were determined by genotyping the SNP on exon 4 (A/G) using the primer set (5′-TGC TAC AAG CGT GGA GTG GA-3′ and 5′-ACC AGT ACA AGG ACG CTT GG-3′) and sequencing of the product.

The polymerase chain reaction (PCR) was performed using a G-Storm Thermal Cycler (model GS1, Gene Technologies Ltd, Essex, UK). The PCR reaction mixture contained 10 ng DNA extract, 0.16 mM deoxynucleoside triphosphates, 2 mM magnesium chloride, 0.4 mM each of forward and reverse primers, 1.2 mM allele-specific primers and 0.04 units of Intron Taq DNA polymerase (Shiga, Japan). The amplification of the targeted region was conducted under the following conditions: 5 min at 94°C; 35 cycles of 40 s at 94°C; 40s at 55°C; and a final extension step of 72°C for 10 min. The amplified products were resolved in 2% native agarose gel, stained with SybrSafe nucleic acid stain (Invitrogen, Carlsbad, CA, USA) and visualised with a UV transilluminator (Alpha Imager).

Statistics

Box and whisker plots were generated to identify outliers in the association between *Waxy* allele and GI using *R* v. 2.13.1. Outliers extending beyond the interquartile ranges were not removed before further analysis because there were so few of them. The correlation between predicted GI and each *Waxy* allele was carried out by pairwise comparisons using the Welch's *T* test in *R*. Differences were significant when $p<0.05$.

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