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Original Paper

Rapid analysis of transgenic rice straw using near-infrared spectroscopy

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Abstract A rapid near-infrared (NIR) spectroscopy method was established to predict the lignin and starch contents and enzymatic saccharification efficiency of transgenic rice (*Oryza sativa* cv. Nipponbare) straw wherein expression of genes encoding lignin synthetic enzymes are regulated. Strong correlations were obtained between laboratory wet chemistry values and the NIR-predicted values. This method is useful to develop transgenic rice where cell-wall formation is engineered.

Key words: Lignin, near-infrared spectroscopy, starch, enzymatic saccharification efficiency

Lignin is one of the major components of the secondary cell wall of vascular plants. It confers mechanical strength and imperviousness to the cell wall, allowing vascular plants to grow upwards (Vanholme et al. 2008). Thus, lignin for vascular plants is an essential component of the cell wall, but it is not a storage material. These characteristics present obstacles to chemical pulping, forage digestion and the enzymatic hydrolysis of polysaccharides of plant cell walls or lignocellulosic biomass. To improve these processes, metabolic engineering of lignocellulosic biomass plants with modified amounts and structures of lignins has attracted a great deal of attention (Chiang 2006; Gressel 2008; Hisano et al. 2009; Vanholme et al. 2008). The production of a large number (dozens or hundreds) of transgenic plant lines by manipulating the expression of a single gene necessitates a rapid analytical method to characterize the resulting transgenic plant materials.

Near-infrared (NIR) spectroscopy is a powerful tool that can provide quantitative information on the chemical and physical properties of various biomaterials (Shenk et al. 1992). NIR spectroscopy is nondestructive, rapid and inexpensive, and involves "relatively simple" sample preparation. NIR spectroscopy has been successfully applied to characterization of lignocellulosic materials (Agarwal and Atalla 2010; Schimleck 2008; Tsuchikawa 2007). Thus, various correlations between chemically measured traits and NIR spectra have been established for numerous plant species of different origins, because the correlation between the chemically-determined values and NIR spectra must be established independently for each plant species: lignin content (Hou and Li 2011; Huang et al. 2008; Huang et al. 2011; Jin and Chen 2007; Liu et al. 2010; Poke et al. 2004; Yao et al. 2010; Yeh et al. 2004; 2005), cellulose or glucan content (Hou and Li 2011; Huang et al. 2011; Liu et al. 2010; Lomborg et al. 2010; Schimleck et al. 2004), extractives content (Poke et al. 2004), microfibril angle (Schimleck and Evans 2002), sugar release (Horikawa et al. 2011; Lindedam et al. 2010), and digestibility (Hou and Li 2011). Despite the usefulness of NIR, only a few studies have reported on the application of NIR analysis to

Abbreviations: NIR, Near-infrared; RNAi, RNA interference; PCR, polymerase chain reaction; r.t., room temperature; RSM, rice straw meal; MeOH, methanol; MFR, MeOH-extracts-free residue; SDG, starch-derived glucose; SFR, starch-free residue; EHG, enzymatically hydrolysable glucan-derived glucose from SFR; GDG, glucan-derived glucose by acid saccharification from SFR; PLS, Partial Least Squares; R^2, coefficient of determination; RMSEE, root mean square errors of estimation; RMSEP, root mean square errors of prediction; RMSECV, root mean square errors of cross-validation; 4CL, 4-coumarate:CoA ligase; HCT, hydroxycinamoyl-CoA:shikimate/quinate hydroxycinnamoyltransferase; CoAOMT, caffeoyl-CoA *O*-methyltransferase; CAOMT, caffeic acid *O*-methyltransferase; CAD, cinnamyl alcohol dehydrogenase.

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transgenic lignocellulosics (Horvath et al. 2011; Yamada et al. 2006).

To establish quantification system using NIR spectra, usually two steps are required: the first is calibration and the second is validation. Partial least squares (PLS) is one of the chemometoric methods and is used to develop a chemometric model using representative samples that cover a whole range of the measured values, so that fitted values are calculated for measured values in the spectra. Next, as validation, reliability of the developed calibration model is evaluated by comparing the calculated, predicted values with the corresponding measured values. The prediction model is evaluated by the coefficient of determination (R^2), root mean square errors of prediction (RMSEP) or root mean square errors of cross-validation (RMSECV), and numbers of PLS components (rank).

Among lignocellulosic biomass plants, grasses are a major resource in the emerging cellulose-to-ethanol strategy of biofuels (Anderson and Akin 2008), and rice is a powerful primary model for identifying desirable genes relevant to new energy grasses, because, along with its tremendous history of improvement and cultivation, it has a deep pool of genetic and genomic resources (Bush and Leach 2007). Herein, we report the establishment of models based on NIR spectroscopy to predict the lignin and starch contents and enzymatic saccharification efficiencies of straw of transgenic rice (*Oryza sativa* cv. Nipponbare) lines in which the expressions of genes encoding lignin biosynthetic enzymes in the cinnamate/monolignol pathway (Umezawa 2010) are down-regulated.

Materials and methods

Preparation of transgenic rice plants

Seeds from a T_0 population of RNA interference (RNAi) lines of rice plant (*O. sativa* L. cv. Nipponbare) were generated as follows. Partial cDNA sequences of the seven lignin biosynthetic enzyme genes annotated by The Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp/) were amplified by polymerase chain reaction (PCR). These genes were, Os08g0448000 [4-coumarate:CoA ligase (4CL)], Os04g0500700 [hydroxycinamoyl-CoA:shikimate/quinate hydroxycinnamoyltransferase (HCT)], Os06g0165800 [caffeoyl-CoA *O*-methyltransferase (CoAOMT)], Os08g0498400 (CoAOMT), Os08g0157500 [caffeic acid *O*-methyltransferase (CAOMT)], Os02g0187800 [cinnamyl alcohol dehydrogenase (CAD)], and Os09g0400300 (CAD). PCR used the rice full-length cDNA clones, AK120964, AK072528, AK065744, AK061757, AK064768, AK105011, and AK067085 (National Institute of Agrobiological Sciences, Japan), respectively as templates, and the primer sets in Table 1. PCR products were subcloned into the entry vector pENTR/D-TOPO (Invitrogen, Grand Island, NY, USA). The insert sequences of the entry vectors were fully sequenced. The RNAi vector was produced by an LR clonase reaction between the entry vector and the pANDA vector (Miki and Shimamoto 2004; Miki et al. 2005). Transgenic rice plants were generated by *Agrobacterium*-mediated transformation of rice calli based on the protocol of Hiei et al. (Hiei et al. 1994). Regenerated transgenic rice plants were grown to maturation in an incubator (RTC-100, Phytoculture, Osaka, Japan) at 27°C under a 12 h-light (ca. 10,000 lx at ground level): 12 h-dark photoperiod for about six months. Plants were kept in ceramics-based supports with hydroponic medium (Mae and Ohira 1981) refreshed every two weeks.

Experimental scheme

A comprehensive scheme for sample preparation and analyses, including NIR spectroscopy, determination of lignin and starch contents, and saccharification efficiencies are summarized in

Table 1. Primers used for RNAi trigger sequence amplification by PCR.

Primer name	Sequence*
Os08g0448000 for.	5′-caccTTGTCATCACGCGCACTTCCATTC-3′
Os08g0448000 rev.	5′-CGCTCACCTTGGCGACGAA-3′
Os04g0500700 for.	5′-caccTTGCCATCATGCCCTTCATTGACC-3′
Os04g0500700 rev.	5′-TCTTGGATGACTGCTGCTCCATCT-3′
Os06g0165800 for.	5′-CGAGCGAGAGATCGAAGCAA-3′
Os06g0165800 rev.	5′-caccCAGGTTCAGGAATTGCCC-3′
Os08g0498400 for.	5′-caccTGGCCTACGACAACACGCTCT-3′
Os08g0498400 rev.	5′-GTTCTCAGACACAAACCTGCATGA-3′
Os08g0157500 for.	5′-caccTGATGAAGTGGATCCTCCACGACT-3′
Os08g0157500 rev.	5′-TACAACACAAGCAGCAGCAGCATC-3′
Os02g0187800 for.	5′-CGACCAGAAGGTTTGTGGTGAAGA-3′
Os02g0187800 rev.	5′-caccTTGTCGACGCAGAAGTTGAGCA-3′
Os09g0400300 for.	5′-caccATGCGCGTCACCGTCATCA-3′
Os09g0400300 rev.	5′-TGACACACAACAAGCAAGCTAGGC-3′

for., forward; rev., reverse. * The "cacc" sequence at the 5′-terminus of each forward primer was added to subclone the PCR product directionally to a pENTR/D-TOPO vector.

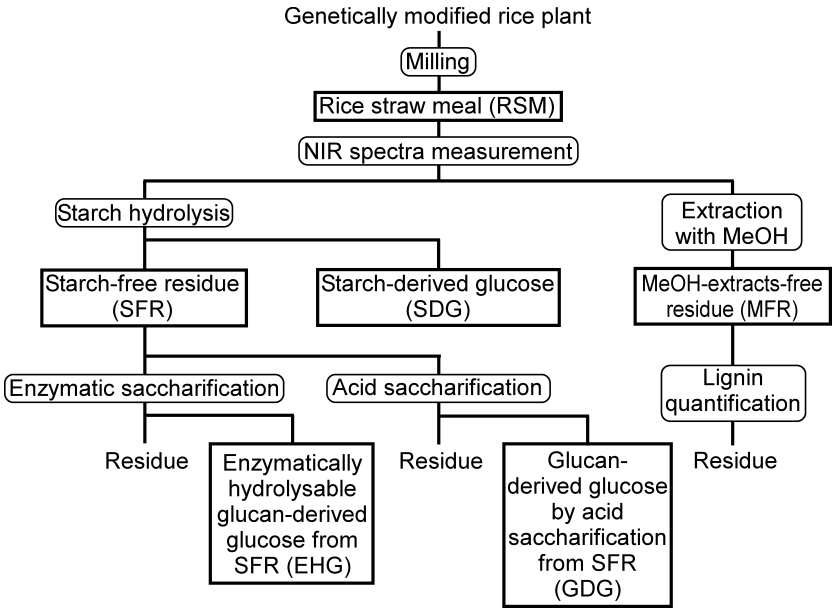

Figure 1. Scheme for sample preparation and analyses. Reactions and analyses are shown in squares with rounded corners. Samples obtained are shown in squares. NIR spectra were recorded for RSM. Lignin content (wt%) was measured on MFR. Lignin contents in RSM and SFR were calculated values. Starch content (wt%) in RSM was determined based on amount of SDG. Starch content in MFR was calculated value. Saccharification efficiencies were the ratio EHG/GDG (%) in glucose amounts measured.

Figure 1. Each step is described below.

Rice straw meal preparation

Genetically modified rice plants were harvested by cutting a few cm above the ground and hung upside down for at least one month under low humidity at room temperature (r.t.). Entire plants, except panicles and seeds, were cut into ca. 0.5 cm lengths with scissors and the pieces were dried over silica gel *in vacuo* at r.t. overnight. Dried samples were milled into rice straw meal (RSM, Figure 1) using a TissueLyser (QIAGEN GmbH, Germany, Hilden) equipped with a stainless jar and balls (20 mm in diameter) at 25 Hz for 1 min, three times with 10-s interval.

RSM (20 mg) was transferred to a dried 2-ml polypropylene tube, and the meal in the tube was dried in an oven at 60°C for 1 h. After cooling and weighing, the sample was vortexed with 1.8 ml of distilled water and centrifuged for 10 min at 16,100×*g* at r.t., and the supernatant was discarded. The residue was vortexed with 1.5 ml of methanol and the solution was incubated at 60°C for 20 min stationary. The solution was centrifuged at 16,100×*g* for 10 min at r.t., and the supernatant was discarded. The extraction with methanol was repeated. The pellet was dried *in vacuo* for 1h and weighed to give methanol (MeOH)-extracts-free residue (MFR, Figure 1).

Three replicates of RSM (18 mg) were put into 2-ml polypropylene tubes, and starch was degraded enzymatically using a Total Starch Kit (Megazyme, Co. Wicklow, Ireland). The degraded starch liberated to the solution was separated by centrifugation at 10,000×*g* for 2 min at r.t., and the supernatants were transferred to new microcentrifuge tubes. The starch content was determined as starch-derived glucose (SDG, Figure 1) in the supernatants with Glucose C-II Test Wako (Wako, Osaka, Japan). Starch content (wt%) was calculated on RSM but also was estimated for MFR.

The pellets were further washed twice with 1 ml of distilled water and twice with methanol, and dried *in vacuo* for 3 h at r.t., to give starch-free residue (SFR, Figure 1) and weighed. The SFR in tubes were directly used for enzymatic saccharification (see ***Enzymatic saccharification***).

Thioglycolic acid lignin determination

Thioglycolic acid lignin in the MFR (Figure 1) was determined as previously reported (Suzuki et al. 2009) with triplicate samples. Lignin content (wt%) was calculated for RSM and SFR.

Enzymatic saccharification

Enzymatic saccharification was done by a modification of the previous protocol of Park et al. (Park et al. 2009) with triplicate samples. To the SFR suspended in 500 μl of 50 mM sodium citrate (pH 4.8), 1 ml of enzyme solution composed of Celluclast 1.5 L (Novozymes, Bagsvaerd, Denmark) (1.1 FPU), Novozyme 188 (Novozymes, Bagsvaerd, Denmark) (2.5 CbU) and Ultraflo L (Novozymes, Bagsvaerd, Denmark) (65 μg) in the same buffer. The suspension was incubated in a rotary reactor (Heatblock Rotator SN-48BN, Nissin, Saitama, Japan) at 12.5 rpm at 50°C for 2, 6 and 24 h. After each saccharification period, the reaction mixture was centrifuged at 10,000×*g* for 2 min at r.t. The residues were washed twice with 1 ml of distilled water. Then the first supernatant and the washings were combined and diluted to 5 ml with distilled water, and the amounts of glucose therein (enzymatically hydrolysable glucan-derived glucose from SFR, EHG, Figure 1) were determined as

described above.

Acid saccharification

Acid saccharification was done by a slight modification of the procedure of Park et al. (Park et al. 2009) with triplicate samples. After the digestion of starch as described above, the pellets were washed twice with 1 ml of distilled water, twice with ethanol, and twice with *n*-hexane, giving rise to the SFR. The SFR thus obtained was dried and weighed as described above. To the SFR in a microcentribuge tube, 1 ml of 72% H_2SO_4 (w/w) was added and the reaction mixture was incubated at 30°C for 1 h using a rotary reactor at 6 rpm (Heatblock Rotator SN-48BN, Nissin). After the reaction, a 150 μl aliquot was diluted with 1,050 μl of distilled water, and the resultant mixture was heated at 100°C for 2 h. Then, the mixture was centrifuged at 10,000×*g* for 3 min at r.t., and 1 ml of the supernatant was neutralized with 1 ml of 25% (w/w) $CaCO_3$ in water to give glucan-derived glucose by acid saccharification from SFR (GDG, Figure 1). Separately, 1.2 mg glucose was treated with H_2SO_4 in the same manner to estimate the amount of glucose degraded during the acid saccharification. The glucose liberated from the meal was measured as described above.

Calculation of saccharification efficiency

The enzymatic saccharification efficiency (%) was determined by the following equation:

$$\text{Saccharification efficiency (\%)} = (\text{EHG}/\text{GDG}) \times 100$$

EHG: Enzymatically hydrolysable glucan-derived glucose from SFR

GDG: Glucan-derived glucose by acid saccharification from SFR

Near-infrared spectroscopy

RSM (0.6 g) was put into glass vessels and dried at 60°C overnight under air-flow. The dried samples were further dried over P_2O_5 *in vacuo* for 2 h at r.t.

All NIR analyses were performed on a MPA Multi Purpose FT-NIR Analyzer (Bruker Optics, Ettlingen, Germany). Reflectance spectra totaling 32 scans (16 each for sample and background) were recorded at 8 cm^{-1} intervals over the range 125000–3600 cm^{-1}. Spectra were recorded five times for each of all RSM samples, and all spectra were used to construct prediction models.

Construction of prediction model

The OPUS software ver. 6.5 (Bruker Optics, Ettlingen, Germany) was used to construct prediction models. Cross-validation with forty RSM samples was employed for the prediction of saccharification efficiency, while for the construction of prediction models for lignin and starch contents, roughly two-third of the samples were used for calibration, and the remaining ones were used for test set validation. Each spectrum was preprocessed via PLS regression. Spectra judged to be an "outlier" by the software were not used for analyses. Numbers of PLS components (rank) recommended by the software was used. The R^2 and the root mean square errors of estimation (RMSEE), RMSEP and RMSECV were used to evaluate the performances of calibration and validation.

Results and discussion

To establish prediction models for lignin and starch contents and saccharification efficiencies, we selected the 94 lines of transgenic rice plants consisting 17 *4CL*-, 13 *HCT*-, 23 *CoAOMT*-, 12 *CAOMT*-, 24 *CAD*-knockdown lines and 5 wild type ones. They cover a wide range of lignin and starch contents and saccharification efficiencies as summarized in Table 2. Detailed characterization of the transgenic rice plants will be reported elsewhere.

NIR spectra were recorded only for RSM samples which retain whole organic components, because the only pretreatment for whole component analysis is pulverization, allowing for rapid analysis. Rice straw often contains significant amounts of starch. Starch content can vary substantially with developmental stage and with other factors, including growth conditions (Matsuki et al. 2010; Park et al. 2011). Starch content strongly affects total glucan content in rice straw. Hence, the enzymatic saccharification efficiency of cell wall residue was determined with SFR.

Details of the preprocessing type and method of validation used for construction of prediction models are summarized in Table 3. Sixty four RSM samples with lignin content in the range of 7.9–22.4% were chosen for development of calibration for lignin contents (Table 2). Most (95%) of the NIR spectra measured for them were usable to develop the calibration. For validation, 97% of the spectra recorded for 30 samples (RSM) in the test set fit the calibration. The prediction model for lignin content in the RSM samples (Figure 2A), covered

Table 2. Summary statistics of the ranges of selected samples for lignin and starch contents, and saccharification efficiencies (%).

	Values (%)	
	Min	Max
Lignin in		
rice straw meal (RSM)	7.9	22.4
MeOH-extracts-free residue (MFR)	9.5	25.7
starch-free residue (SFR)	10.2	25.5
Starch in		
rice straw meal (RSM)	0.24	24.4
MeOH-extracts-free residue (MFR)	0.28	22.8
Saccharification efficiencies at		
2-h-incubation	20.4	38.4
6-h-incubation	25.8	45.5
24-h-incubation	30.0	53.8

Table 3. Preprocessing and validation used for constraction of prediction models for lignin and starch contents and saccharification efficiencies.

Interests for NIR prediction	Lignin contents (%) in			Starch contents (%) in	
	RSM	MFR	SFR	RSM	MFR
Pre-processing[a]	First derivative +SNV	First derivative +SNV	Second derivative	Second derivative	Second derivative
	Wave number: 6125.4–5411.8	Wave number: 8046.3–4563.2	Wave number: 7263.3–5465.8 and 4698.2–4208.3	Wave number: 7001–3799.4	Wave number: 6029–5755.1 and 5342.4–4667.3
Method of validation	Test set validation				

Interests for NIR prediction	Saccharification (%) at		
	2-h-incubation	6-h-incubation	24-h-incubation
Pre-processing[a]	MSC	First derivative +MSC	First derivative +MSC
	Wave number: 7456.2–5454.2 and 4597.9–4378	Wave number: 9982.7–7483.2 and 5353.9–4594	Wave number: 7506.3–6121.5 and 5377.1–4516.9
Method of validation	Cross varidation		

RSM: Rice straw meal. MFR: MeOH-extracts-free residue. SFR: Starch-free residue. Wave number: cm^{-1}. SNV: Vector nomalization. MSC: Multiplicative scatter correction. [a]SNV is a nomaliazation technique of a spectrum by first calculating the averabe intensity value and subsequent subraction of this value from the spectrum. MSC is a technique to remove some effects of scattering on NIR spectra caused by different particle sizes of ground grain of samples (Manley et al. 1994; Ilari et al. 1988), enabling a linear transformation of each spectrum for it to best match the mean spectrum of the whole set.

the range of 9.1–21.5% and correlated well with the validation set (R^2=0.89, RMSEP=1.01).

Correlations for lignin content were also established for MFR and SFR samples, which had lignin content ranging 9.5 to 25.7% and 10.2 to 25.5%, respectively (Table 2). Most of the spectra for the calibration samples (97% for MFR and 95% for SFR samples) could be used to develop the calibrations. For validation, 94% and 97% in the test sets fit the calibration. Figures 2B and 2C show the prediction models, which cover the lignin amount in the ranges of 10.6–25.6% and 11.6–25.3%, respectively. Both showed good correlation with the validation sets (MFR: R^2=0.85, RMSEP=1.32; and SFR: R^2=0.82, RMSEP=1.3).

PLS regression was done between NIR spectra and measured lignin content in MFR (Figure 1). In addition, PLS regression was done between NIR spectra and calculated lignin content values in RSM and SFR samples (Figure 1).

The calibration regions used for preprocessing (Table 3) included the wave numbers between 6098 and 5480 cm^{-1}, and 7143 and 6579 cm^{-1}: the former range spans the first and second overtones of lignin aromatic and aliphatic carbon-hydrogen vibration, while the latter spans the first overtone of lignin hydroxyl vibration (Maranan and Laborie 2008). In addition, the regions included 5980 cm^{-1}, which is also assigned to lignin (Yamada et al. 2006; Yeh et al. 2004) as the first overtone of the aromatic skeletal vibration of lignin (Sandak et al. 2010).

The values of R^2 and rank indicate that the prediction model for lignin content in RSM was the most accurate, followed, in order, by the models for MFR and SFR. The lower accuracy of the latter two might be due to experimental error caused by additional sample processing, i.e. MeOH extraction and starch hydrolysis.

Starch content of RSM and MFR samples was in the ranges of 0.24–24.4% and 0.28–22.8%, respectively (Table 2). Sixty four RSM samples were chosen for development of calibration. Most (95%) of the NIR spectra for the calibration sample met the criterion for calibration of RSM and MFR. For validation, 97% and 100% in the test sets fit the calibration for RSM and MFR.

Figures 3A and 3B show the starch content prediction models for RSM and MFR, which ranged from 0.27 to 18.9% and 0.29 to 21.3%, respectively. Both showed good correlation with the validation sets (RSM: R^2=0.94, RMSEP=1.28; and MFR: R^2=0.92, RMSEP=1.70). As for model for prediction of saccharification efficiencies, most (97%) of the recorded NIR spectra for 40 RSM samples were used to develop the calibration. By cross-validation, the predicted model (Figure 4A) covered the saccharification efficiency for the 2-h incubation from 20.4-38.4%. The predicted model correlated well to the validation set (R^2=0.77, RMSECV=2.05). The predicted models for saccharification efficiency for the 6-h and 24-h incubations covered ranges of from 25.8–45.5% (Figure 4B) and 30.0–53.8% (Figure 4C), respectively.

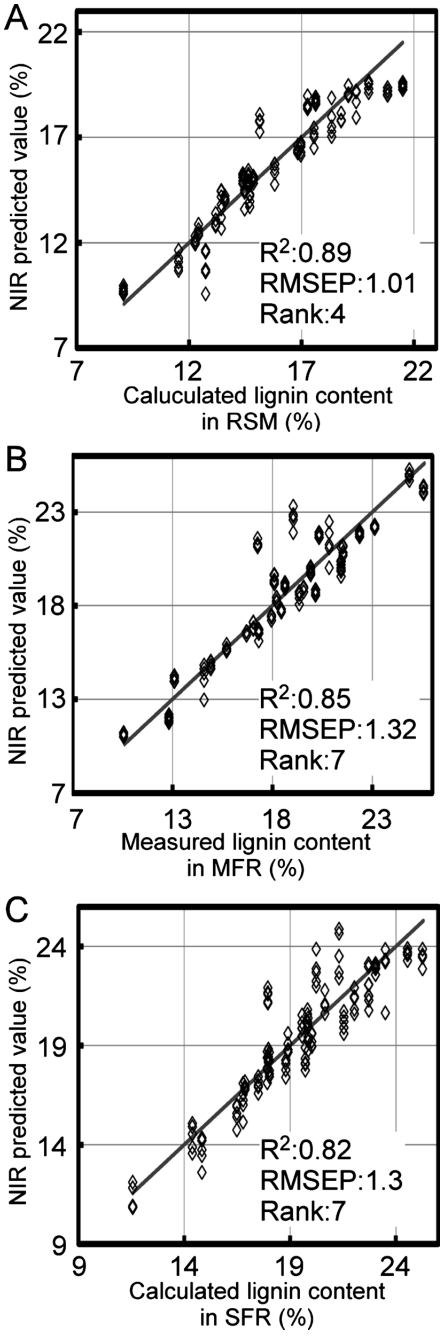

Figure 2. NIR predicted versus measured plots for lignin contents based on A, RSM; B, MFR; and C, SFR (%). Solid lines represent the line of equivalence.

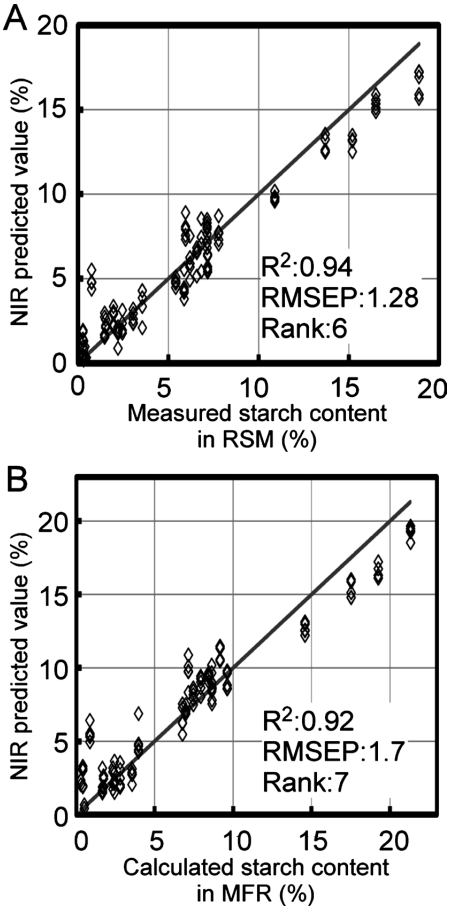

Figure 3. NIR predicted versus measured plots for starch contents based on A, RSM; and B, MFR (%). Solid lines represent the line of equivalence.

They correlated well with the validation sets (6-h incubation: $R^2=0.80$, RMSECV=2.09; 24-h incubation: $R^2=0.84$, RMSECV=2.26). The values of the R^2 and rank indicated that the prediction models for saccharification efficiencies became more accurate as the saccharification period increased.

Generally, saccharification efficiency negatively correlates with lignin content (Chen and Dixon 2007; Hisano et al. 2009). In this study, the most negative correlation coefficients were obtained between the lignin content of samples and the 24-h saccharification efficiencies: 0.63, 0.59 and 0.65 for RSM, MFR, and SFR, respectively (data not shown). By contrast, the negative correlations were smaller for samples incubated for shorter periods; the values for the 6- and 2-h saccharifications, respectively, were: 0.55 and 0.53 for RSM; 0.48 and 0.45 for MFR; and 0.49 and 0.46 for SFR. In the initial stages of the enzymatic saccharification, i.e. up to 6 h under the present conditions, the reaction rate (glucose formed per minute) was high (0–2 h, 7.1–4.0 nmol·mg sample^{-1}·min^{-1}; 2–6 h, 0.8–0.4 nmol·mg sample^{-1}·min^{-1}). Then it decreased as the reaction proceeded (6–24 h, 0.3–0.07 nmol·mg sample^{-1}·min^{-1}). This phenomenon is generally observed in enzymatic saccharification of lignocellulosic materials (Park et al. 2009). At around 24 h of incubation under the present conditions, moieties which are recalcitrant to enzymatic saccharification are being slowly degraded, a process that probably fluctuate little among samples. Therefore, the strong negative correlation between lignin content and 24-h saccharification efficiency may reflect the slow and stable enzymatic reaction occurring at around 24 h.

In summary, a NIR spectroscopy method was

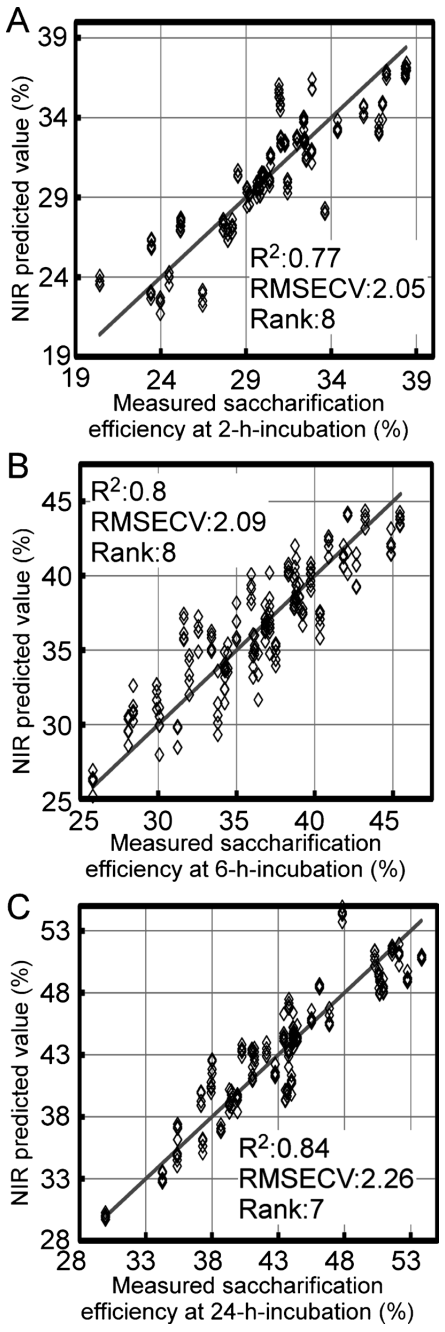

Figure 4. NIR predicted versus measured plots for saccharification efficiencies at 2, 6 and 24-h-incubations (%); A, B and C, respectively. Solid lines represent the line of equivalence.

developed to predict the variation in lignin and starch contents and enzymatic saccharification efficiency of transgenic rice (*O. sativa* cv. Nipponbare) straw in which the expression of genes encoding enzymes in the cinnamate/monolignol pathway were manipulated. This method is very rapid, nondestructive, and high-sensitive, and can be expanded to the screening of a large mutant collections, such as *Tos* mutant panel (Hirochika 2001, 2010).

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