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Estimation for Different Genotypes of Plants based on DNA Analysis using Near-infrared (NIR) and Fourier-transform Infrared (FT-IR) Spectroscopy

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Near-infrared (NIR) spectroscopy and Fourier-transform infrared (FT-IR) spectroscopy were applied for the discrimination of genomic DNAs from different genotypes of plants. From the results of NIR and FT-IR analyses for the detection of genetically modified (GM) maize, GM and non-GM maize genotypes were distinguished based on the absorbance wavelength or wave number. Statistical analysis of the data obtained suggested that FT-IR gave clearer and more reproducible results than NIR for the discrimination of a GM maize line from a non-GM one. Based on these findings, FT-IR was used to discriminate much smaller variation such as allelic differences at one locus in isogenic lines of rice, and was also applied to the characterization of higher levels of genomic variation using typical *indica* and *japonica* rice varieties. In the case of isogenic lines, no obvious difference was observed. On the other hand, *indica* and *japonica* varieties could be clearly distinguished, and two varieties belonging to the *indica* varietal group could also be separated. It was concluded that this method may enable to discriminate among different varieties, and could become an easy and effective method of plant genotyping.

Key Words: FT-IR, GM crops, genotype discrimination, NIR.

Introduction

In recent years, techniques for the development of genetically modified organisms (GMOs) have been improved and applied to agricultural crop production worldwide. The increased distribution of genetically modified (GM) crops in the market, however, has become not only an economic but also an ethical issue. Campaigns led by consumers and a series of public demands for the introduction of mandatory labeling of GMOs in foods, have expanded significantly in many countries, including Japan. Consequently, the development of precise and convenient methods for discriminating between GM and non-GM crops has become essential for both producers and consumers (Ahmed 2002).

Methods for the identification of GM crops can be roughly classified into two categories, 1) evaluation based on DNA information such as polymerase chain reaction (PCR) (Wurz *et al.* 1999, Matsuoka *et al.* 2001, Matsuoka *et al.* 2002), and 2) protein-based methods such as enzyme-linked immunosorbent assay (ELISA) (Lipp *et al.* 2000, Fagan *et al.* 2001). For the detection of GM crops, the major advantage of these methods is their high degree of accuracy. However, every method is associated with limitations such as the difficulty in applying protein-based methods to processed foods (Chiueh *et al.* 2001). In general, PCR is the most popular method used to characterize GM crops worldwide (Meyer 1999, Lau *et al.* 2004). Although, both PCR- and ELISA-based methods have been found to be accurate and reliable, they require complicated procedures, specialized techniques and laboratory equipment and are both time-consuming and costly. These methods are not sufficiently convenient and rapid for application anywhere and by anyone.

In addition to GM crops, false labeling of ordinary non-GM crops in the market using the name of a sperior variety or a particular brand name has also become a serious problem that often causes economic damage and distrust. For example, in Japan, the commercial distribution of inferior rice varieties under the name of an elite variety, "Koshihikari", has become a public issue. For commercial distribution of crops such as rice, since the cultivar name, year and place of production must be displayed, the development of reliable methods of identifying crop varieties has become essential. For the identification of ordinary crop varieties, DNA fingerprinting based on methods such as PCR and sequencing, has been commonly applied for genotyping analysis, but unlike in the case of GM and non-GM crops, since it is often difficult to detect subtle differences among ordinary cultivars using only one PCR marker, laborious preliminary experiments for marker construction and selection are required (Ohtsubo *et al.* 1999, Akagi 2000, Nasu *et al.* 2002).

Recently, spectroscopic methods have been applied to

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the detection of GMOs and the characterization of crop varieties (Roussel *et al.* 2001, Delwiche and Graybosch 2002, Mehinagic *et al.* 2003). In these studies, emphasis was placed on differences in the ingredients (chemical components) and physical structure between GM and non-GM crops, and among varieties. The advantages of spectroscopic methods include the non-destructive and simple nature of the procedures, reasonable cost and negligible environmental impact. However, spectroscopy-based genotyping of plants by the analysis of their DNAs has not been reported.

In the present study, both near-infrared (NIR) and Fourier-transform infrared (FT-IR) spectroscopic methods were tested for their suitability for the discrimination of genomic DNAs from three different categories of plant genotypes, namely, 1) GM and non-GM maize (testing for the presence or absence of the transgene); 2) isogenic lines of rice (testing for allelic differences at the same locus); and 3) typical *indica* and *japonica* rice varieties (analysis of genome-wide variations).

Materials and Methods

Plant materials and DNA extraction

For the plant materials, the three categories of genotypes mentioned above were investigated as follows: 1) the Bt11 maize (line: N79-L3) as a representative GM sample and corresponding non-transgenic maize (line: N79-P4) as the non-GM control (Syngenta Seed Co. Ltd., Japan); 2) rice strains of Taichung 65 (T65), its waxy (*wx*) isogenic line of T65*wx* and double isogenic line carrying liguleless (*lg*) and long sterile lemma (*g1*), T65*lg g1* (Oka and Morishima 1974); and 3) T65 as a typical *japonica* variety, Ac. 130 (Tan-kuang-hwa-lo, from Taiwan) and Ac. 419 (P.T.B.7, from India) as typical *indica* varieties revealed to be genetically largely different from T65 (Sato *et al.* 1990). DNA was extracted from fresh leaves of each sample by the method of Fulton *et al.* (1995). To test the reproducibility of the DNA samples prepared, five (NIR) and ten (FT-IR) independent DNA extractions from each material were conducted at different times, respectively. The quality and quantity of the DNAs were checked by conventional spectrophotometry and agarose gel electrophoresis.

NIR analysis

Five independently extracted DNA samples, each from GM and non-GM maize lines were prepared. After dilution of the extracted DNA to 5.0 μg/ml, NIR measurements were conducted for 10 sub-samples of each DNA isolate for NIR spectrometry, using NIRSystems model 6500 (USA). Measurements were performed using VISION Ver. 1.11 software (NIRECO, Japan) for 850 μl of each sub-sample at the temperature of 25°C and in the wavelength range of 400 to 2500 nm. Data were recorded with 50 scans at 2 nm intervals.

FT-IR analysis

For FT-IR analysis, ten independently extracted DNA samples were prepared and adjusted to a concentration of 5.0 μg/ml for maize (1.0 μg/ml for rice samples, genome size difference between maize and rice was taken into account). FT-IR measurements were conducted using the FT-IR spectrometer, IRPrestige-21 (Shimadzu, Japan), with 80 μl of diluted DNA in a BaF_2 cell. Measurements were conducted using the IRsolution Ver. 1.10 software (Shimadzu, Japan) with 40 scans/sample taken at 25°C and in the wave number range of 400 to 4600 cm^{-1} at 4 cm^{-1} intervals.

Results and Discussion

Discrimination between GM and non-GM maize lines using NIR and FT-IR

We determined whether the NIR and FT-IR spectroscopic methods could be successfully used for the genotyping GM maize line, Bt11, carrying the *cryIA(b)* gene (Bt gene), and its corresponding non-GM maize control line. To enhance the accuracy of the measured NIR spectral difference between Bt and control maize, both smoothing and differentiation treatments (Savitzky and Golay 1964) of the absorbed spectra were applied, using the software Pirouette Ver3.02 (GL Science). After these treatments, significant differences between the Bt and control maize lines were observed at 788 nm ($P<0.01$), as shown in Figure 1A.

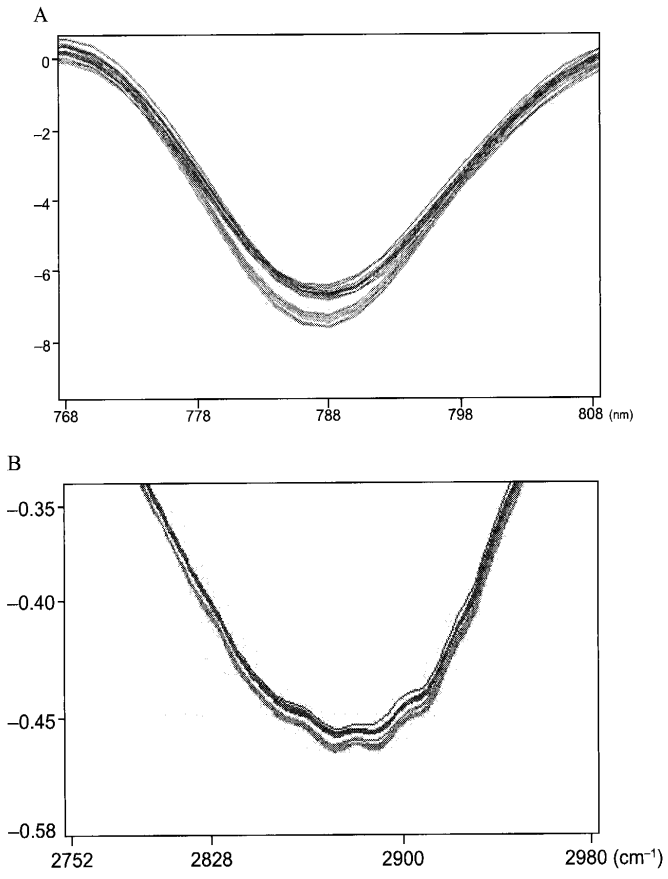

Fig. 1. Original spectrum data based on smoothing and primary differentiation treatments (Savitzky and Golay 1964) in NIR (A) and FT-IR (B) analyses. Spectra of Bt and Control maize lines were shown as gray and black lines, respectively.

Characterization of the data-set at around 788 nm (700 to 800 nm) was then conducted by principal component analysis (PCA, Fig. 2). The results in Figure 2 showed that both the Bt and control spectra were plotted as different clusters, indicating that the Bt and non-GM maize lines displayed different specific spectral characteristics at 700–800 nm. However, no significant correlation between the data obtained from different measurement times was recoeded, and it was difficult to compare data-sets from independent NIR experiments (differences in days and/or times).

For the FT-IR analysis, similar smoothing and differentiation treatments were applied to the data, and significant differences between the spectra obtained for the Bt and control maize lines could also be observed at 2893 cm^{-1} ($P < 0.01$), as shown in Figure 1B. PCA was then conducted for the spectra obtained at 2800 to 3000 cm^{-1} (Fig. 3), and results much more clearly separable than in the case of NIR were obtained. Furthermore, there was a significant correlation ($r = 0.758$, $P < 0.05$) for the data from independent measurement times. It was, therefore, concluded that FT-IR provides much more stable and reliable genotyping data than NIR.

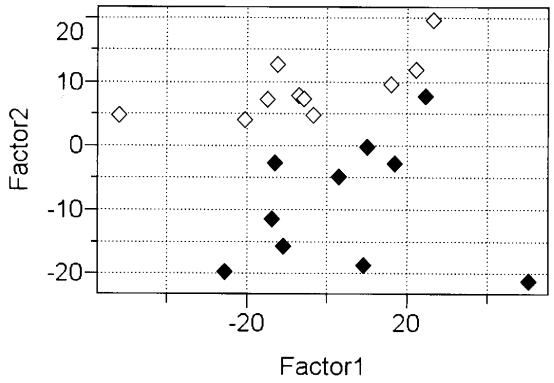

Fig. 2. Principal Component Analysis showing differences between Bt (◆) and Control (◇) maize lines based on NIR data measured at 700–800 nm. (Contribution rate, Factor 1: 49.3%, Factor 2: 13.4%)

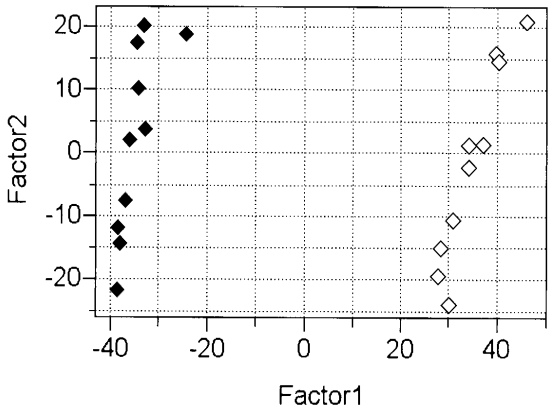

Fig. 3. Principal Component Analysis showing differences between Bt (◆) and Control (◇) maize lines based on FT-IR data measured at 2800 to 3000 cm^{-1}. (Contribution rate, Factor 1: 60.8%, Factor 2: 9.5%)

Generally, it had been recognized that the wave number around 2800 to 3000 cm^{-1} corresponded to the specific absorbance areas of the C-H stretch in the methyl (CH_3-) group (Naumann 2000). It was also possible that this result reflected the DNA methylation involved in the transgene.

Application of the FT-IR method to isogenic lines of rice

The results obtained from the FT-IR analysis of the Bt and control maize lines showed that the lines could be distinguished by spectroscopic methods. However, it was important to determine whether spectroscopy may reflect only structural differences like presence/absence of the gene, or could be used to identify much smaller variation such as allelic differences at one locus. Therefore, isogenic lines with only allelic differences were used. However, the resulting FT-IR spectra treated by smoothing and differentiation did not show any obvious differences among closely related isogenic lines and no clear patterns of the plotted data by PCA were observed (Fig. 4). It was also suggested that there was no clear difference between single (T65*wx*) and double (T65 *lg g1*) isogenic lines. Hence small variations such as difference between alleles may be below the threshold of detection by spectroscopy. In the case of rice isogenic lines, the genetic differences between T65 and T65*wx* had previously been found to correspond to a 23 bp duplication of the 2nd exon and a single nucleotide polymorphism (SNP) at the 6th exon of the waxy gene (Inukai *et al.* 2000).

Genome-wide differences among rice varieties

To evaluate the suitability of FT-IR for detecting wider gross genomic variations, rice varieties with genome-wide differences in genetic backgrounds were used. A typical *japonica* (T65) and *indica* (Ac. 130 and Ac. 419) varieties were examined as *indica-japonica* differentiation had previously well documented (Oka 1958, Sato *et al.* 1990). It was expected that the differences between the *indica* and *japonica* varieties would be genome-wide and indeed, significant FT-IR spectral differences among these varieties were observed at 1450 cm^{-1}. Results of PCA of these spectra from 1350 to 1500 cm^{-1} also revealed the presence of different

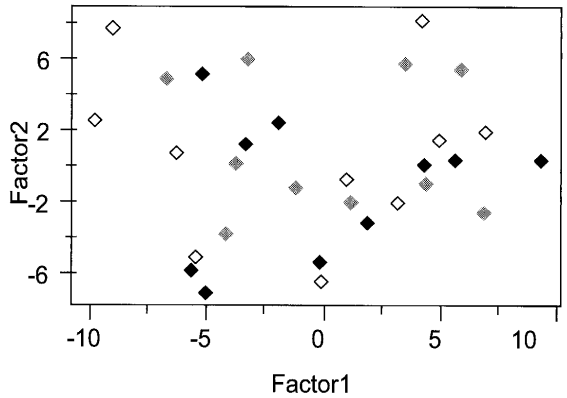

Fig. 4. Principal Component Analysis of FT-IR data from rice isogenic lines. (◇: T65, ◈: T65*wx*, ◆: T65*lg g1*, Contribution rate, Factor 1: 31.7%, Factor 2: 20.2%)

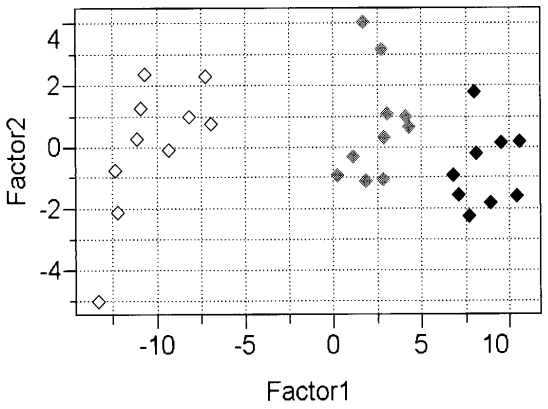

Fig. 5. Principal Component Analysis of FT-IR data from different rice varieties. (◇: T65, ◈: Ac.130, ◆: Ac.419, Contribution rate, Factor 1: 64.0%, Factor 2: 18.5%)

plot patterns between the *indica* and *japonica* varieties (Fig. 5). It was reported that the main vibrations of nucleic acids were observed in the spectral range between 700 and 1800 cm^{-1}, and that spectra associated with the base-sugar moieties strongly depending particularly on the glycosidic torsion angle, were observed between 1350 and 1500 cm^{-1} (Taillandier and Liquier 1992). These finding suggested that the differences in the spectra observed in our study reflected the differences in the DNA structure between *indica* and *japonica* varieties.

Furthermore, the two different varieties, Ac. 130 and Ac. 419, that belonged to same *indica* group, could also be clearly distinguished from one an other. These data strongly suggest that FT-IR spectroscopy can successfully discriminate between genotypes at the variety level. This spectroscopic method also has a distinct advantage over the PCR method, as direct genome-wide scanning from extracted genomic DNA can be achieved with only one measurement. On the other hand, in the PCR method, preliminary studies on marker construction and selection showing polymorphisms among samples should be carried out for each marker locus. In particular, it is necessary to conduct several preliminary marker combinations to examine samples closely related genetically (e.g. Japanese rice varieties), as well as a large number of preliminary experiments and to collect basic data.

We concluded that spectroscopy has could become as a new conventional and rapid tool for the genotyping of GM crops and identification of different genotypes, without need for sophisticated molecular techniques. We further predict that spectroscopy-based methods will become a powerful tool, in particular, for the testing of a large number of samples, providing that relevant data-profiling could be obtained on the spectroscopic database.

Acknowledgements

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