Metabolome profile of Negi-Nira chive, an interspecies hybrid of green spring onion (*Allium fistulosum*) and Chinese chive (*A. tuberosum*)

Takeshi Ara1,*,a, Kunihiro Suda1, Masayuki Amagai2,3,b, Kiyoshi Namai2,c, Hideyuki Suzuki1,d, Nozomu Sakurai1,e, Daisuke Shibata1,3

1Kazusa DNA Research Institute, 2-6-7 Kazusa Kamatari, Kisarazu, Chiba 292-0818, Japan; 2Tochigi Agricultural Experimental Station, 1080 Kawaraya, Utsunomiya, Tochigi 320-0002, Japan; 3Department of Biological Sciences, Kisarazu Campus of Tohoku University, 2-6-7 Kazusa Kamatari, Kisarazu, Chiba 292-0818, Japan
*E-mail: ara@rish.kyoto-u.ac.jp Tel: +81-774-38-3653 Fax: +81-774-38-3682

Received January 22, 2020; accepted June 5, 2020 (Edited by S. Takahashi)

**Abstract** Metabolome analysis of flavored vegetables, green spring onion (*A. fistulosum*), Chinese chive (*A. tuberosum*), and their interspecies hybrid Negi-Nira chive, was conducted using liquid chromatography-Fourier transform ion cyclotron resonance-mass spectrometry, with ca. 2 ppm mass accuracy. Ion peaks in the chromatograms of four biological replicates of the vegetable leaves were processed using the alignment software PowerGet for metabolite comparison, from which we obtained the potential chemical formulae. In total, 860 ion peaks were reproducibly detected; of these, 506, 525, and 336 peaks were found in the hybrid, *A. tuberosum*, and *A. fistulosum*, respectively. There were 130 peaks specific to the hybrid; from these, 31 metabolites were annotated by searching compound databases. The sulfur-containing compounds and flavonoids were further analyzed using bioinformatics, to examine the sulfur metabolism of *Allium* volatiles and the flavonoid pathways in these species. In conclusion, our metabolome analysis of this interspecies hybrid and its parents provides a unique opportunity to elucidate their metabolic background.

**Key words:** Fourier transform ion cyclotron resonance-mass spectrometry, interspecies hybrid, metabolome.

The genus *Allium* includes economically important vegetables such as onions, garlic, leeks, and chives, which possess characteristic aromas, and which are cultivated extensively worldwide. Green spring onion (*A. fistulosum*) and Chinese chive (*A. tuberosum*), known as "Negi" and "Nira" in Japanese, have a long history of cultivation as flavored vegetables in Japan. Various cultivars of these vegetables are used in Japanese cuisine. Their aromatic compounds, especially sulfides, have been well studied (Yoshimoto and Saito 2019).

Interspecies crossing of *A. fistulosum* and *A. tuberosum* was achieved in 1991 at Tochigi Agricultural Experimental Station (Tochigi, Japan) (Amagai et al. 1995, in Japanese): when *A. tuberosum* was pollinated using *A. fistulosum* pollen, the resulting seeds contained genetic material only from the *A. tuberosum* parent, due to apomixis (Kojima et al. 1991; Nakazawa et al. 2006). However, *A. fistulosum* pollinated by *A. tuberosum* produced no seeds (Amagai et al. 1995): the embryos from 1,276 ovules of these *A. fistulosum* specimens were cultivated under sterile conditions on callus induction medium, and the calluses were placed on regeneration medium: two calluses obtained from pollination of *A. fistulosum* ‘Nissato’ using pollen from *A. tuberosum* ‘Kinumidori’ (an F1 hybrid cultivar) regenerated whole plants, one of which grew normally. This plant had 24 chromosomes, while the *A. tuberosum* ‘Kinumidori’ cultivar has 32 chromosomes (tetraploid), and the *A. fistulosum* cultivar has 16 (diploid); further, its esterase isozymes exhibited a mixed pattern of the isozymes of its parents (Amagai et al. 1995), thereby confirming that this plant, named “Negi-Nira chive” as *A. fistulosum*×*A. tuberosum*, was an interspecies hybrid between its parent cultivars. The overall morphological phenotype of the

**Abbreviations:** GC-MS, gas chromatography-mass spectrometry; LC-FT/ICR-MS, liquid chromatography-Fourier transform ion cyclotron resonance-mass spectrometry.

*a* Present address: Research Institute for Sustainable Humanosphere, Kyoto University, Gokasho, Uji, Kyoto 611-0011, Japan

*b* Present address: Department of Agriculture, Tochigi Prefectural Government, 1-1-20, Hanawada, Utsunomiya, Tochigi 320-8501, Japan

*c* Present address: Tochigi Agricultural College, 1145-1 Kamikomoriya, Utsunomiya, Tochigi 321-3233, Japan

*d* Present address: Department of Research and Development, Hirata Corporation, 111 Hitotsugi, Ueki, Kumamoto, Kumamoto 861-0198, Japan

*e* Present address: Bioinformation and DDBJ Center, National Institute of Genetics, 1111 Yata, Mishima, Shizuoka 411-8540, Japan

This article can be found at http://www.jspcmb.jp/ Published online September 17, 2020

Copyright © 2020 Japanese Society for Plant Biotechnology
hybrid was more similar to that of the paternal parent *A. tuberosum*, although with the hollow leaf cross section that is characteristic of *A. fistulosum* (Figure 1). The hybrid was registered as a new type of “Nira” cultivar, and was named ‘Nakamidori,’ in accordance with the plant variety protection system of Japan. The cultivar is sterile, and has been propagated vegetatively for commercial purposes, mainly by farmers of Tochigi Prefecture (Japan). This hybrid has a garlic-like flavor, unlike its parental cultivars.

To compare the aromatic composition of the interspecies hybrid and its parental cultivars, Kobayashi et al. (1997) analyzed the leaf volatiles using gas chromatography-mass spectrometry (GC-MS): the chromatographic intensities of diallyl disulfide and other sulfur-containing compounds were much higher for the hybrid than for its parents, which may explain the garlic-like aroma in the hybrid. There were 41 volatiles, including five unknown chemical peaks; 11 volatiles were common to the hybrid and parental cultivars; 25 were specific to the hybrid and *A. tuberosum*; and four were specific to the hybrid and *A. fistulosum*. Methyl (Z)-1-propenyl disulfide was specific to the hybrid; this suggests that the mixing of the genomes of the parents produced sulfur metabolism in the hybrid that was different from that in the parents.

To gain an insight into the metabolism of the hybrid in comparison with those of its parents, we conducted metabolome analysis using liquid chromatography coupled with FT/ICR mass spectrometry (LC-MS) and bioinformatics. The cultivars *A. fistulosum* ‘Nissato,’ *A. tuberosum* ‘Kinumidori’ and *A. fistulosum*×*A. tuberosum* ‘Nakamidori’ were grown in a greenhouse of Tochigi Agricultural Experimental Station (Figure 1). Mature leaves were harvested (four biological replicates per three cultivars). The ion peaks of the parents occurred in the chromatogram of the hybrid (Figure 2). For the hybrid-specific ion peaks, the gas chromatograms of this hybrid seems unlikely that the relative intensities of the ions occurring in the hybrid reflect those of its parents when they were comparable ions. However, the chromatogram of the hybrid generally resembles that of the paternal *A. tuberosum*, rather than that of *A. fistulosum*. Consistent with this finding, the gas chromatograms of this hybrid and *A. tuberosum* were similar (Kobayashi et al. 1997). These results reflect that the metabolism of the hybrid is influenced by complex interactions between the parents’ genomes.

Twelve chromatograms were generated (from the four biological replicates for three cultivars). The ion peaks were extracted and aligned using ion information with the *m/z* value and retention time of each ion by the software PowerGet (Sakurai et al. 2014). A possible chemical formula (or formulae) for each ion was calculated from its *m/z* value. Ions that occurred reproducibly in all four replicates in any of the cultivars were analyzed further. In total, 860 reliable ion peaks were selected, and are depicted in a Venn diagram: 506, 525, and 336 ions occurred in the hybrid, *A. tuberosum*, and *A. fistulosum*, respectively; 130 ions were specific to the hybrid (Figure 3). For the hybrid-specific ion peaks, we then used the calculated mass values to search the compound databases, KEGG (https://www.genome.jp/
kegg/), KNAPSacK (http://www.knapsackfamily.com/KNAPSacK/), LIPIDMAPS (https://www.lipidmaps.org/), and HMDB (http://www.hmdb.ca/) yielded 31 matching metabolites, which belonged to metabolic categories including aminocarboxylic acids, fatty acid derivatives, flavonoids, iridoids, phenolics, and steroids (Table 1). Although none of the predicted molecules was identified by using authentic chemicals on the same chromatographic conditions, this metabolomic profile information helps to elucidate the metabolism of Allium species. The datasets analyzed here are publicly available at Metabolonote (http://metabolonote.kazusa.or.jp/SE43:).

As sulfuric volatiles characterize Allium flavors (Kusano et al. 2016), we searched for sulfur-containing compounds among the predicted formulae, and annotated 18 sulfides, 19 disulfides, and 6 trisulfides; of these, 24 ion peaks were confirmed as $^{34}$S isotope-containing compounds, using the software MassChroViewer (Table 2) (Sakurai and Shibata 2017). Most of the predicted sulfuric compounds had $m/z$ values of less than 240; some of these compounds might relate to flavor production. There were also two large disulfides (C$_{26}$H$_{45}$NO$_{8}$S$_{2}$ and C$_{56}$H$_{84}$O$_{23}$S$_{2}$).

There were three isomers of C$_{6}$H$_{10}$OS$_{2}$ with distinct retention times on the chromatogram; one of these might be the main constituent of the flavor of garlic, namely allicin. C$_{6}$H$_{11}$NO$_{3}$S, which occurred in all three cultivars, was annotated as alliin, a precursor to allicin, although the possibility that it indicated the presence of S-acetylcysteine or other compounds cannot be excluded without further targeted chemical analysis. C$_{14}$H$_{23}$N$_{3}$O$_{8}$S occurred in all three cultivars; it was annotated as S-(2-Carboxypropyl)glutathione, a precursor peptide of the sulfur-containing flavor molecules. C$_{6}$H$_{11}$NO$_{2}$S$_{2}$, which was specific to the hybrid and A. fistulosum, was annotated as S-(Allylthio)-L-cysteine, a sulfur-containing amino acid. Future analysis targeting these compounds will clarify their chemical structures.
Metabolome profile of an interspecies hybrid Negi-Nira chive (A. fistulosum × A. tuberosum).

| No. | Retention time (min) | Detected m/z | Adducts | Chemical category | Annotation                      |
|-----|----------------------|--------------|---------|-------------------|---------------------------------|
| 1   | 6.5                  | 231.134      | [M+H]^+ | Aminocarboxylic acids | Peptide C_{10}H_{16}N_{2}O_{2} |
| 2   | 17.9                 | 233.15       | [M+H]^+ | Aminocarboxylic acids | Peptide C_{10}H_{16}N_{2}O_{2} |
| 3   | 33.7                 | 330.264      | [M+H]^+ | Fatty acid derivatives | Fatty acid derivative C_{6}H_{14}N_{2}O_{4} |
| 4   | 34.4                 | 348.274      | [M+H]^+ | Fatty acid derivatives | Fatty acid derivative C_{6}H_{14}N_{2}O_{4} |
| 5   | 39.1                 | 227.128      | [M+H]^+ | Fatty acid derivatives | Fatty acid derivative C_{6}H_{14}N_{2}O_{4} |
| 6   | 15.8                 | 873.204      | [M+H]^+ | Flavonoids         | Flavonoid (trimmer) C_{9}H_{16}O_{2} |
| 7   | 17.3                 | 1,111.312    | [M+H]^+ | Flavonoids         | Flavonoid (+4Hex) C_{9}H_{16}O_{2} |
| 8   | 17.8                 | 771.198      | [M+H]^+ | Flavonoids         | Flavonoid (+2 or 3Hex) C_{9}H_{16}O_{2} |
| 9   | 17.9                 | 757.218      | [M+H]^+ | Flavonoids         | Flavonoid (+3Hex) C_{9}H_{16}O_{2} |
| 10  | 18.8                 | 697.161      | [M+H]^+ | Flavonoids         | Flavonoid (+2Hex) C_{9}H_{16}O_{2} |
| 11  | 19.4                 | 757.219      | [M+H]^+ | Flavonoids         | Flavonoid (+3Hex) C_{9}H_{16}O_{2} |
| 12  | 22.3                 | 595.166      | [M+H]^+ | Flavonoids         | Flavonoid (+2Hex) C_{9}H_{16}O_{2} |
| 13  | 23.2                 | 625.176      | [M+H]^+ | Flavonoids         | Flavonoid (+2Hex) C_{9}H_{16}O_{2} |
| 14  | 24.6                 | 757.198      | [M+H]^+ | Flavonoids         | Flavonoid (+2Hex) C_{9}H_{16}O_{2} |
| 15  | 24.9                 | 787.208      | [M+H]^+ | Flavonoids         | Flavonoid (+2Hex) C_{9}H_{16}O_{2} |
| 16  | 25.6                 | 609.181      | [M+H]^+ | Flavonoids         | Flavonoid (+2Hex) C_{9}H_{16}O_{2} |
| 17  | 27.9                 | 595.145      | [M+H]^+ | Flavonoids         | Flavonoid (+0 or 1Hex or dimer) C_{9}H_{16}O_{2} |
| 18  | 28.2                 | 625.155      | [M+H]^+ | Flavonoids         | Flavonoid (+1Hex) C_{9}H_{16}O_{2} |
| 19  | 17.8                 | 421.17       | [M+H]^+ | Iridoids           | Iridoid (+1Hex) C_{6}H_{12}O_{4} |
| 20  | 20.4                 | 391.16       | [M+H]^+ | Iridoids           | Iridoid (+1Hex) C_{6}H_{12}O_{4} |
| 21  | 19.1                 | 558.255      | [M+H]^+ | Phenolics           | Phenolic C_{6}H_{12}O_{4} |
| 22  | 22.2                 | 269.102      | [M+H]^+ | Phenolics           | Phenolic C_{6}H_{12}O_{4} |
| 23  | 22.2                 | 431.155      | [M+H]^+ | Phenolics           | Phenolic (+1 or 2Hex) C_{9}H_{16}O_{2} |
| 24  | 22.6                 | 443.155      | [M+H]^+ | Phenolics           | Phenolic (+1Hex) C_{9}H_{16}O_{2} |
| 25  | 24.2                 | 627.244      | [M+H]^+ | Phenolics           | Phenolic C_{9}H_{16}O_{2} |
| 26  | 26.6                 | 503.212      | [M+H]^+ | Phenolics           | Phenolic (+2Hex) C_{9}H_{16}O_{2} |
| 27  | 33.6                 | 269.102      | [M+H]^+ | Phenolics           | Phenolic C_{9}H_{16}O_{2} |
| 28  | 35.8                 | 303.123      | [M+H]^+ | Phenolics           | Phenolic C_{9}H_{16}O_{2} |
| 29  | 36.9                 | 253.107      | [M+H]^+ | Phenolics           | Phenolic C_{9}H_{16}O_{2} |
| 30  | 41.3                 | 273.112      | [M+H]^+ | Phenolics           | Phenolic C_{9}H_{16}O_{2} |
| 31  | 33.7                 | 769.4        | [M+H]^+ | Steroids           | Steroid (+3Hex) C_{9}H_{16}O_{2} |

Abbreviations: Hex: hexose.

Table 2. The list of predicted sulfur-containing compounds in the three vegetables.

| Detected vegetables | Formula of predicted sulfur-containing compounds |
|---------------------|-----------------------------------------------|
| A. tuberosum        | C_{6}H_{12}N_{2}O_{2}S_{6}, C_{6}H_{14}O_{2}S_{6}^* |
| A. fistulosum × A. tuberosum | C_{6}H_{14}NO_{5}S_{6}, C_{6}H_{14}N_{2}O_{5}S_{6}^*, C_{6}H_{14}N_{2}O_{5}S_{6}, C_{6}H_{16}NO_{5}S, C_{6}H_{16}N_{2}O_{6}S |
| A. fistulosum       | C_{6}H_{14}NO_{5}S_{4}, C_{6}H_{14}O_{5}S_{4}, C_{6}H_{16}NO_{5}S_{4}, C_{6}H_{16}N_{2}O_{6}S, C_{6}H_{16}N_{2}O_{6}S, C_{6}H_{18}N_{2}O_{6}S, C_{6}H_{18}N_{2}O_{6}S |
| A. fistulosum and A. fistulosum × A. tuberosum | C_{6}H_{14}NO_{5}S_{4}, C_{6}H_{14}O_{5}S_{4}, C_{6}H_{16}NO_{5}S_{4}, C_{6}H_{16}N_{2}O_{6}S, C_{6}H_{16}N_{2}O_{6}S, C_{6}H_{18}N_{2}O_{6}S, C_{6}H_{18}N_{2}O_{6}S, C_{6}H_{18}N_{2}O_{6}S, C_{6}H_{18}N_{2}O_{6}S, C_{6}H_{20}N_{2}O_{6}S, C_{6}H_{20}N_{2}O_{6}S, C_{6}H_{20}N_{2}O_{6}S, C_{6}H_{20}N_{2}O_{6}S |

The same formula in the table means the isomers that have distinct retention times on the chromatography. * 3S peak was confirmed by the software MassChromViewer.

We were able to annotate five sulfides, including two isomers of C_{6}H_{12}N_{2}O_{2}S_{6} and C_{6}H_{14}NO_{5}S_{6}, C_{6}H_{14}N_{2}O_{5}S_{6}, and C_{12}H_{20}N_{2}O_{8}S_{6}, which were specific to the hybrid. Methyl (Z)-1-propenyl disulfide (C_{4}H_{6}S_{2}, M.W. 120.0067), which was found only in the hybrid when analyzed by GC-MS (Kobayashi et al. 1997), was not found in this study, probably due to the low sensitivity of FT/ICR mass spectrometry at the mass range of ca. 100 to 150 m/z. Interestingly, we annotated two molecules with the formula C_{6}H_{14}OS_{3}; this is also the formula of ajoene, an antioxidant found in garlic (Naznin et al. 2010). These annotations of sulfides provide potential avenues for further study of the flavor of this hybrid.

We compared the distribution of the compounds annotated as flavonoids in the three cultivars (Supplementary Table S1). Interestingly, only C_{22}H_{26}O_{6}S was common among the three cultivars. Three were common between the hybrid and A. fistulosum, and four between the hybrid and A. tuberosum. Thirteen were specific to the hybrid, suggesting that the genomic mixing results in significant alternation of flavonoid synthesis. Targeted analyses of flavonoids, using MS/
MS/MS analysis, for example, will provide greater clarity about the effects of genomic mixing on flavonoid pathways in *Allium*.

This study provided a comparative metabolome profile of the interspecies hybrid and its parents. Although the chemical structure of each molecule cannot be described from the metabolome profiles that we present here, they nonetheless provide a basis for hypotheses about the potential metabolomic differences that can occur in hybrid genomes. In particular, knowing which molecules are found in the parents but not in the hybrid makes it possible to hypothesize about the origins of the molecules that are specific to the hybrid. Genomic and transcriptomic information about interspecies hybrids and their parents will stimulate future research in this regard.

**Acknowledgements**

This work was supported by a grant from the Kazusa DNA Research Institute Foundation.

**Accession numbers**

The raw data were deposited in the MassBase metabolome database (http://webs2.kazusa.or.jp/massbase/; accession numbers MDLC1_32601-32607 and 32611-32618), and are publicly available.

**References**

Amagai M, Ohashi K, Kimura S, Oguri N, Kojima A (1995) Breeding of interspecies hybrid between *Allium fistulosum* and *A. tuberosum* by embryo culture. *Bull Tochigi Agr Exp Stn* 43: 87–94 (in Japanese)

Ara T, Enomoto M, Arita M, Ikeda C, Kera K, Yamada M, Nishioka T, Ikeda T, Nihei Y, Shibata D, et al. (2015) Metabolonote: A wiki-based database for managing hierarchical metadata of metabolome analyses. *Front Bioeng Biotechnol* 3: 38

Iijima Y, Nakamura Y, Ogata Y, Tanaka K, Sakurai N, Suda K, Suzuki T, Suzuki H, Okazaki K, Kitayama M, et al. (2008) Metabolite annotations based on the integration of mass spectral information. *Plant J* 54: 949–962

Kobayashi A, Amagai M, Kubota K, Morisawa C (1997) Aroma composition of an interspecies hybrid cultivar named “Nakamidori” between *Allium fistulosum* and *A. tuberosum* by Embryo Culture. *Nippon Nogeikagaku Kaishi* 71: 1273–1279 (in Japanese)

Kojima A, Nagato Y, Hinata K (1991) Degree of apomixis in Chinese chive (*Allium tuberosum*) estimated by esterase isozyme analysis. *Japan J Breed* 41: 73–83

Kusano M, Kobayashi M, Iizuka Y, Fukushima A, Saito K (2016) Unbiased profiling of volatile organic compounds in the headspace of *Allium* plants using an in-tube extraction device. *BMC Res Notes* 9: 133

Nakazawa Y, Namai K, Kojima A, Kobayashi T, Tasaki K, Amagai M (2006) Estimating the inheritance for parthenogenesis in tetraploid Chinese Leek (*Allium ramosum*, syn. *A. tuberosum*). *Ikushugaku Kenkyu* 8: 89–98 (in Japanese)

Naznin MT, Maeda T, Morita N (2010) Antioxidant functions of E- AND Z-Ajoene derived from Japanese garlic. *Int J Food Prop* 13: 821–829

Sakurai N, Ara T, Enomoto M, Motegi T, Morishita Y, Kurabayashi A, Iijima Y, Ogata Y, Nakajima D, Suzuki H, et al. (2014) Tools and databases of the KOMICS web portal for preprocessing, mining, and dissemination of metabolomics data. *BioMed Res Int* 2014: 1–11

Sakurai N, Shibata D (2017) Tools and databases for an integrated metabolite annotation environment for liquid chromatography-mass spectrometry-based untargeted metabolomics. *Carotenoid Science* 22: 16–22

Yoshimoto N, Saito K (2019) S-Alk(en)ylcysteine sulfoxides in the genus *Allium*: Proposed biosynthesis, chemical conversion, and bioactivities. *J Exp Bot* 70: 4124–4138