Title of research project	Elucidation of Safety Assessment Points for the Foods Obtained from the Transgene-free, Phenotype-transformed Plants
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Abstract/Summary

Many genetically modified (GM) transgenic crops harboring foreign genes with useful traits, such as herbicide tolerance and insect-resistant traits, have been developed, and a wide variety of foods obtained from GM plants is eaten worldly. Risk assessments of GM foods have been conducted in accordance with guidelines published in each country. New Plant Breeding Technologies (NPBTs) are now under development. With NPBTs, transgenic technology is used throughout the process of food production to utilize the useful traits of transgenes. However, foods produced from crops under NPBTs do not contain any transgene sequences. Grafting is a traditional technique, but the grafting using GM and non-GM plants is expected to be a promising technique as one of the NPBTs. Many molecular and physiological research studies have been done on improving agronomic traits and increasing yields through grafting. However, there are few studies on the risk evaluation of grafting technique by using GM plants. Under the current safety assessment guidelines for GM foods, such foods can be recognized as conventional (non-genetically modified) foods. However, the traits of transgene-free foods obtained from grafted plants could be influenced by grafting with GM plants. The aim of this research is to assess the safety of foods obtained from the transgene-free plant body that had been grafted with GM plants.

The point of evaluation is the mobile substances produced in the GM plant body in the grafted plants. RNA molecules, protein, and metabolites are the potentially transported substances from GM plant body to the transgene-free grafted plant parts, and these mobile substances may give unintended influences on the grafted plants. For the current study, the effect of such mobile factors were investigated by using multi-omics analyses.

(1) The transport of siRNA molecules.

RNA molecules at lengths of 21 ~ 24 nt are called siRNAs and play facilitating roles to mediate RNA-directed DNA methylation (RdDM). Transmission of siRNAs from a rootstock to a scion has been observed as well as the reverse direction transmission. A transgenic tobaccoline, end2, produces siRNAs that target the Cauliflower mosaic virus 35S promoter (CaMV 35S promoter) sequences. The LUC line is a transformed tobacco plant harboring the firefly

luciferase gene under the control of CaMV 35S promoter. We produced a chimera plant by grafting the LUC scion onto the end2 rootstock. The methylation of the CaMV 35S promoter region was weakly detected in the LUC scion. Then, the wild-type tobacco scion was grafted onto the end2 rootstocks, and the leaf tissues of the scion cultivated for 4 to 6 weeks after grafting were subjected to the multi-omics analyses. Analyses of transcriptome, proteome, and metabolome showed that there were no essential differences between the scions grafted onto end2 rootstocks and the scions grafted onto non-GM rootstocks. Therefore, even though siRNA molecules were transported from GM rootstocks to non-GM scions, the possible risks of such grafted plants were expected to be very limited.

(2) Grafting with a GM plant, in which the transgene function is independent from the host plant metabolism.

The useful traits of GM crops, such as herbicide tolerant and insect-resistant traits, have been commercialized, and the functions of most of the transgenes for such useful traits are independent of plant metabolism. As a model case, we produced a grafted tomato plant that consists of a GM rootstock with a *GUS* transgene and a non-GM scion. The *GUS* gene encodes a reporter protein, β-glucuronidase, and is independent of plant metabolism. The Micro-Tom tomato plants transformed with the *GUS* transgene were used as a rootstock and an edible tomato cultivar, Stella Mini Tomato, was used as scion. The tomato fruits on the scion were used for detection of alteration of transcriptomic, proteomic, and metabolomic traits with food ingredients. The resultant data showed no significant differences in the fruits of Stella scions obtained from the grafting with GM and non-GM Micro-Tom rootstocks, indicating no apparent risk for food safety.

(3) Grafting with a GM plant, in which the transgene encoding a mobile protein, FloweringLocus T (FT), has been introduced.

There are only a few proteins that are clearly demonstrated to move over long distance in plants. One of such mobile protein is FT, which and whose orthologs are known as a long-distance signaling component, florigen. Anorthologgene of FT, SP6A, was introduced into the potato plant and the resultant GM strain (SP6Aox#7) was grafted onto non-GM rootstock potato as scion. The potato tubers on the rootstocks were subjected to the multi-omics analyses and determination of food ingredient levels. The resultant data showed no significant differences in the potato tubers, suggesting no apparent risk for food use. The SP6A protein itself is expected to be transported into potato tubers from the GM scion. Therefore, safety assessment of such mobile protein would be required from the perspective of the allergenicity and toxicity.

(4) Possible long-distance movement of proteins.

Here, we tried to clarify whether proteins other than the FT proteins could move across the graft junction. For this purpose, a grafted tobacco plant was produced by using the LUC transgenic plants as a rootstock and the non-GM tobacco plants as a scion. The estimated molecular weight of the LUC protein is approximately 62 kDa. Interestingly the LUC activity was detected in the scion's plant body, especially in the petioles approximately 4 to 6 weeks after grafting. Then we next produced a chimera plants by grafting cucumber (Cucurbitaceae) scion onto the tobacco (Solanaceae) rootstocks. By the proteome analysis of the cucumber leaf proteins on the scion, a total of 1096 peptides were annotated and 16 proteins were identified as tobacco proteins not cucumber proteins. In the cucumber petioles, a total of 1607 peptides were annotated and 27 tobacco proteins were detected. The range of molecular weights of these tobacco proteins was mainly 30 to 60 kDa, and a tobacco protein with 100 kDa or more was also detected in the cucumber scion.

Conclusion.

The grafting with GM plants can confer the useful traits such as a stress- and insect-resistance to the rootstocks, and foods can be obtained from the transgene-free scion that have a superior trait for food use. We here investigated the effects of grafting with GM plants on the non-GM plant parts by using multi-omics analyses. When the transgenes encode proteins that are independent of plant metabolism, the tomato fruits on the non-GM scion and potato tubers on the non-GM rootstock showed no apparent risks for food use. However, attention should be paid to the movement of transgene-encoded proteins through the graft junction in the light of allergenicity and toxicity. Further detailed investigation is required to understand the long-distance movement of transgene-encoded proteins. In addition, when the transgene products interact with plant metabolism, the effects of transport of low-molecular-weight metabolites into foods cannot be ruled out from moving into transgene-free foods. Assessment points of the latter case of grafting remains to be clarified.

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1. List of papers published on the basis of this research

   Kodama, H., Miyahara, T., Oguchi, T., Tsujimoto, T., Ozeki, Y., Ogawa, T., Yamaguchi, Y., Ohta, D. (2021) Effect of transgenic rootstock grafting on the omics profiles in tomato. **Food Safety** (in press).

2. List of presentations based on this research

   1) Study for the transgene influences in leaves of non-GM tobacco scion grafting on GM tobacco rootstock. Miyahara T., Umeyama Y., Tsujimoto T., Ozeki Y., Ogawa T., Yamaguchi Y., Ohta D., Oguchi T., Kodama H. The 26th Annual Meeting of the Japanese Society of Food Chemistry.
   2) The metabolic analysis of the tomato fruit of non-GM scion grafting on the GUSgene transformed tomato rootstock. Ogawa T., Yamaguchi Y., Oguchi T., Miyahara T., Tsujimoto T., Ozeki Y., Kodama H., Ohta D. The 26th Annual Meeting of the Japanese Society of Food Chemistry.
   3) The research for the influences on the tobacco scion by the siRNAs targeting promoter sequences expressing in the grafting tobacco rootstock. Umeyama Y., Miyahara T., Oguchi T., Ohta D., Ozeki Y., Kodama H. 2020 Annual Meeting of Japan Society for Bioscience, Biotechnology, and Agrochemistry Kanto Branch

3. The number and summary of patents and patent applications:

   None.

4. Others (awards, press releases, software and database construction):

   None.