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Development of a Screening System for the Evaluation of Soybean Volatiles

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Flavor properties are important factors of soybean seeds in their utilization as food materials. In order to isolate novel varieties and mutants of soybean having preferable flavor properties, a simple and efficient screening system was established using an automated headspace sampler coupled to gas chromatography. With this system, five major volatile compounds were analyzed within 12 min. By applying this screening system to 626 soybean varieties collected worldwide, we isolated four soybean varieties that showed unique compositions of volatile compounds. Through biochemical analysis, it was found that the uniqueness of three of them was possibly independent of lipoxygenase enzyme, and thus perhaps this screening system can expand the subject of flavor properties beyond lipoxygenase and thus be useful in discovering new types of soybeans.

Key words: soybean (*Glycin max* L. Merril) seed; flavor; lipoxygenase; hydroperoxide lyase; hexanal

Soybean production and use has been increasing dramatically (1.78×10^{11} kg in 2001 to 2.10×10^{11} kg in 2005 according to FAOSTAT, http://faostat.fao.org) because of its high nutritional value and potential health benefits, but utilization of soybean seeds as a food material has sometimes been limited because of their unique beany flavor. The beany off-flavor, especially in soymilk, puts off many consumers. Therefore, flavor properties are one of the foremost targets for soybean breeders, among others, such as agronomic traits, protein, and fat content, and a lot of effort has been given in order to reduce the off-flavor. It is known that oxidative products derived from fats, such as *n*-hexanal and *n*-hexanol, contribute to the beany flavor.[1)] Lipoxygenase (LOX) oxygenizes linoleic and linolenic acids either in their free forms or in their esterified forms to yield their hydroperoxides.[2)] If the hydroperoxide locates at the C13 positions of fatty acids, hydroperoxide lyase (HPL) can catalyze a cleavage reaction at the C12–C13 bonding to form carbon 6 aldehydes and carbon 12 oxo acids (the latter can be in free or esterified form).[2)] Because linoleic acid is abundant in soybean seeds, *n*-hexanal is a predominant product of the LOX-HPL action. Part of *n*-hexanal can be reduced by alcohol dehydrogenase to form *n*-hexanol. This series of reactions takes place essentially only after water-imbibition and subsequent homogenization of the seeds.

Progress in breeding efforts came when LOX-less varieties were brought about.[3,4)] The LOX-less varieties were isolated through comprehensive screening of soybean cultivars by analyzing the amounts of LOX proteins in the seeds.[5)] Soymilk made from LOX-less varieties showed low amounts of *n*-hexanal and *n*-hexanol, and the varieties were widely accepted by consumers because of low beany off-flavor in the soymilk processed from them. Tofu made from these LOX-less soybeans lacks a rich flavor, and thus the volatile products derived from LOX actions are thought to be important ingredients in the case of tofu.[6)] Other than the volatile compounds derived from the usual LOX-HPL system, there exists an array of compounds that have impact on the flavor of soybean foods. 1-Octen-3-ol has earthy and mushroom-like flavor properties, and it is usually recognized as an off-flavor for soymilk.[7)] This C8 compound might be a precursor to form 1-octen-3-one, which has high CHARM value.[8)] Another important volatile compound is 1-penten-3-ol, because it can also be converted into raw-bean volatile 1-penten-3-one (ethylvinylketone).[9)]

Previous screening efforts largely relied on the amounts of LOX proteins, but there must be other factors that affect on the flavor profiles of soybean seeds. The impact of these still unknown factors on the quality of processed food derived from soybean can be confirmed using a variety or a mutant of soybean that lacks the factor in question. Such a variety or mutant might be preferable as a food material, or it might be a useful genetic resource for breeding a soybean variety showing better flavor properties. In order to uncover such a useful soybean, it is important to establish a simple and efficient system to evaluate volatile profiles. In this study, we developed a volatile analysis system suitable to comprehensive screening, and we isolated soybean varieties having unique volatile compositions.

Materials and Methods

Soybeans. A normal soybean variety containing all three seed LOX isozymes, Fukuyutaka, and ones lacking all of them, L-Star and Ichihime, were grown in the experimental field of the National Institute of Crop Science, Tsukuba, Japan, and harvested in 2007. The other soybean varieties were harvested in the same field in 2004. The seeds were stored at 4 °C under dry conditions.

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Abbreviations: HPL, hydroperoxide lyase; LOX, lipoxygenase

Head space GC analysis of soybean volatiles. Soybean seeds (0.5 to 0.6 g, corresponding to 2 to 8 grains depending on size) were soaked in 5 ml of distilled water in a headspace vial (22 ml, Perkin Elmer, Waltham, MA) at room temperature (25 °C) overnight. After removing the seed coat, the imbibed seeds were homogenized thoroughly with a Polytron homogenizer (PT10-35, Kinematica, Littau, Switzerland) for 1 min. The homogenate was incubated at 25 °C for 30 min in order to facilitate the enzyme reaction, and then 5 ml of saturated solution of $CaCl_2$ was mixed in order to kill the enzymes. The vial was sealed tightly with a butyl stopper and crimp top seal (National Scientific, Rockwood, TN). Automated headspace volatile analysis was carried out with a headspace sampler (HS 40XL, Perkin Elmer) equipped with a GC (GC-2014, Shimadzu, Kyoto). The vial was incubated at 80 °C for 30 min, and pressurized for 3 min with N_2 gas set at 100 kPa. Headspace gas was introduced to the GC system for 0.2 min. GC was performed with a Stabiliwax column (30 m × 0.25 mm i.d., Restek, Bellefonte, PA) with 40 °C (1 min) to 180 °C (1 min) at 10 °C/min. Detection was performed with a FID detector.

Enzyme assay. n-Hexanal formation from linoleic acid (99% pure, Sigma, St. Louis, MO) or from linoleic acid 13-hydroperoxide (prepared with soybean LOX1 as reported previously[2]) was determined essentially as described previously.[10,11] In brief, soybean seeds were imbibed overnight at 25 °C and homogenized with 98 vol (v/w) of distilled water with a Polytron mixer on ice. After centrifugation at 6,000 rpm (T15A36 rotor, Hitachi, Tokyo) for 10 min at 4 °C, the supernatant was taken as crude enzyme solution. The enzymatic reaction was carried out with the enzyme solution (equivalent to 2.5 mg of seeds) in the presence of 1 mM linoleic acid (prepared as 50 mM solution in 0.2% Tween 20, emulsified before use with a tip-type sonicator) or 0.4 mM linoleic acid 13-hydroperoxide (prepared as 50 mM solution in ethanol) in 0.1 M Na phosphate (pH 7.0, with 1 ml of total volume of the reaction mixture) at 25 °C for 10 min. After the reaction, 2 ml of 0.1% 2,4-dinitrophenylhydrazine in ethanol containing 0.5 M acetic acid and 0.1% butylated hydroxytoluene were added with 100 nmol of *n*-heptanal as an internal standard, and this was incubated for 30 min at 25 °C. The hydrozone derivatives were extracted with 2 ml of hexane, and the hexane extract was washed once with brine. After drying *in vacuo*, the residue was dissolved with 100 µl of CH_3CN. A portion (2 µl) of the solution was separated with a HPLC system (L-2130, Hitachi) equipped with a Mightysil RP-18 column (250 × 4.6 mm, Kanto Chemicals, Tokyo). The solvent was comprised of CH_3CN:H_2O:tetrahydrofurane (80:19:1, v/v) at a flow rate of 1 ml/min. Detection was performed with absorbance at 350 nm. *n*-Hexanal was quantified using a calibration curve constructed with *n*-heptanal as an internal standard.

1-Octen-3-ol forming activity. For photosensitized oxygenation, linoleic acid (20 mg) was dissolved in 2 ml of methanol containing 75 µg ml^{-1} of methylene blue (Wako Pure Chemicals, Osaka). The solution was cooled to 4 °C and irradiated for 3 h with a 250 W sodium lamp (Panasonic, Osaka) with continuous introduction of oxygen gas to the solution. The formation of linoleic acid hydroperoxides was monitored by TLC (Silica gel 60, Merck, Whitehouse Station, NJ) with a developing solvent consisting of hexane:2-propanol:acetic acid (110:10:10, v/v). The hydroperoxides were detected by spraying *N*,*N*′-dimethyl-*p*-phenylene diaminedihydrochloride solution (10 mg/ml in methanol:water:acetic acid, 28:25:1, v/v). After photosensitization reaction, unreacted linoleic acid was removed using a silica gel (Wakogel C-300, Wako Pure Chemicals) column with hexane:ether (9:1, v/v). The crude hydroperoxide was eluted with hexane:ether (6:4, v/v). The solvent was removed *in vacuo*, and the hydroperoxides were emulsified in 0.2% (w/v) Tween 20 in 0.1 M Na phosphate (pH 6.5) to a final concentration of 10 mM. The concentration of hydroperoxides was calculated from conjugated diene absorption at 234 nm (ε = 25,000 M^{-1} cm^{-1}).

The activity to form 1-octen-3-ol was determined at 25 °C in a reaction mixture containing 0.1 ml of the hydroperoxides and 2 ml of crude enzyme solution. The reaction mixture was incubated for 15 min; thereafter, 0.2 ml of 0.1 N NaOH was added to stop the reaction. Nonenzymatic activity was estimated by using boiled crude enzyme instead. The amount of 1-octen-3-ol was determined by headspace GC analysis, essentially as described above. One unit of enzyme activity was defined as the ability to form 1 µmole of 1-octen-3-ol per min.

SDS–PAGE. Protein profiles were analyzed by a modification of the procedure of SDS–PAGE developed by Kitamura.[5]

Results and Discussion

Headspace volatile screening

A simple and efficient headspace sampling procedure was established with a headspace sampler coupled to a GC system. It relies on the formation of volatile compounds during homogenization and subsequent incubation of the soaked soybean seeds. The amounts of volatiles in dry soybean seeds are usually low, but they increase rapidly during homogenization, which affects the flavor properties of soybean-derived foods.[2] Because the seed coat hampers efficient homogenization and flavonoids accumulated in it are known to be inhibitory to lipoxygenase activity,[12] it was removed before homogenization. With this system, five major volatile compounds formed in the homogenized soybean seeds, *viz.*, *n*-hexanal, 1-penten-3-ol, *n*-pentanol, *n*-hexanol, and 1-octen-3-ol, were analyzed within 12 min (Fig. 1).

The peaks corresponding to these volatile compounds found with Fukuyutaka (having all three isozymes of LOX) were much bigger than those found with L-Star (LOX-less variety), except for 1-octen-3-ol. This is in good accordance with the volatile compositions examined in our previous study.[2] This indicates that this system is useful to identify a soybean variety that has lower (or higher) ability to form volatile compounds. Almost the same amount of 1-octen-3-ol was found with Fukuyutaka and L-Star because it was formed independently on LOXs.[2,13]

Because the homogenate was heated to 80 °C in order to facilitate vaporization of volatiles during headspace sampling, it was anticipated that heat degradation of some nonvolatile compounds (such as lipid hydroperoxides) might result in overestimation of the amounts of volatiles. Because heat degradation of hydroperoxides can be largely prevented by the addition of lipophilic antioxidants, we evaluated the effects of the addition of butylated hydroxytoluene. When headspace GC analysis was performed in the presence of 1 mM butylated hydroxytoluene, little difference was found (data not shown). Hence, we concluded that the volatiles detected in this system were those mostly formed in the homogenate but not those formed during headspace analysis. With this sampling system, about 72 samples were routinely analyzed per day.

Selection of soybean varieties having modified volatile formation

We evaluated ability to form volatile compounds after homogenizing soybean seeds of 626 varieties collected worldwide. The first screening resulted in 29 candidates, then repeated analyses of these candidates confirmed four varieties, Laredo, Daizu B, BRS213, and Santa Maria, as the ones having unusual volatile-forming properties (Fig. 2).

The amounts of volatile compounds formed from homogenized soybean seeds of BRS213 were much lower than those found with a normal soybean variety, Fukuyutaka (Fig. 3). The profile was quite similar to that of L-Star, which had no LOX. Santa Maria formed C6 and C5 volatiles in amounts approximately half of those

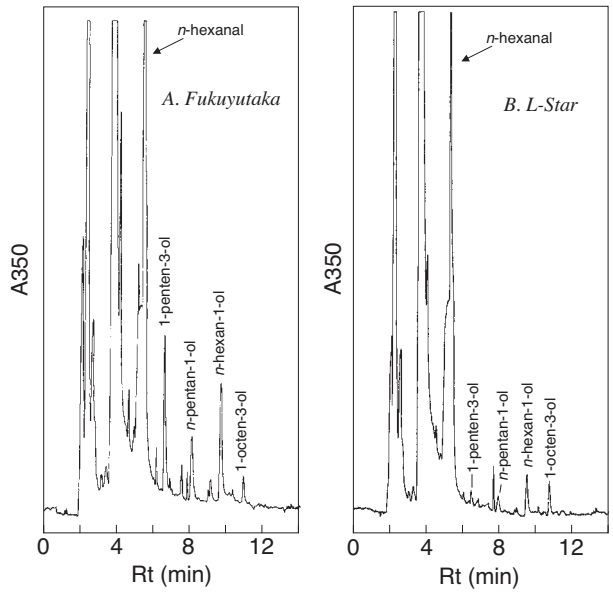

Fig. 1. Representative Chromatograms of Headspace GC Analyses.
Volatiles formed in the homogenates of Fukuyutaka (containing all the seed LOXs) and L-Star (containing no LOX) were analyzed by the headspace GC system.

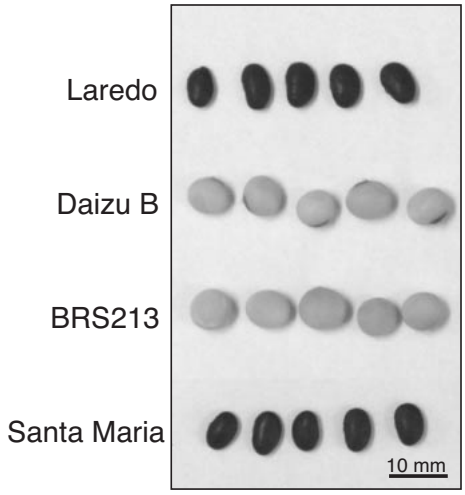

Fig. 2. Appearance of Soybean Seeds Selected by Screening.

found in Fukuyutaka; on the contrary, this variety formed 1-octen-3-ol in 3 times higher amounts than that found in Fukuyutaka. Laredo also showed volatile profiles similar to Santa Maria, even though it showed an intermediate profile between Santa Maria and Fukuyutaka. Daizu B showed volatile profiles similar to Fukuyutaka, but showed higher amounts of 1-penten-3-ol.

Activities of enzymes involved in volatile formation

n-Hexanal forming activity from linoleic acid 13-hydroperoxide (corresponding to HPL activity) or from linoleic acid (corresponding to a sum of LOX and HPL activities) was determined with the four selected soybean varieties (Fig. 4). As expected, *n*-hexanal formation from linoleic acid was lower in L-Star than in Fukuyutaka, but there was still significant activity forming *n*-hexanal in L-Star. This might be due to nonenzymatic oxygenation of linoleic acid and subsequent spontaneous degradation of the resulting hydro-

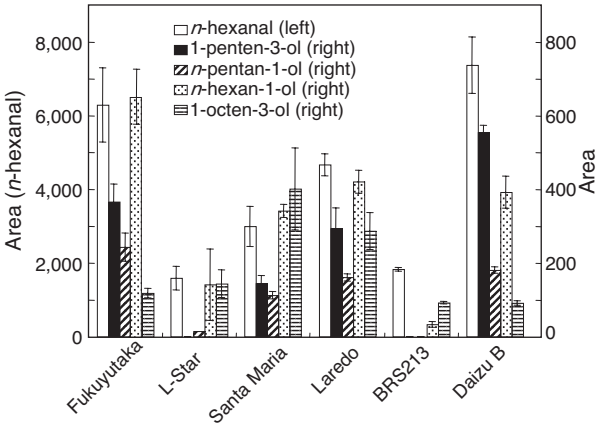

Fig. 3. Volatiles Detected in Soybean Seeds Selected by Screening.
Volatiles formed in the homogenates of soybean seeds selected by screening are shown with those from Fukuyutaka and L-Star. The left vertical bar is for *n*-hexanal, and the right one is for the other volatiles. The bars represent standard deviations (n = 3).

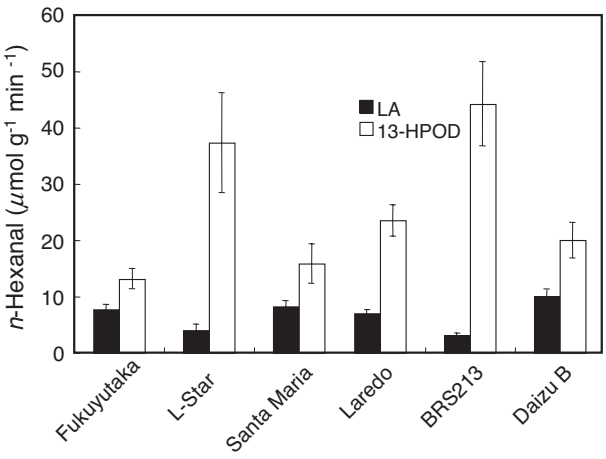

Fig. 4. *n*-Hexanal Forming Activity of Selected Soybean Seeds.
The amounts of *n*-hexanal formed from linoleic acid (LA, solid square) and from linoleic acid 13-hydroperoxide (13-HPOD, open square) are shown. The bars represent standard deviations (n = 3).

peroxides, as reported previously.[14] On the contrary, HPL activity in L-Star was much higher than that found in Fukuyutaka. This variety might have high HPL activity, but it has been found that LOX3 inhibits *n*-hexanal formation by converting linoleic acid hydroperoxide into forms unavailable to HPL catalysis.[15,16] Hence, it was assumed that HPL activity in Fukuyutaka and in the other soybean variety that had LOX3 was underestimated as long as crude soybean homogenate was used.

The activity profile found in BRS213 was similar to that in L-Star, that is, low activity from linoleic acid and high activity from its hydroperoxide as compared with Fukuyutaka. This result is consistent with the volatile profiles, and it can be assumed that the low amounts of C6 and C5 compounds found in the homogenate of BRS213 were largely due to little LOX activity, because the ability to form C6 and C5 volatiles in the soybean homogenate was mostly attributable to LOX activity, but only slightly to HPL activity.[10] The amounts of *n*-hexanal formed from linoleic acid in Santa Maria and Laredo were similar to Fukuyutaka, but HPL activity in

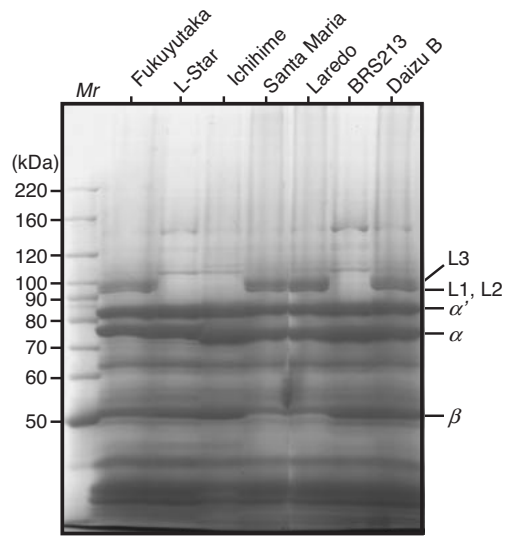

Fig. 5. Protein Profiles of Homogenates of Selected Soybean Seeds.

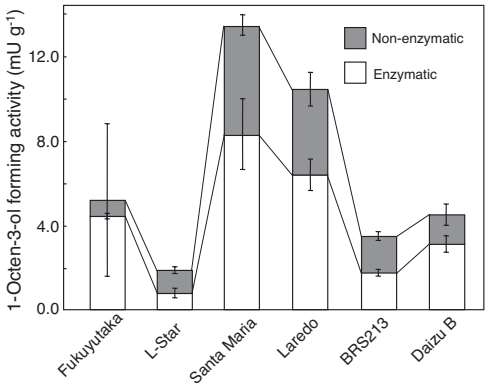

Fig. 6. 1-Octen-3-ol Forming Activity of Selected Soybean Seeds.
Activity was determined with and without heat denaturation of the homogenate, and the value after subtraction of that found with denatured homogenate (shown as non-enzymatic, gray) from that without denaturation is shown as enzymatic activity (open bar). The bars represent standard deviations (n = 3).

Laredo was significantly higher. In Daizu B, both activities were slightly higher than those found in Fukuyutaka.

We analyzed protein profiles using a specialized SDS–PAGE developed by Kitamura[5] in order to visualize LOX proteins. As expected, Fukuyutaka showed distinct bands at around 95 kDa, but L-Star and Ichihime (another LOX-less variety) showed no band around that region (Fig. 5). From this result, comparing the profile reported by Kitamura,[5] it is suggested that the protein bands around 95 kDa corresponded to LOX1, 2, and 3. When BRS213 was analyzed, it showed no band around the LOX region, and hence it is suggested that BRS213 is a LOX-less variety. Instead of the lack of LOX1, 2, and 3, BRS213 had distinct protein bands at 107 and 140 kDa. These two protein bands were also apparent in L-Star and Ichihime; on the contrary, Fukuyutaka showed only faint bands corresponding to these proteins. These proteins might be common among LOX-less soybeans even though their identities have not been determined. Santa Maria, Laredo, and Daizu B showed protein profile at around 95 kDa similar to Fukuyutaka, so, it can be concluded that these varieties contained all three LOXs. Among them, Santa Maria and Laredo showed lower amounts of the β-subunit of 7S globulin (β-conglycinin).

A preliminary experiment indicated that 1-octen-3-ol might be formed either enzymatically or nonenzymatically. Therefore, both the activities were evaluated. When Fukuyutaka homogenate was used as the enzyme source, 1-octen-3-ol was largely formed by enzymatic activity, and only one-eighth of total activity could be attributed to nonenzymatic formation (Fig. 6). Reducing reagents and transition metals in the seed homogenate might be involved in the nonenzymatic formation of 1-octen-3-ol from linoleic acid hydroperoxides.[17] Both the enzymatic and nonenzymatic activities forming 1-octen-3-ol were significantly higher in Santa Maria and Laredo than in Fukuyutaka. These high activities might account for the higher amounts of 1-octen-3-ol formed in the homogenates of these two varieties. It has been reported that 1-octen-3-ol was formed independent of LOX.[2,5] Recently, a patent was issued for the identification of a novel cytochrome P450 monooxygenase that catalyzes conversion of linoleic acid to 1-octen-3-ol.[18] It is still uncertain whether this monooxygenase was involved in the formation of the 1-octen-3-ol detected in soybean homogenate in this study. On the contrary, the activities found in BRS213 and Daizu B were similar to that found in Fukuyutaka. These profiles of 1-octen-3-ol forming activities agreed well with the amounts of the C8 compound in the seed homogenates, but disagreed with the amounts of 1-penten-3-ol, even though obvious similarities were found between this secondary alcohol and 1-octen-3-ol. The amounts of 1-penten-3-ol correlated to the LOX activities, *i.e.*, highest amount was found in Daizu B, which showed distinct bands of LOXs on the SDS–PAGE gel and high activity to form *n*-hexanal from linoleic acid. It has been reported that 1-penten-3-ol is formed through anaerobic side reaction of LOX1 and 2 from linolenic acid 13-hydroperoxide.[19] Therefore, the higher amounts of 1-penten-3-ol might be due to higher LOX1 and/or 2 activities or to modified catalytic activities of LOXs in Daizu B.

Conclusion

Soybean seeds are an important food material, and their chemical composition, including proteins, fats, amino acids, and flavor compounds, account for the quality of soybean food. Therefore, many studies to isolate and develop soybean varieties suitable for processing have been reported.[7,20,21] Among them, the isolation of soybean seeds that lack seed-specific LOX is a great achievement, and it has widened the use of soybeans as a food material worldwide, essentially because soymilk made from them showed preferable flavor properties.[3,4] Based on this success, one might expect to uncover another variety or mutant of soybean that has preferable flavor properties. Until now, the other enzymes involved in the formation of *n*-hexanal and *n*-hexanol, *e.g.*, HPL, lipid hydrolyzing enzyme, and alcohol dehydrogenase, have not been subjected to screening. Furthermore, it is known that a wide array of compounds other than *n*-hexanal and *n*-hexanol account for the flavor properties of soybean food.[7–9] Some of these flavor compounds are formed through an enzyme system independent of LOX. In this context, the

introduction of a new screening strategy is promising to uncover a novel variety or mutant of soybean that might be valuable for direct consumption or as a resource for further breeding. In this study, we developed a simple and efficient procedure to perform flavor analyses for screening soybean seeds. This system can extend the range of subjects of investigation as to the factors affecting the flavor properties of soybean varieties. A preliminary screening of 626 varieties using this screening system identified four soybean varieties that showed modified activities to form volatile compounds. One of them, BRS213, was found to be a LOX-less variety, but the others contained LOXs, and showed different volatile profiles. They might be useful in further breeding to develop varieties having favorable flavor properties. Also, this simple screening system can be widely used in further attempts to discover novel soybean varieties and mutant lines.

Acknowledgments

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