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## Research Communication

# Simple and Rapid Method for the Selection of Individual Rapeseed Plants Low in Glucosinolates

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**A simple and rapid colorimetric method for the selection of individual rapeseed plants with low glucosinolate contents was developed using one of a pair of germinated cotyledons of a single seedling and a glucose-measuring method employed in clinical tests (CII-Test method). The CII-Test method can be used to estimate the amount of glucose released by the hydrolysis of glucosinolates extracted from a germinated cotyledon. In the present experiment, HPLC analysis showed that the total glucosinolate content of germinated cotyledons was not significantly different from that of seeds three days after seed sowing. When the glucosinolate content of the seeds and/or cotyledons was estimated by the glucose-measuring CII-Test method and HPLC method, high positive correlations were recognized between both types of measurements. The CII-Test method exhibits some clear advantages in that 1) the glucosinolate content can be estimated in an individual plant, and 2) 150–500 samples can be estimated by one person in one day. These advantages indicate that the glucose measuring CII-Test method is suitable for efficient and accurate selection of rapeseed plants with low glucosinolate contents in the $F_2$ generation.**

**Key Words:** ***Brassica napus*, glucosinolates, glucose release, cotyledon, selection.**

## Introduction

Glucosinolates are sulphur-containing glycosides found in cruciferous plants. Compounds derived from glucosinolates that accumulate in seeds of rapeseed (*Brassica napus* L.) have been implicated in the goitrogenic and toxic effects of some animal feeds (Underhill 1980, Fenwick *et al.* 1983). International breeding programs aimed at reducing the levels of glucosinolates in rapeseed in order to utilize the meal as livestock feed have been successfully carried out. Low-glucosinolate cultivars with contents of less than 30 μmol/g in the meal, which remain after the oil has been extracted from the seeds, are now available (Uppström 1995).

The contents of total and/or individual glucosinolates of rapeseed can be accurately analyzed by high-performance liquid chromatography (HPLC) (Møller *et al.* 1985, Bjorkqvist and Hase 1988). However, the determinations were performed in a few samples only because of the time-consuming sample preparation and long analytical time required for HPLC. In order to efficiently breed low-glucosinolate rapeseed cultivars, several simple methods of screening for low-glucosinolate lines have been developed. Some of these methods are based on the measurement of equimolar amounts of glucose released from glucosinolates by hydrolysis with the endogenous enzyme myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1) (Heaney *et al.* 1988, Mithen *et al.* 2000). The glucose release methods (Van Etten *et al.* 1974, Smith and Dacombe 1987) based on a colorimetric glucose oxidase/peroxidase assay including the clinical glucose test paper (Tes-Tape) method for urine sugar (Lein 1970, McGregor and Downey 1975), and the TRUBLUGLU meter method (Tholen *et al.* 1993), have since been developed. Palladium method (Thies 1982, Møller *et al.* 1985) and nondestructive quality evaluation methods using X-ray fluorescence spectroscopy (Schnug and Haneklaus 1988) and near-infrared reflectance spectroscopy (Renard *et al.* 1987, Biston *et al.* 1988) have also been developed. Specific ELISA assays have been developed for the determination of sinigrin and progoitrin that are major glucosinolates in Brussels sprouts (Van Doorn *et al.* 1998). However, except for the Tes-Tape method, all of these methods require a specialized equipment or complex pretreatments. Although the Tes-Tape method is very useful for the initial screening of a large number of samples in rapeseed breeding, it is difficult to apply it to the evaluation of the total glucosinolate content of a single seed.

In this study, we report a simple and rapid colorimetric method for selection of individual low-glucosinolate $F_2$ plants of rapeseed using one of a pair of cotyledons of a seedling and a clinical kit for measuring the glucose concentration.

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## Materials and Methods

*Plant materials*

Glucosinolates and glucose were analyzed in seeds and germinated cotyledons of 20 Japanese high-glucosinolate rapeseed cultivars and 10 exotic low-glucosinolate rapeseed cultivars of *Brassica napus* L. (Table 1). Seeds were harvested from the plants grown in the field at the National Agricultural Research Center for Tohoku Region at Morioka, Iwate, from September to July.

Fresh cotyledons were obtained from germinated seeds on filter paper moistened with distilled water in Petri dishes incubated at 25°C in the dark. One day after seed sowing, half of the Petri dishes were transferred to light conditions (16-h photoperiod, 5,000 lux) and maintained at 25°C. Cotyledons were collected from seedlings 1, 2, 3, 5 and 7 days after seed sowing, and were freeze-dried, ground, and stored in tubes at room temperature until pretreatment for HPLC analysis.

*Glucosinolate analysis*

Extraction of glucosinolates from seeds and cotyledons, and preparation of desulphoglucosinolates by sulphatase digestion were carried out according to the method of Bjerg and Sorensen (1987), with minor modifications. Glucosinolates were extracted from ground, freeze-dried samples of 0.2 g seeds and one cotyledon or 0.2 g cotyledons in boiling 70% aqueous methanol at 70°C, and sinigrin (Nacalai Tesque, Kyoto, Japan) was added as an internal standard. The extracts were adsorbed onto anion exchange resin DEAE-Sephadex A-25 (Amersham Biosciences, Buckinghamshire, UK) and subsequently converted to desulphoglucosinolates by treatment with aryl sulphatase (E.C. 3.1.6.1, type H-1, Sigma-Aldrich, St. Louis, MO). Individual desulphoglucosinolates were detected by using an L-7000 HPLC system (Hitachi, Tokyo, Japan) with UV absorption at 229 nm, as described previously (Ishida *et al.* 1997). Individual and total glucosinolate contents were calculated by integration of the individual desulphoglucosinolate peak areas, taking into account the recovery of the internal standard and the specific response factors reported at The International Organization for Standardization (1992).

**Table 1.** Cultivars used in the measurements of seed glucosinolate content

| Glucosinolate type | Cultivar name |
|---|---|
| High-glucosinolate cultivars | Norin 2, Norin 5, Norin 7, Norin 8, Norin 11, Norin 12, Norin 16, Norin 18, Murasaki-natane, Isuzu-natane, Chisaya-natane, Miyuki-natane, Asahi-natane, Kogane-natane, Oomi-natane, Dairyu-natane, Kamikita-natane, Asakano-natane, Kizakino-natane, Aomori 1, |
| Low-glucosinolate cultivars | Altex, Arabella, Bridger, Bronowski, Global, Hanna, Lergo, Loras, Topas, SV0212 |

*Glucose estimation using a medical glucose-measuring kit*

Two days after seed sowing, one of a pair of germinated cotyledons from a seedling was ground in a microtube (1.5 ml) with 100 µl distilled water using a homogenizer (Model S-205, Ikeda-rika, Tokyo, Japan) to release glucose. Endogenous myrosinase was allowed to act for 5 min at room temperature, and then the homogenate was treated or was not treated with 100 µl of ethanol. After centrifugation (10,000 × g for 5 min), 100 µl of the supernatant was decanted into a test tube. One milliliter Glucose CII-Test Wako reagent (Wako Pure Chemical Industries, Osaka, Japan) was added to the test tube, mixed, and then incubated for 20 min at 25°C. The glucose content was determined colorimetrically at 505 nm using a spectrophotometer (Model U-2000A, Hitachi, Tokyo, Japan). The glucose content was determined for 6–18 replicate cotyledon samples per cultivar.

## Results and Discussion

*Changes in total glucosinolate content after germination*

In order to develop a simple selection method to estimate the glucosinolate content of single seeds, we attempted to measure the glucosinolate content of single cotyledons after germination. The differences between the total glucosinolate content of seeds and cotyledons under both dark and light conditions were not appreciable in the low-glucosinolate 'SV0212' cultivar as well as in the high-glucosinolate 'Kizakino-natane' cultivar (Fig. 1). Differences in the contents of total glucosinolate and specific glucosinolates were particularly small within three days after seed sowing. These results are consistent with those reported by McGregor (1988). Removal of a single cotyledon from the seedling is easier than the removal of part of a cotyledon still within the seed. Furthermore, almost no extension of the radicle had occurred at this stage and little damage to the roots was found in transplanted seedlings. These results indicate that germinated cotyledons sampled 2–3 days after seed sowing are a suitable alternative material for the estimation of seed glucosinolates.

*Adaptation of the glucose measuring CII-Test method for evaluation of glucosinolates*

By measuring the glucose content in the germinating cotyledon using Glucose CII-Test Wako reagent, we attempted to develop a simple and rapid method to estimate the glucosinolate content of a seed. Glucose CII-Test Wako reagent is used clinically for measuring glucose concentrations in the serum and plasma. Glucose content can be estimated qualitatively by the intensity of the red color and quantitatively by the absorbance at 505 nm.

Initially, samples were prepared by homogenization in distilled water. However, we failed to evaluate accurately the glucosinolate content by the enhancement of color development. It is possible that the lipid layer formed onto the water surface interfered with the accurate measurement of the color development, because the reagent may become turbid

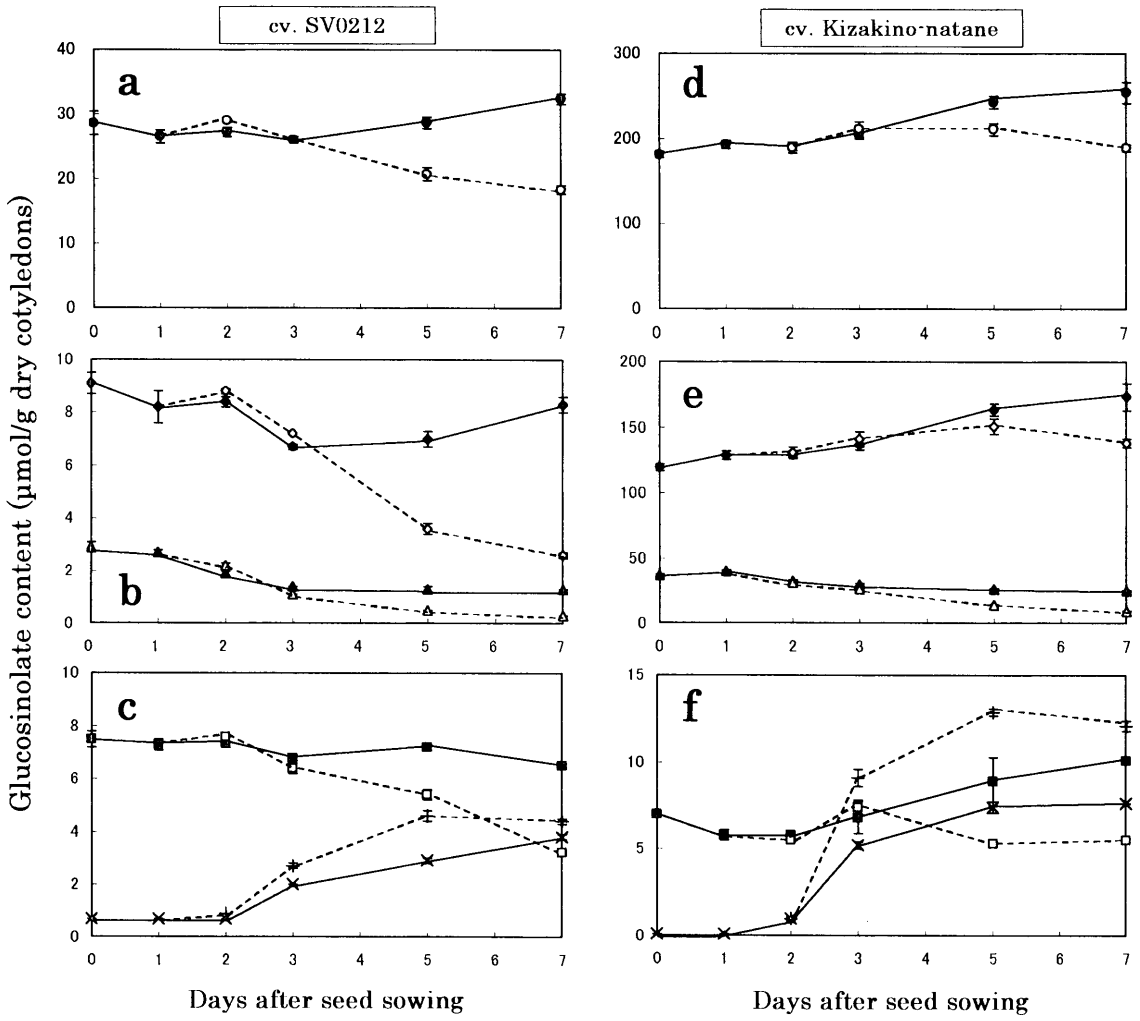


**Fig. 1.** Contents of total glucosinolates (●,○), progoitrin (◆,◇), gluconapin (▲,△), 4-hydroxy glucobrassicin (■,□) and glucobrassicin (×,+) in the cotyledons of rapeseed cv. SV0212 (a, b, c) and cv. Kizakino-natane (d, e, f) seedlings grown in darkness (—), and under a 16h/day photoperiod (···) 2 days after seeding.

when the lipid layer mixes with the reagent. When the homogenized solution was treated with ethanol, the intensity of the red color clearly changed depending on the glucosinolate type in the cultivars. Thus, we concluded that the addition of ethanol was indispensable to evaluate accurately the glucosinolate content by using the glucose measuring CII-Test method. The protocol developed in the present study was as follows:

1. Removal of a cotyledon from the seedling 2–3 days after seed sowing, and placement into a 1.5 ml microtube.
2. Addition of 100 μl of distilled water and homogenization.
3. Standing for 5 min at room temperature to release glucose.
4. Addition of 100 μl of ethanol and centrifugation at 10,000 × g for 5 min.
5. Transfer of 100 μl of supernatant to a test tube, and addition of 1 ml of Glucose CII-Test Wako solution.
6. Mixing, incubatation for 20 min at 25°C, and measurement of absorbance at 505 nm.

*Analytical accuracy of the glucose measuring CII-Test method for glucosinolate determination*

To confirm the analytical accuracy of the glucose measuring CII-Test method, variations among individual seeds were investigated using three cultivars with different glucosinolate contents: 'SV0212', 'Norin 18' and 'Kizakino-natane'. The glucosinolate content was determined by HPLC for one of a cotyledon pair, while the glucose content was determined by the glucose measuring CII-Test method for the remaining cotyledon. There was a close relationship between the total glucosinolate content measured by HPLC and the glucose content measured by the glucose measuring CII-Test method (Fig. 2), although the high-glucosinolate 'Kizakino-natane' and 'Norin 18' cultivars showed a relatively larger variation in the measurements of both compounds. The large variation in the high-glucosinolate cultivars was attributed to the variation in the glucosinolate content among seeds produced in a plant. Kondra and Downey

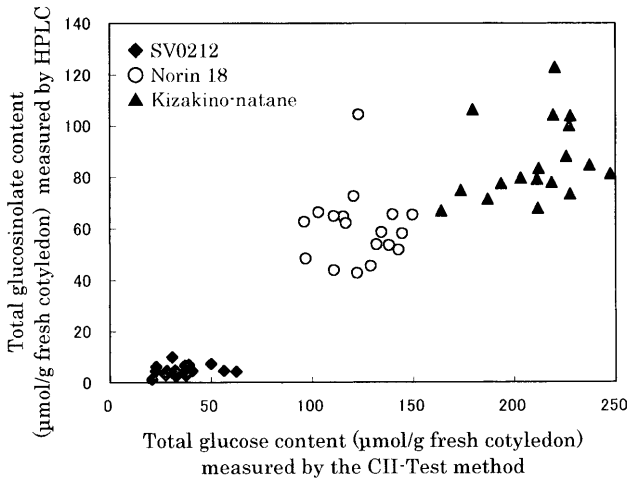


**Fig. 2.** Relationship between the concentration of glucose determined by Glucose CII Test Wako in one of a pair of fresh rapeseed cotyledons and HPLC in the remaining cotyledon.

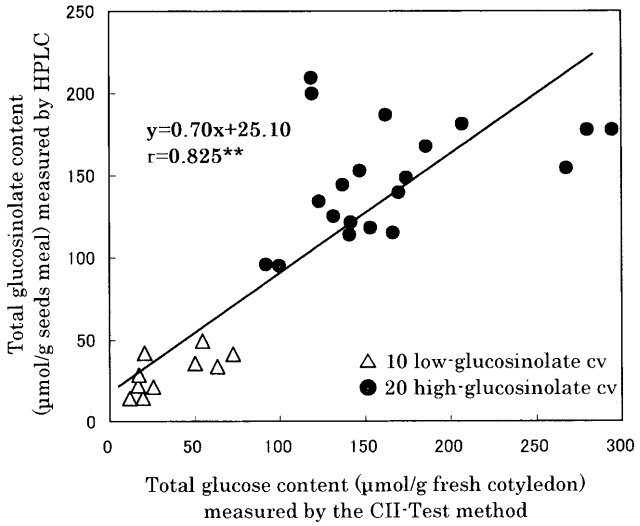


**Fig. 3.** Relationship between the concentrations of glucose determined by Glucose CII Test Wako in fresh cotyledons and HPLC in seeds of 30 rapeseed cultivars. **; Significant at the 1% level.

(1970) and Booth *et al.* (1990) reported that seeds from pods at a lower position on the terminal raceme displayed higher glucosinolate content than the seeds from the other part of the raceme on a high glucosinolate cultivar.

In order to determine how precisely the glucose content measured by the glucose measuring CII-Test method reflected the seed glucosinolate content, the relationship between the glucose content of the cotyledon and the seed glucosinolate content determined by HPLC techniques was examined using 30 cultivars. As shown in Fig. 3, the contents of both compounds showed a highly significant correlation ($r=0.825^{**}$, $P<0.01$). The low-glucosinolate cultivars with a glucosinolate content of less than 49.4 μmol/g showed a concentration below 72.6 μmol/g glucose, while the high-glucosinolate cultivars with more than 94.9 μmol/g glucosinolate showed a concentration above 92.0 μmol/g glucose over a wide range. Although theoretically 1.0 mol glucose is released by enzymatic hydrolysis of 1.0 mol glucosinolate, the regression line deviated from the theoretical line to some extent, presumably due to the influence of free glucose. We observed that free glucose interfered with the determination of the amount of glucose released in the total glucosinolate assay. Parts of the rapeseed plant contain endogenous glucose, although at a low level (Van ETTEN and Daxenbichler 1977, Smith *et al.* 1985). In addition, the free glucose that was released by other glycosidases may be attributed to the increase in the glucose content (Tholen *et al.* 1993). However, in the present study, the low-glucosinolate cultivars were clearly distinguishable from the high-glucosinolate cultivars when the glucose measuring CII-Test method was used. These results indicate that the method developed in the present study is sufficiently accurate for the screening of single low-glucosinolate seeds.

Several glucose detection methods (e.g. Tes-Tape method, Glucose oxidase/peroxidase method, TRUBLUGLU meter method) based on the release of glucose by enzymatic hydrolysis of glucosinolates are available for the estimation of the content of total glucosinolates. However, all these methods require several seeds as sample material. The development of a simple and reliable method for estimating the content of total glucosinolates in single seeds may enable to select low glucosinolate individuals in the $F_2$ segregating generation. We were able to successfully use the palladium method (Thies 1982, Møller *et al.* 1985), which utilizes the formation of a colored complex of palladium with glucosinolate, to estimate the glucosinolate content of single seeds using the half-seed method (Downey and Harvey 1963). However, this method was associated with some drawbacks, including the requirement of a high level of technical skill to remove cotyledons from seeds, a high seedling mortality when the cotyledon was removed and the difficulty in extracting crude glucosinolates. Therefore, glucosinolate levels in crossing progeny are usually estimated using bulked $F_3$ seeds. Since the total glucosinolate content is considered to be controlled by 2 to 3 (Gland 1985, Siebel and Pauls 1989) or 4 to 5 genes (Kondra and Stefansson 1970, Rücker and Röbbelen 1994), the frequency of appearance of the low-glucosinolate genotype is relatively low. For the selection of low-glucosinolate plants in the $F_2$ generation by the analysis of $F_3$ seeds, $F_3$ seeds must be harvested from a large number of $F_2$ plants. If it is possible to determine the seed glucosinolate content in an individual $F_2$ plant, selection of low-glucosinolate plants in the $F_2$ generation may be achieved efficiently.

Glucose CII-Test Wako which is the clinical reagent for the quantitative determination of glucose in the serum and plasma is an enzymatic solution containing mutarotase (EC 5.1.3.3), glucose oxidase (EC 1.1.3.4), peroxidase (EC 1.11.1.7) and 4-aminoantipyrine as chromogen. The principle

of the action of this reagent is as follows. Glucose oxidase reacts only with β-D-glucose. However, in the presence of mutarotase, α-D-glucose is rapidly converted to β-D-glucose, so that all glucose (not only β-D-glucose but also α-D-glucose) is always oxidized completely by using mutarotase in combination with glucose oxidase to produce hydrogen peroxide. Hydrogen peroxide induces oxidative condensation between phenol and 4-aminoantipyrine in the presence of peroxidase, so that a red color is produced. The amount of glucose contained in the test sample is determined by measuring the absorbance of the red color (Miwa *et al.* 1972, Okuda *et al.* 1973).

The method developed in the present study using Glucose CII-Test Wako enabled to determine simply and rapidly the total glucosinolate content at a the single seed level. By using this method, more than 150 samples could be analyzed by one person in one day. Furthermore, it is possible for one person to analyze more than 500 samples per day if color changes are estimated approximately visually rather than more precisely by the use of a spectrophotometer. This method would be really advantageous in breeding programs for low-glucosinolate rapeseed cultivars. Selection of individual low-glucosinolate plants can be carried out with the $F_2$ seeds, so that the field area and/or cultivation labor of the $F_2$ population can be reduced substantially. We anticipate that this convenient method will be employed for broad initial screening for the selection of low-glucosinolate rapeseed plants.

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