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Gas chromatography-mass spectrometry metabolomics-based prediction of potato tuber sprouting during long-term storage

Tomohiko Fukuda,[1,*] Kiyofumi Takamatsu,[1] Takeshi Bamba,[2,3] and Eiichiro Fukusaki[2]

Agricultural Research Institute, HOKUREN Federation of Agricultural Cooperatives, 375 Kita-6, Higashi-7, Higashi-ku, Sapporo 060-0906, Japan,[1] Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan,[2] and Division of Metabolomics, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan[3]

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In order to supply potato (*Solanum tuberosum* L.) tubers for the processed food industry throughout the year, suppliers should provide consistent quality potatoes even after long-term storage. Despite being one of the most important foods, there is no simple way to control tuber quality and, in particular, controlling sprouting. Chemical suppression such as chlorpropham is used to inhibit sprouting, however, the regulatory status of such chemical inhibition differs in each country. Gas chromatography–mass spectrometry-based metabolomics was applied to identify the applicable biomarkers for prediction of potato sprouting during long-term storage. Sprouting was measured in chipping potatoes, and these were also subjected to metabolite profiling to develop a predictive model. The model was based on projections to latent structures (PLS) regression calculated from a metabolome data set obtained before storage and was consistent with actual measured sprouting values. Sucrose, phosphate, and amino acids were selected as valid contributing biomarkers for prediction in a validation field experiment. These biomarkers will contribute to the development of a successful novel method for prediction and control of potato tuber quality during long-term storage.

[**Key words:** Potato; Sprouting; Metabolomics; Gas chromatography–mass spectrometry; Storage; Prediction]

Potato (*Solanum tuberosum* L.) is one of the most important food crops worldwide. Potato tubers are an excellent staple food and represent the fourth highest with respect to worldwide production, after maize (*Zea mays* L.), rice (*Oryza sativa* L.), and wheat (*Triticum aestivum* L.). Tubers are consumed fresh or in the form of processed foods such as chips or fries, leading to a continual year-round demand and making long-term storage of tubers after harvest necessary. However, the largest obstacle to the use of potatoes is the difficulty in controlling sprouting traits during storage (1). Improvements in this regard would benefit the potato industry not only by preventing quality loss due to tuber shrinking following starch collapse but also by preventing the production of toxic potato steroid glycoalkaloids, which are synthesized in tuber sprouts. Although chemical treatment such as chlorpropham (CIPC) is used to inhibit sprouting (2), the regulatory status of such chemical inhibition differs in each country, and thus, it is not a universally applicable tool for the potato supply chain. On the other hand, prediction of sprouting using near-infrared spectroscopy had been achieved (3); however, the key metabolites playing important roles in predicting the degree of sprouting have not been described. Therefore, practical methods based on biochemical markers for prediction of tuber sprouting during storage are needed.

Metabolomics can be a powerful tool in elucidating a variety of biological phenotypes, including the study of human disease, nutrition, drug discovery, and plant physiology (4). In potato, the metabolomics approach has been applied in one of the first successful examples for the simultaneous analysis of a large number of metabolites using gas chromatography–mass spectrometry (GC/MS) (5). Subsequently, the approach has been used to evaluate the metabolic changes that occur following various genetic modifications involved in environmental stress tolerance (6), inducing carbohydrate insulin synthesis enzymes (7) and repression of 14-3-3 signaling protein (8). Additionally, GC/MS (9) and liquid chromatography-mass spectrometry (LC/MS) (10) have also been used for the identification of useful molecular markers for selection of breeding lines resistant to late blight disease using nontargeted analyses. Furthermore, nuclear magnetic resonance (NMR)-based metabolic profiling has also been applied for potato leaves in relation to resistance to this important potato disease (11). In particular, the metabolomics approach has been applied to monitoring metabolites during the tuber life cycle (12). This work can link metabolites to important temporal phenotypic changes in the process of potato tuber maturation from a period of dormancy to the appearance of a sprout. Therefore, in this study, a metabolomics methodology was employed to screen and choose applicable biomarkers for prediction of potato sprouting during storage.

Gas chromatography coupled with quadrupole mass spectrometry (GC-Q/MS)-based metabolomics was selected as it provides reproducible results with high sensitivity and is able to quantify a large number of metabolites (13). To identify applicable

* Corresponding author at: Field Crop Section, Plant Breeding & Production Division, Hokuren Agricultural Research Institute, 829 Shimo-Shimamatsu, Eniwa 061-1364, Japan. Tel.: +81 123 36 3356; fax: +81 123 39 6156.
E-mail address: fukuda-tomohiko@hokuren.jp (T. Fukuda).

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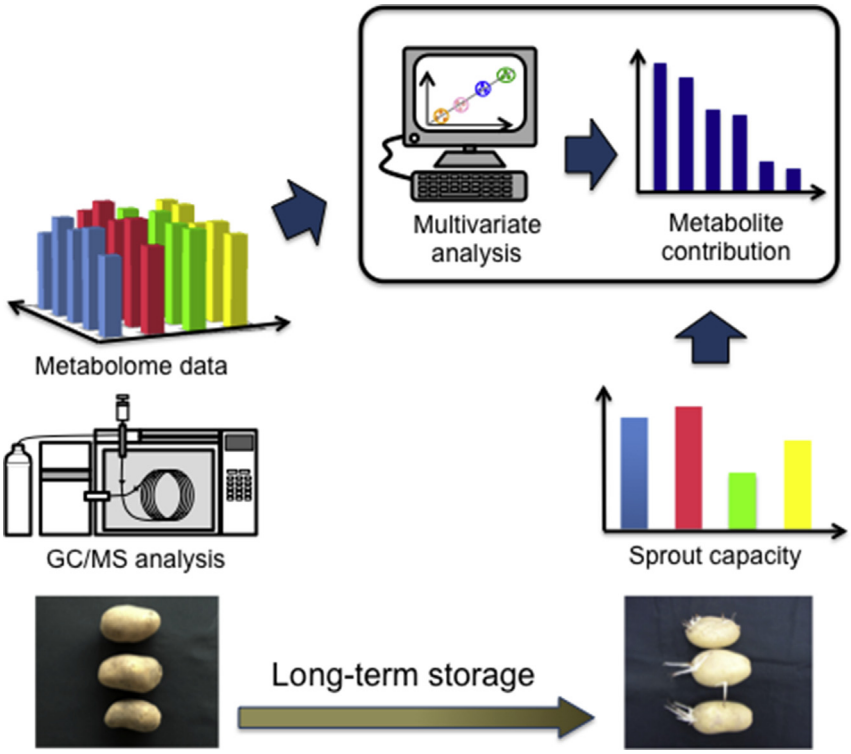

FIG. 1. GC/MS metabolomics-based strategy to identify molecular markers able to predict potato tuber sprouting during long-term storage.

biomarkers that can be used to predict sprouting, potato tuber samples were collected from outdoor experimental fields rather than from a controlled growth environment.

The objective of this study was to identify the biochemical markers, based on metabolomics techniques, that could be used to predict appropriate length of storage in the industrial potato supply chain (Fig. 1). In the first year, a total of 60 potato samples, including commercial varieties and breeding lines, were used to develop a predictive model using projections to latent structures (PLS) regression analysis. Prediction markers, which were selected on the basis of their contribution value to the PLS model, were verified for reproducibility and applicability using 34 potato samples in the second year. Our results will contribute to the development of novel tools for determining appropriate storage conditions for the potato supply industry.

MATERIALS AND METHODS

Plant material Chipping potato varieties, including breeding lines and commercial varieties, were used in this study. The potato breeding lines were chosen to roughly represent a wide range of possible sprout growth during storage. Atlantic and Snowden are cultivars in the United States that are famous for their higher yield, processing ability, and chipping quality. Toyoshiro and Kitahime are the leading chipping cultivars in Japan. Each variety or breeding line was corrected from two ridges in 2014, and from three ridges in 2015 (except for Snowden in 2015).

All the potato varieties used in this study were grown in 2014 and 2015 and obtained from the experimental field of the Eniwa Research Station (Hokuren Agricultural Research Institute, Eniwa, Japan). Seed potatoes were cultivated between May 8 to October 20 in 2014 and May 1 to October 14 in 2015.

Thirty-five tubers were collected from every ridge and were divided into 15 and 20 tubers for use in GC/MS metabolome analysis and evaluation of sprouting capacity during storage, respectively. Tubers for metabolome analysis were frozen and lyophilized a few days after harvest. Freeze-dried tubers were ground with an ultra-centrifugal mill (ZM 200, Retsch, Haan, Germany) and then stored in a deep freezer at −20 °C until measurements were performed. Tubers to be used for evaluation of sprouting capacity were set aside for a curing period of 2 weeks to toughen their skins. All the tubers were weighed before storage and then stored for 7 months at 8.0 °C and 95% relative humidity.

Sprouting capacity After 7 months of storage, the sprouts on each tuber were removed and the tuber was weighed to determine the total mass of sprouts per tuber. The ratio of sprout weight to initial tuber weight was calculated for each tuber.

Reagents Methanol and chloroform were used as extraction solvents, ribitol was used as an internal standard, pyridine (ultra-pure grade) was used as a solvent, and all were purchased from Wako (Osaka, Japan). Methoxyamine hydrochloride was purchased from Sigma (St. Louis, MO, USA). *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) and hydrocarbon mixtures were purchased from GL Sciences, Inc. (Tokyo, Japan).

Extraction Potato powder (10 μg) was placed in a 2 mL Eppendorf tube, cooled with liquid nitrogen, ground again with a Retsch ball mill (20 Hz, 5 min), and then extracted with 1 mL of extraction solvent consisting of 5:2:2 (v/v/v) methanol, distilled water, and chloroform containing an internal standard (ribitol at 12 μg mL^{-1}). Extraction mixture was shaken for 30 min and then centrifuged at 16,000× *g* for 3 min at 4 °C. Supernatant (900 μL) was transferred to a 1.5 mL Eppendorf tube, and 400 μL of Milli-Q water (Wako) was added before vortexing. Following centrifugation (16,000× *g*, 3 min, 4 °C), 400 μL of supernatant was transferred to a fresh 1.5 mL Eppendorf tube and capped. Additionally, 200 μL of supernatant was collected from each sample and pooled to produce a mixture of all samples to be used as quality control (QC) samples, and this pooled mixture was then divided into individual aliquots in 1.5 mL Eppendorf tubes. Extracts were evaporated to remove methanol in a centrifuge vacuum concentrator (CVE-3110, EYELA, Tokyo, Japan) for 2 h, followed by freeze-drying overnight.

Derivatization Derivatization for GC/MS analysis was performed by oximation and trimethylsilylation. Methoxyamine hydrochloride (100 μL, 20 mg/mL in pyridine) was added as the first derivatizing agent. The mixture was incubated at 30 °C for 90 min. After addition of the second derivatizing agent, 100 μL of MSTFA was added and incuvated at 37 °C for 30 min. One microliter of sample was injected in split mode (25:1, v/v), and QC samples were analyzed every five injections.

GC/MS analysis GC/MS analysis was performed on a GCMS-QP2010 Ultra (Shimadzu, Kyoto, Japan) equipped with an InertCap 5MS/Sil column (0.25 mm × 30 m, 0.25 μm, GL Sciences Inc.) and coupled to an injector (AOC-20i/s, Shimadzu). The injection temperature was 230 °C. The carrier gas (helium) flow was 1.12 mL/min with a linear velocity of 39 cm/s. The column temperature was held at 80 °C for 2 min isothermally, increased by 15 °C/min to 330 °C, and then held for 6 min. The transfer line and ion source temperatures were 250 °C and 200 °C, respectively. Ions were generated by electron ionization at 70 eV with electron ionization. Spectra were recorded at 20 spectra per second over the mass range *m/z* 85–500. A standard hydrocarbon mixture (C8–C40) was injected at the beginning and end of the analysis to obtain a retention index (RI).

Data processing Raw chromatographic data were converted into an AIA file (ANDI files: Analytical Data Interchange Protocol, *.cdf) by the GC–MS Solution software package (Shimadzu). Processing of GC/MS scan data was performed on the ANDI files using the MetAlign software package (14), and alignment of peak retention times were normalized to the ribitol internal standard. Peak intensities were normalized using the LOWESS software package against the intensities of QC samples (15).

Metabolite annotation Metabolites were annotated using Aloutput2 (version 1.29) (16,17) and AMDIS (18), based on comparison of each MS spectrum with an in-house library prepared from authentic standard chemicals referring the RI. Other peaks were tentatively annotated on the basis of the National Institute of Standards and Technology (NIST) library.

Multivariate data analysis Metabolite data sets were autoscaled as pretreatment for multivariate analysis in order to adjust for the differences in concentration among metabolites. Principal components analysis (PCA), Ward's hierarchical cluster analysis (HCA), and PLS regression analysis were performed. PLS with a 7-fold cross-validation was carried out to assess the accuracy of the prediction model in practice. The goodness-of-fit (R^2) and predictability (Q^2) parameters were then determined. Analysis was performed with commercial software, The Unscrambler (v10.2; Camo Analytics, Trondheim, Norway).

RESULTS AND DISCUSSION

Potato plant growth and measurement of tuber sprouting during storage Corresponding to the genetic diversity in the 60 potato samples, there was a high degree of difference in fresh weight. Sprouting capacities of potato tubers after 7 months of storage are summarized in Table S1. The score in sprouting capacities varied between 0.37 and 16.39.

GC/MS metabolome analysis GC/MS metabolomics analysis was performed on hydrophilic metabolites, because established protocols for single-step extraction, derivatization, and measurement were available (13). Fifteen potato tubers were used to obtain metabolome data on every variety and breeding line. One hundred and eight peaks were extracted from the MetAlign data based on GC/MS metabolite profiling, from which 57 compounds were annotated using the in-house library, whereas 13 peaks were tentatively annotated using the NIST library (Table S2). A total of 70 compounds consisting of amino acids, organic acids, sugars, and other compounds were used for the analysis described below. Harmful steroidal glycoalkaloids such as α-solanine and α-chaconine were not detected in this study.

To reduce the complexity and visualize the information in the GC/MS data sets, unsupervised PCA analysis was utilized. However, the sum of first two principal components explained only 30.7% (PC1 and PC2 accounted for 19.5% and 11.2%, respectively, data not shown). Therefore, another unsupervised method, HCA was employed. Seventy metabolites data sets on the HCA plot showed that the same varieties or breeding lines generally clustered together (Fig. 2). Cultivar Snowden, shown in cluster A in Fig. 2 possessed the maximum distance because it has no genetic connections with the other samples used in this study. Genetic relationships described appropriately similar metabolic traits and could be consequently merged into clusters (e.g., breeding lines K and L with cv. white flyer—the latter is the parent of the former, shown in cluster D in Fig. 2). This suggests that genetic relationship strongly influences metabolic profiles compared with environmental factors.

PLS regression modeling The unsupervised HCA provided an overview of the tuber metabolome data; however, selection of

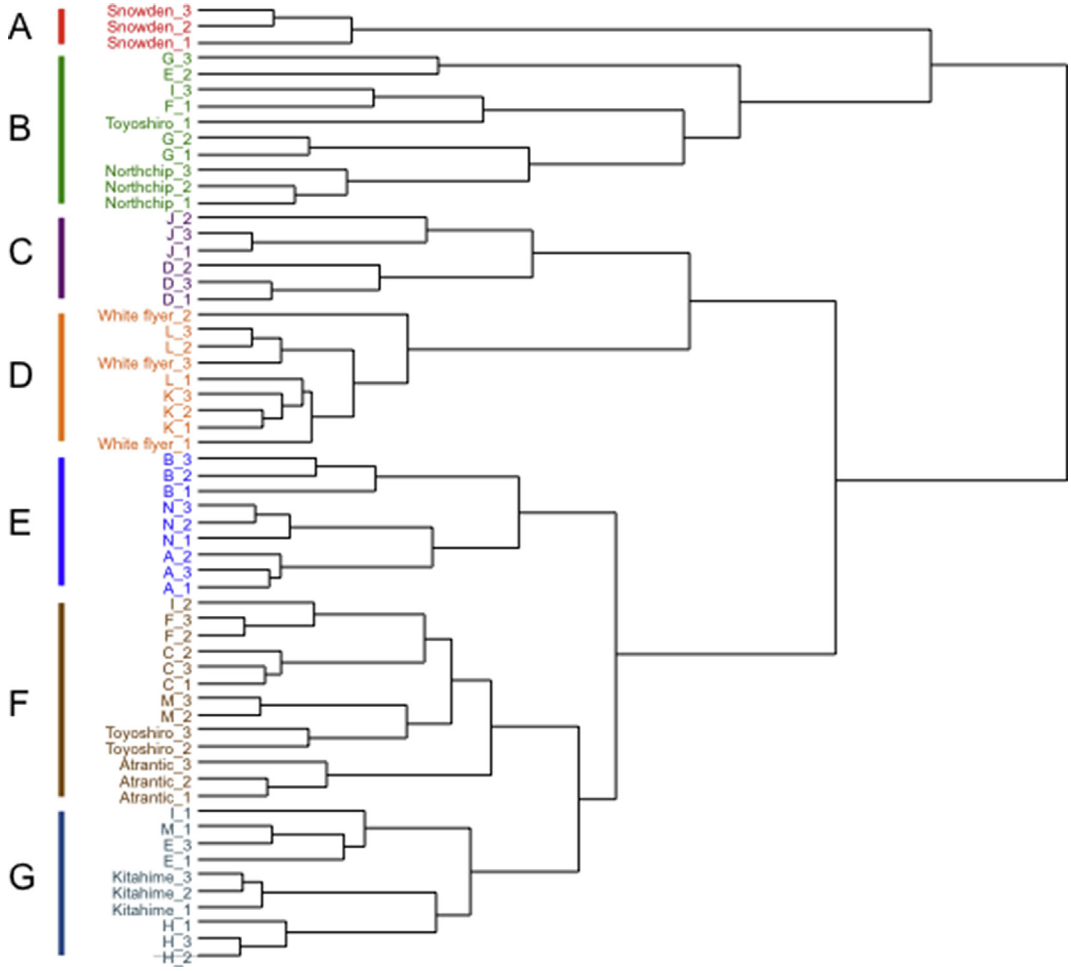

FIG. 2. Hierarchical cluster analysis of potato varieties and breeding lines based on tuber metabolome data, which varieties and breeding lines divided into clusters A–G.

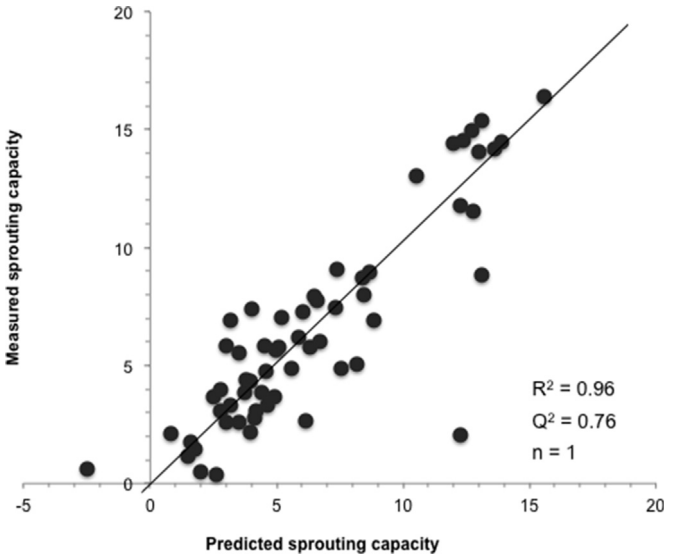

FIG. 3. PLS regression model for sprouting capacity based on measured score vs predicted in the first year (samples harvested in 2014).

useful biochemical markers to predict tuber sprouting capacity was needed. PLS regression is a well-known and widely employed method that is used to model the relationship between two matrices, X and Y (19). In this case, metabolite peak intensities obtained by GC/MS analysis were used as the prediction variables (x variables), whereas measured sprouting capacity values during 7 months of storage were used as the response variables (y variables). Metabolites with a robust and substantial contribution to the PLS regression model could be converted into widely applicable biochemical markers for the prediction of sprouting. A PLS regression model was constructed from 60 samples and is shown in Fig. 3. The model described the response variable well with $R^2 = 0.96$ and $Q^2 = 0.77$ (R^2 and Q^2 denote goodness-of-fit and cross-validated fitting, respectively). Sprouting capacity predicted by the tuber metabolome obtained shortly after harvest coordinated sufficiently with actual measurements. To identify metabolites possessing the greatest relationship to sprouting capacity, variable importance in projection (VIP) values were calculated (20). Large VIP values (>1.0) are more relevant for model construction (20). As shown in Table 1, the metabolites with high VIP scores were sucrose, phosphate, and several amino acids including valine, lysine and isoleucine. Potato tuber sprouting is regulated by hormonal activities, in particular, a drop in abscisic acid below a certain threshold level triggers the sprouting process, which is linked to an increased level of trehalose-6-phosphate (T6P) in a positive correlation with sucrose content in transgenic potatoes (21,22). On the other hand, tuber-specific expression of a bacterial pyrophosphate results in acceleration of sprouting, which pyrophosphate (PPi) hydrolysis produces two inorganic phosphates (23). This suggests that increasing levels of sucrose and decreasing levels of phosphate reduce sprouting capacities and so result in longer dormancy phenotypes, which is consistent with the results of transgenic tuber experiments. In tobacco leaves, supplying sucrose results in activating amino acid synthesis depending on sucrose content in association with nitrate or ammonium assimilation (24,25), which corresponds with this study. On the other hand, increases in specific amino acids are required conditions for decreased level of supplying sucrose to the potato tuber. (26) Therefore, the biological correlation between amino acids and sprouting requires further investigation.

TABLE 1. Metabolite with high VIP values in 2014.

Metabolite	VIP	Correlation
Sucrose	1.986	Negative
Phosphate	1.826	Positive
Valine	1.673	Negative
Lysine	1.665	Negative
Isoleucine	1.635	Negative
Unknown (RT = 4.163)	1.570	Positive
Proline	1.487	Negative
Chlorogenic acid	1.419	Positive
Phenylalanine	1.387	Negative
Tyrosine	1.374	Negative
Glycine	1.341	Negative
Threonine	1.340	Negative
Unknown (RT = 8.920)	1.333	Positive
2-Hydroxypyridine	1.327	Negative
Mannose	1.322	Negative
Galactose	1.287	Negative
Fructose 6-phosphate	1.239	Negative
Glutamine	1.233	Negative
Tryptophan	1.219	Negative
β-Alanine	1.217	Negative
Unknown (RT = 15.763)	1.180	Negative
Unknown (RT = 6.608)	1.164	Negative
Inositol	1.159	Negative
Histidine	1.144	Positive
Allantoin	1.129	Positive
Mannitol	1.108	Positive
Glucose	1.075	Positive
2-Aminoethanol	1.030	Positive
Fructose	1.024	Positive

Validation of the prediction model using potato tubers produced in different year In this study, our initial motivation was to identify the applicable biochemical markers that could predict, at or shortly after harvest, the degree of tuber sprouting during storage. The power of the predictive marker candidates obtained in 2014 was tested in a PLS regression model in tubers grown under different environmental conditions in 2015. Thirty-four potato samples, including commercial varieties and breeding lines, were cultivated between April 28 and October 4, 2015 (Table S3). As with the first year, 15 tubers were selected randomly before storage for use in metabolite analysis and 20 tubers were stored for 7 months prior to measurement of the sprouting capacities of each tuber. In the second year, 53

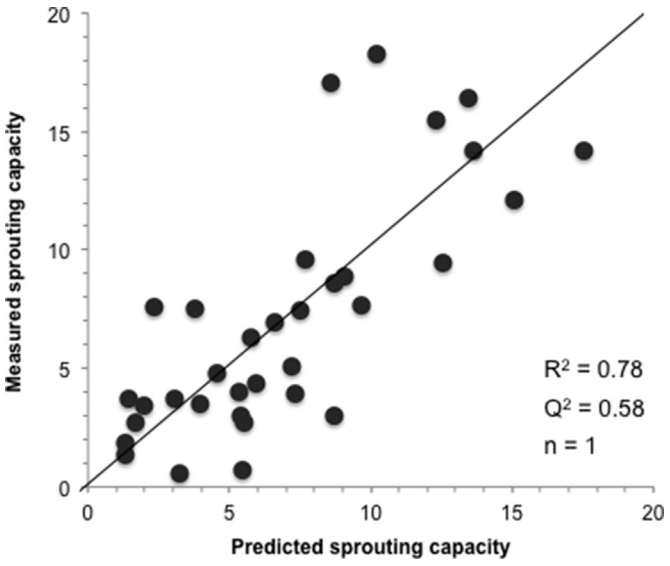

FIG. 4. PLS regression model for validation in second year (samples harvested in 2015).

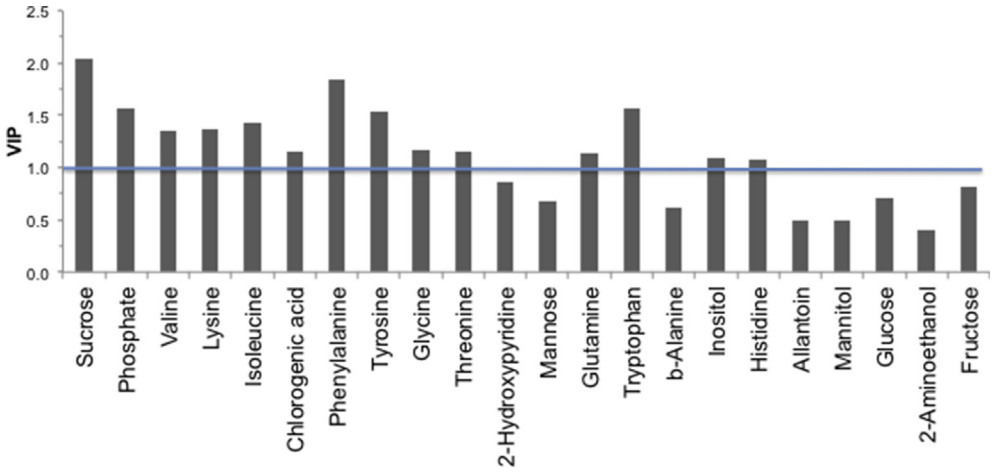

FIG. 5. Contribution of prediction markers that had high VIP values (>1.0) in the second-year experiment. Proline, galactose, and fructose 6-phosphate were not detected in the experiment in 2015.

metabolites annotated with the authentic standard were used for validation of the PLS regression model (Table S4). The model described the response variable well with $R^2 = 0.78$ and $Q^2 = 0.58$ (Fig. 4). Although, when compared with the first year, correlation values decreased slightly because the number of x variables was altered, the prediction model was able to provide a sufficient indication of the prediction. Therefore, our prediction model was successfully validated.

To display the applicability of the selected biochemical markers to the potato tubers harvested in the second year, VIP values were calculated from the validation data set. Fourteen compounds had higher VIP values (>1.0) in both years (Fig. 5). Moreover, the selected predictive markers, including sucrose, phosphate, and several amino acids including valine, lysine and isoleucine exhibited large VIP values that corresponded with the first year. Hence, we confirmed that these metabolites contribute to predicting potato sprouting traits during long-term storage. Level of each candidate metabolite marker in 2014 and 2015 is listed in Tables S5 and S6, respectively.

To summarize, in this study, a GC/MS metabolomics-based applicable tool for the prediction of the potato tuber sprouting capacity during storage was established. Commercial cultivars and breeding lines were subjected to measurement of sprouting capacities during long-term storage. A PLS regression model was constructed on the basis of metabolome data obtained from tubers soon after harvest, and it corresponded well with actual sprouting capacities measured after 7 months of storage. Several metabolites, including sucrose, phosphate, and amino acids, were verified as robust biochemical markers for prediction. The use of employing applicable prediction markers for controlling potato storage strategies will be of considerable economic importance to those involved in the potato supply chains from farmers to consumers. The potential advantages of using predictive biomarkers based on GC/MS metabolomics technique are expected to achieve viable, cost-effective, and environmentally acceptable post harvest agricultural industries.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2019.01.016.

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