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Note

Radish sprouts as an efficient and rapidly available host for an agroinfiltration-based transient gene expression system

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Abstract Agroinfiltration, the infiltration of plants with *Agrobacterium* harboring a plasmid that contains a specific gene, is used to transiently express a gene in a heterologous organism. Using the "Tsukuba system", greater amounts of target protein accumulate compared with usual expression plasmids. Reported host plants, including *Nicotiana benthamiana*, a common plant for agroinfiltration, need several weeks after sowing to grow enough for infection. To shorten the culture period and, thereby, improve target protein production, we tested sprouts as host plants. Sprouts were grown in the dark to encourage elongation so that vacuum infiltration becomes easier, and this was followed by a few days of exposure to illumination before infection with pBYR2HS-EGFP, the EGFP expression plasmid of the Tsukuba system. Among six tested species of *Fabaceae* and *Brassicaceae*, radish showed the highest transient expression. Among six tested radish cultivars, Kaiware, Hakata, and Banryoku provided the best results. Culturing for 5 day, including 1 day of imbibition and 1 to 2 day of exposure to illumination resulted in EGFP fluorescence in 80% of the cotyledon area. Thus, a remarkable amount of EGFP was obtained only 8 day after seed imbibition. The EGFP amount in Kaiware cotyledons was comparable with Rubisco at ~0.7 mg/g fresh weight. Kaiware sold in supermarkets could also be used, but resulted in lower expression levels.

Key words: agroinfiltration, *Raphanus sativus*, seedling, vacuum infiltration.

Agroinfiltration, the infiltration of plants with *Agrobacterium* harboring a plasmid that contains a specific gene, is used to transiently express a gene. Using the new "Tsukuba system", developed by Yamamoto et al. (2018) at the University of Tsukuba, a protein of interest accumulates at a remarkably high level, compared with that produced by the usual expression plasmids, owing to the new plasmid's construct, in which tandemly arranged double terminators are integrated downstream of the gene of interest and a geminiviral replication system-related DNA fragment is present. The latter enables the amplification of the introduced gene of interest in the host cells. Using the Tsukuba system, when pBYR2HS-EGFP (Yamamoto et al. 2018) was introduced as the expression plasmid, the amount of EGFP reached ~4 mg g^{-1} fresh weight in *Nicotiana benthamiana* leaves.

In addition to *N. benthamiana*, which is widely used for agroinfiltration because of the resulting high levels of gene expression and ease of handling during infiltration, the Tsukuba system is applicable in other plants, including tomato, eggplant, lettuce, hot pepper, melon, and orchid (Hoshikawa et al. 2019; Yamamoto et al. 2018), as well as some *Fabaceae* plants (*Lotus japonicus*, *Glycine max*, and *Phaseolus vulgaris*; Suzaki et al. 2019), although the accumulation levels of the target proteins were quite variable depending on the host species, variety, and organ.

In contrast to these host plants that required several weeks of cultivation for agroinfiltration after sowing seeds, the production of amounts of proteins comparable with those produced in *N. benthamiana* in inexpensive sprouts would remarkably improve the productivity of useful proteins, in terms of energy cost, and the production amount per unit time and per unit area. In this report, to broaden the utilization of this protein production system in plants, we tested the usability of sprouts as a host in the Tsukuba system.

To determine which species' sprouts were the most useful, three *Fabaceae* [pea (*Pisum sativum*), mung bean (*Vigna radiate*), and alfalfa (*Medicago sativa*)] and three *Brassicaceae* [rape (*Brassica napus* cv. Kizakino), radish (*Raphanus sativus* cv. Kaiware), and broccoli (*B. oleracea* var. *italicam*)] were tested. Their seeds are commercially available at low prices, and their sprouts are suitable

Abbreviations: EGFP, enhanced green fluorescent protein.
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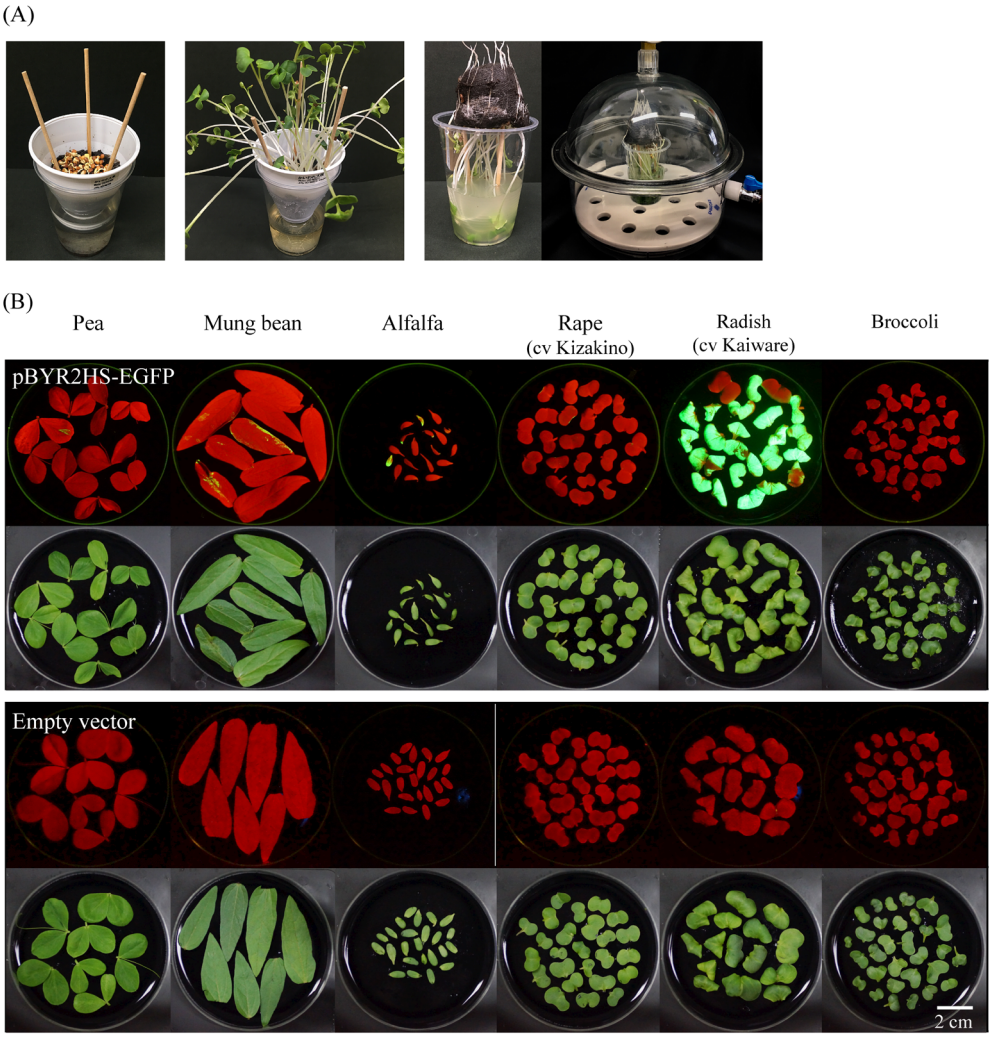

Figure 1. EGFP expression levels in sprouts of *Fabaceae* and *Brassicaceae*. A, radish sprouts used for the agroinfiltration. From the left, seeds after 1 day of imbibition, sprouts after 5 day of cultivation, including of 1 day of imbibition and 1 day of illumination, and sprouts dipped in a suspension of *Agrobacterium* for vacuum infiltration. B, cotyledons or leaves of sprouts 3 day after infection with *Agrobacterium* harboring pBYR2HS-EGFP or the empty vector. Upper panel: EGFP (green) and chlorophyll (red) fluorescence; lower panel: white-light image.

for vacuum-infiltrating and harvesting, because the cotyledons or leaves of the sprouts are larger than those of many other plant species and the hypocotyls can elongate to several cm (with some reaching greater than 10 cm) when grown in the dark. The seeds were imbibed for 1 day in water and put on Jiffy-7 pellets (φ42 mm, Sakata Seed Co., Yokohama, Japan) that absorbed 2 g l^{-1} of Powder Hyponex (Hyponex Japan Co., Osaka, Japan) solution (Figure 1A, left). Then, they were cultivated for 4 day in the dark, followed by 1 day of illumination with a white fluorescent lamp (~1,500 lx) for the purpose of greening (Figure 1A, middle). During cultivation, the hydroponic solution was supplied from underneath the pellets. *Agrobacterium tumefaciens* strain GV3101::pMP90, having the expression plasmid pBYR2HS-EGFP, was cultured and resuspended in the infiltration buffer to adjust OD_{600}=1.0, as described by Yamamoto et al. 2018. The cotyledons or leaves of sprouts were dipped, upside down, in the *Agrobacterium* resuspension and infiltrated by vacuuming four times for 5 min at ~−0.1 MPa (Figure 1A, right). Sprouts were further cultivated in the dark for 1 day, followed by illumination for 2 day. Sprouts were maintained at 25°C during cultivation. EGFP fluorescence was captured using a SC52 filter (Fujifilm, Tokyo, Japan) under excitation by a blue LED (472 nm). Figure 1B shows EGFP fluorescence from the first or second leaves of the three *Fabaceae* and the cotyledons of the three *Brassicaceae* plants. The EGFP fluorescence was observed in widest areas of the radish cotyledons, while it was observed in only small areas of the three *Fabaceae* leaves. No EGFP fluorescence was observed when plant organs were infected with the empty vector lacking the *EGFP* gene. It was reported that recombinant protein was produced in *Agrobacterium* having expression plasmids (Mankin et al. 1997, Vancanneyt et al. 1990).

However, the high EGFP fluorescence in these sprouts were unlikely due to the protein produced in the *Agrobacterium*, because it was under detectable level on the infiltration day (data not shown) although high concentration of *Agrobacterium* (OD_{600}=1.0) was used.

Six radish cultivars, all commercially available in Japan, were tested. The area-based efficiency of EGFP expression levels (EGFP-expressing region area per cotyledon area, determined using ImageJ software ver. 1.52 obtained at https://imagej.nih.gov/ij/) were higher in cultivars Hakata, Kaiware, and Banryoku, than in the other three cultivars (Figure 2).

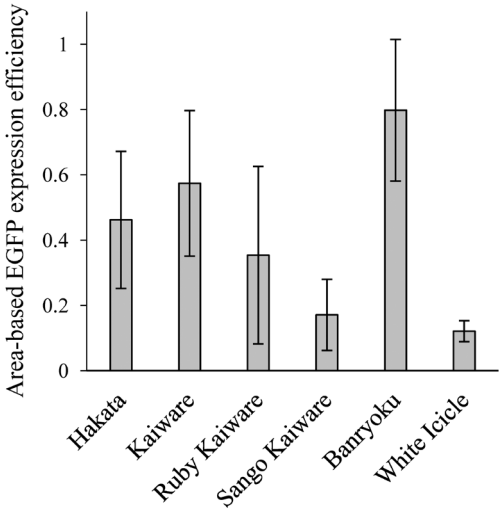

Figure 2. EGFP expression levels in six radish varieties. Sprouts of six radish varieties after 5 day of cultivation, including of 1 day of imbibition and 1 day of illumination, were infected with *Agrobacterium* harboring pBYR2HS-EGFP, and EGFP expression was analyzed after an additional 3 day of cultivation. The area-based efficiencies of EGFP expression (EGFP-expressing region area per cotyledon area) are indicated (means±standard deviations, n=3).

Then, we investigated how the cultivation and illumination periods affect the efficiency of EGFP expression in the three radish cultivars. Cultivation periods, which include 1 day of imbibition and 1 day of illumination, were changed from 4 to 7 day. At least 5 day of cultivation were required to achieve the optimum expression efficiency (Figure 3A). Changing the illumination period prior to infection from 0 to 2 day with a fixed 5-day cultivation period indicated that at least 1 day of illumination was required to achieve the optimum expression efficiency (Figure 3B). EGFP expression occurred in ~80% cotyledon area with the 5-day cultivation period, which included 1 day of imbibition and 1 day of illumination, when using Hakata and Banryoku, and with the 2-day illumination period when using Kaiware. Thus, we obtained recombinant proteins in only 8 day after seed imbibition. The expression efficiencies under each of the optimized culture conditions were similar among these cultivars, but Kaiware would be the most recommended considering the prices; about 1,000 yen (9.4 USD) for 1l Kaiware seeds compared with 5,400 yen (50.5 USD) for 1l Banryoku sees and 1,700 yen (15.9 USD) for 200 ml of Hakata seeds (Nakahara Seed Product Co., Ltd. Hakata, Japan, at 2019).

The level of protein accumulation was analyzed using SDS-PAGE. The soluble protein fraction from Kaiware infected with pBYR2HS-EGFP produced a band of EGFP at approx. 27 kDa, and the intensity was comparable with those of Rubisco large and small subunits (Figure 4). Comparing the band intensity with those of known amounts of purified EGFP indicated that the EGFP protein amounts were ~0.7 mg g^{-1} fresh weight of cotyledon. At present, this productivity ranks second after that of *N. benthamiana* leaves among all the plants tested using the Tsukuba system. Assuming Jiffy-7 pellets

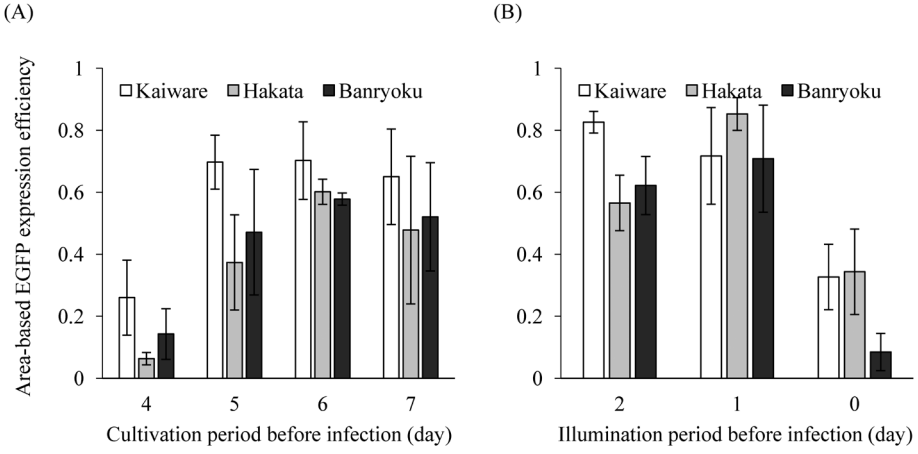

Figure 3. EGFP expression in three radish varieties under various culture conditions. Sprouts of three radish varieties were infected with *Agrobacterium* harboring pBYR2HS-EGFP after 4–7 day of cultivation, including of 1 day of imbibition and 1 day of illumination (A), or after 5 day of cultivation, including 1 day of imbibition and 0–2 day of illumination (B). The area-based efficiencies of EGFP expression (EGFP-expressing region area per cotyledon area) are indicated (means±standard deviations, n=3).

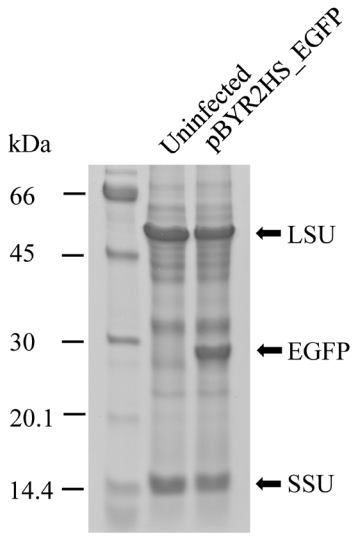

Figure 4. SDS-PAGE analysis of proteins from Kaiware cotyledons uninfected or infected with *Agrobacterium* harboring pBYR2HS-EGFP. The soluble protein fraction obtained from 0.94 mg (fresh weight) cotyledons was loaded per lane and stained with Coomassie Brilliant Blue (CBB). EGFP and Rubisco large (LSU) and small (SSU) subunits are indicated by arrows.

of φ42 mm yield ~6 g fresh weight of cotyledons, EGFP production was estimated to be 3 g m^{-2} over 8 day. Only two 40 W white fluorescent lamps were required for an illumination of 1 m^2.

We can purchase Kaiware sprouts for food, such as salad, every day of the year from supermarkets in Japan. We tested whether they could be used for agroinfiltration. We purchased seven packages of Kaiware sprouts on five different days at a local supermarket and infected them with pBYR2HS-EGFP on the purchase day. The EGFP expression efficiency varied, ranging from ~10% to over 40% cotyledon area, and the efficiency could be affected by the day on which the sprouts were purchased (data not shown). The EGFP expression efficiencies were lower than those obtained using the sprouts grown under well-controlled conditions in the laboratory, but the commercial sprouts would be available for some specific purposes, such as the analysis of the intracellular localization of an EGFP-tagged protein.

To produce another protein, the EGFP-coding region in pBYR2HS-EGFP can be replaced with the encoding cDNA, and the resulting plasmid can be co-introduced into Kaiware sprouts along with a marker plasmid. We used a visual marker plasmid containing the 2×35S promoter: *Arabidopsis thaliana* alcohol dehydrogenase 5′ untranslated region: EGFP: heat-shock protein terminator in the backbone of the binary vector pRI201 (Takara Bio, Shiga, Japan). EGFP expression was not driven by the Tsukuba system, and thus, the expression level was quite low, but its fluorescence was bright enough to determine in which cotyledon regions the infection occurred (data not shown).

The present study is not the first report of agroinfiltration in radish sprouts. This was previously reported by Liu et al. (2011, 2018) in which pBI121-based binary plasmids were used for agroinfiltration. However, our present study is the first report of significant recombinant protein production at a level comparable with that of Rubisco in radish sprout using the newly developed Tsukuba system. This is also the first report showing that the Tsukuba system also works as well in cotyledons as in leaves.

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