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Establishment of an efficient genetic transformation system based on *Agrobacterium rhizogenes* and the *RUBY* marker in *Brassica juncea*

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Abstract

Brassica juncea is an important and widely cultivated vegetable. Current genetic transformation techniques for this crop are limited by low efficiency. To establish a stable and efficient genetic transformation system for *B. juncea*, this study first combined *Agrobacterium rhizogenes*-mediated transformation with the novel visual marker gene *RUBY*. Rootless seedlings from two *B. juncea* genotypes, as explants, were infected by the *A. rhizogenes* strain K599 carrying a *RUBY* overexpression vector, successfully inducing red hairy roots. Subsequently, through exogenous hormone induction, a complete regeneration pathway was achieved, where transgenic positive hairy roots differentiated via callus to form regenerated shoots, establishing an efficient hairy roots–callus–regenerated shoots regeneration system. Ultimately, transgenic *B. juncea* plants carrying the *RUBY* gene, exhibiting distinctly red leaves throughout the plant, were obtained. Quantitative real-time polymerase chain reaction confirmed the high expression levels of the *RUBY* gene in these transgenic plants. It was worth noting that overexpression of *RUBY* gene may lead to growth abnormalities such as leaf wrinkling in some plants. This system using the visible marker of the *RUBY* gene significantly enhanced the efficiency and accuracy of screening for positive transformant plants and provided a new and efficient technical platform for the precise improvement of target traits and the functional analysis of genes in *B. juncea*.

Keywords: *Brassica juncea*, *Agrobacterium rhizogenes*, genetic transformation, *RUBY*

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Brassica juncea is a significant vegetable of the Brassicaceae family, widely cultivated in China. On the basis of its edible parts, it is classified into the root, leaf, stem, and heading types. Its processed products, such as pickled *B. juncea* and preserved *B. juncea* tubers, demonstrate distinct industrial characteristics, holding significant economic value and broad market prospects[1,2]. In the production of *B. juncea*, the common diseases in cruciferous vegetables, such as turnip mosaic virus, downy mildew, and clubroot, can cause serious losses. However, there are very few resistant resource lines among the *B. juncea* germplasm resources. Breeders usually introduce resistance genes from *B. rapa* or *B. nigra* into *B. juncea* through methods of distant hybridization, such as *B. juncea* (AABB genome) × *B. rapa* (AA genome) or *B. juncea* × *B. nigra* (BB genome)[3]. However, this method can take a relatively long time and has very low efficiency. Moreover, with the exploration of functional genes in *B. juncea*, the interpretation of gene functions has become increasingly important. Therefore, an efficient *B. juncea* genetic transformation system is needed to provide a rapid tool for precise improvement of target traits, the creation of characteristic germplasm materials, and interpretation of gene functions, thereby serving the development of the *B. juncea* industry.

Genetic transformation technology could enable targeted improvement of specific traits by directly introducing genes for the desired characteristics or editing endogenous genes. Although this technology has been applied in several brassica crops, including *B. napus*[4], *B. oleracea*[5], and *B. rapa*[6], it currently relies predominantly on the *Agrobacterium tumefaciens*-mediated system and still faces some challenges in *B. juncea*, such as low transformation efficiency and unstable regeneration systems (Supplementary Fig. S1). Therefore, exploring a stable and efficient genetic transformation method is of critical importance. *Agrobacterium rhizogenes* is a Gram-negative soil bacterium from the Rhizobiaceae family that induces the formation of extensively branched hairy roots at wound sites in plants[7], and these hairy roots are widely used for functional gene validation and the production of plant secondary metabolites. To date, hairy root induction has been successfully achieved in various plant species, primarily in herbaceous plants belonging to the Solanaceae[8], Brassicaceae[9], and Fabaceae family[10], as well as in some woody plants such as poplar (*Populus* spp.)[11] and *Citrus*[12]. However, the application of *A. rhizogenes*-mediated transformation in *B. juncea* has not been reported yet.

To improve transformation and screening efficiency, researchers have often used fluorescent genes as markers, such as *YFP*[13], *DsRed2*[14], *mCherry*[15], and *eGFP*[16]. However, these traditional marker methods require observation with a fluorescence microscope. In recent years, the *RUBY* gene has emerged as a new type of visual reporter gene that can be observed directly with the naked eye. This gene originates from plants of the order Caryophyllales[17] and mediates the synthesis of a class of vivid natural pigments called betalains, which can cause plant tissues to visibly appear red or purple, significantly enhancing the screening efficiency of genetic transformation. This marker has been successfully applied in species

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such as *Arabidopsis thaliana*[18], tomato (*Solanum lycopersicum*)[19], cotton (*Gossypium hirsutum*)[20], and *B. rapa*[21]. In addition, the beet pigments coded by the *RUBY* gene exhibit significant antioxidant activity, demonstrating potential health benefits such as cancer prevention, lipid reduction, and anti-inflammatory effects, thereby expanding the possibilities for cultivating new functional germplasm[22]. Consequently, utilizing the *RUBY* gene as a visual marker for genetic transformation not only significantly enhances screening efficiency and accuracy but also provides a novel technical approach for germplasm innovation. However, the application of the *RUBY* gene in *B. juncea* has not been reported yet.

In this study, an efficient transformation system was established based on *A. rhizogenes*-mediated transformation and a visual screening marker, namely the *RUBY* gene, which followed the pathway of hairy roots to callus to regenerated buds. We constructed a *RUBY* overexpression vector (Fig. 1a), introduced it into *A. rhizogenes* strain K599, and transformed two *B. juncea* genotypes, '50#' and '89#', using 6-day-old rootless seedlings as explants (Fig. 1b, c;

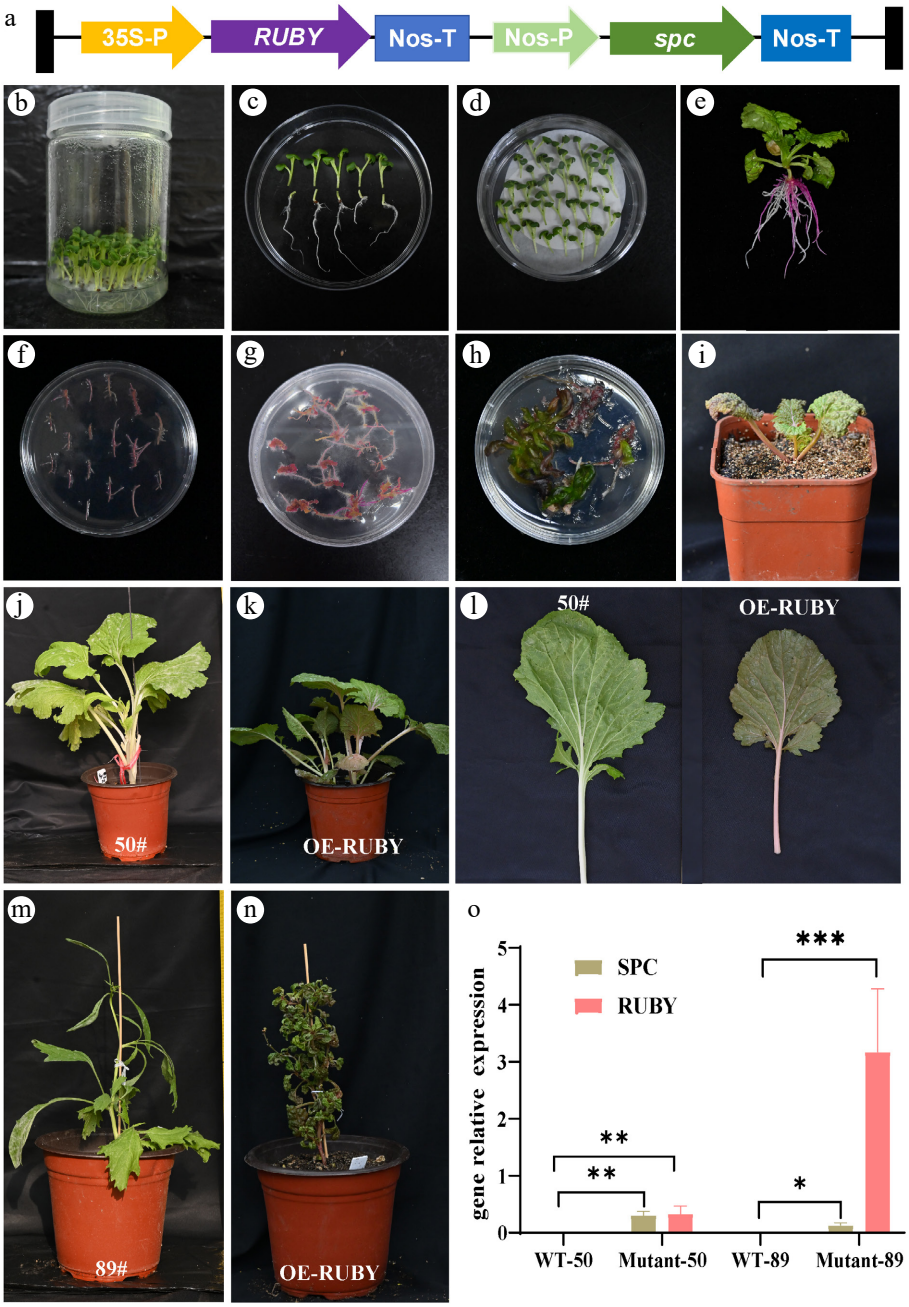

Fig. 1 Protocol for an efficient genetic transformation system in *B. juncea*. (a) The *RUBY* overexpression vector. The *RUBY* gene driven by the 35S promoter is co-expressed with the *spc* gene controlled by the nopaline synthase (NOS) promoter. (b)–(i) Genetic transformation procedure. (b) 6-d-old seedlings of *B. juncea*; (c) explant preparation; (d) cocultivation; (e) seedlings developing positive roots; (f) culture of positive root segments; (g). induction of callus from positive roots; (h) shoot regeneration from positive callus; (i) acclimatization of positive seedlings) (j)–(l) Phenotypic comparison of wild-type and transgenic *RUBY* overexpression plants from Line 50#. (j) Wild-type plant; (k) *RUBY* transgenic plant. (l) Comparison of leaves between the *RUBY* transgenic plant and the wild-type) (m),(n) Phenotypic comparison of wild-type and transgenic *RUBY* overexpression plants from Line 89#. (m) Wild-type plant; (n) *RUBY* transgenic plant). (o) Expression level of the *RUBY* gene in wild-type and the transgenic lines.

Supplementary Table S1). First, the explants were soaked in a resuspension solution with an optical density (OD) value of 0.6 for approximately 15 min. After air-drying, the explants were placed on a cocultivation medium (Fig. 1d) and cultured in the dark for about 40 h. Subsequently, they were transferred to hormone-free Murashige and Skoog (MS) medium containing 300 mg/L of timentin and cultured for about 14 d. During this period, red hairy roots were observed (Fig. 1e). The red hairy roots were cut into 5–8-mm segments (Fig. 1f) and transferred to a callus induction medium (MS + sucrose 30 g/L + 2.7 g/L phytagel + 300 mg/L timentin + 5.0 mg/L 6-BA + 0.5 mg/L naphthyl acetic acid [NAA]). After approximately 20 d, they were transferred to a shoot induction medium (MS + 30 g/L sucrose + 2.7 g/L phytagel + 300 mg/LTimentin + 0.5 mg/L 6-BA + 0.5 mg/L NAA) (Supplementary Table S2), a distinct red callus with red roots was observed (Fig. 1g). After continuing the culture for about 20 d, the positive shoots became visible (Fig. 1h). When the positive shoots developed three to four leaves, they were transferred to nutrient pots for acclimatization (Fig. 1i, Supplementary File 1). Once the seedlings grew seven or eight leaves, they underwent acclimatization and transplantation, ultimately yielding transgenic *B. juncea* plants carrying the *RUBY* gene. Compared with the control, the transgenic plants exhibited obvious red coloration in the entire leaves (Fig. 1j–n). Moreover, quantitative real-time polymerase chain reaction (qRT-PCR) results demonstrated the high expression levels of the *RUBY* and *SPC* gene in the transgenic plants (*SPC*: Approximately 0.30-fold for Line '50' and 0.15-fold for Line '89'; *RUBY*: Approximately 0.35-fold for Line '50' and 3-fold for Line '89') (Fig. 1o).

Plant roots generally have a weaker regenerative capacity than cotyledons and hypocotyls. *A. rhizogenes*-mediated transformation is typically limited to hairy root induction and rapid gene validation, making it difficult to obtain intact plants from hairy roots (Supplementary Fig. S2). However, this study demonstrated that exogenous hormone induction can promote callus formation from positive transgenic roots, leading to plant regeneration. Moreover, with the *RUBY* visual marker, shoots emerging from positive roots are directly identifiable as positive transformants. However, it was worth noting that overexpression of the *RUBY* gene may lead to plant abnormalities; for example, the '89#' line exhibited leaf wrinkling (Fig. 1n). Similar phenomena resulting from the *RUBY* gene, have also been observed in other plants, such as maize (*Zea mays*)[23], carrot (*Daucus carota*)[24], and cotton[25]. This may be related to the synthetic pathway of the *RUBY* gene, which included three enzyme-coding sequences (CYP76AD1, DODA, and glycosyltransferase, expressed in tandem via 2A peptides), mediating betalain synthesis in Caryophyllales, and its heterologous overexpression in other plants could trigger growth abnormalities via tyrosine depletion and intermediate accumulation[26]. According to the qRT-PCR results, it was speculated that exceeding a certain threshold of *RUBY* gene expression may impair normal plant development, indicating that the use of this visual *RUBY* marker for screening has limitations. We will further explore the use of weak or inducible promoters to drive *RUBY*'s expression, aiming to reduce its expression level and thus mitigate its adverse effects on plants while preserving the convenience of visual screening.

In summary, we have established an efficient transformation system for hairy roots–callus–regenerated shoots using *A. rhizogenes* and the *RUBY* visual marker gene, and obtained *RUBY* transgenic plants, providing both technical and resource foundations for the genetic improvement of *B. juncea*. We will clone the marker gene into a clustered clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) expression vector for coexpression with Cas9 and the corresponding sgRNA. This will allow the selection and tracking of successfully transformed cells or individuals using the marker gene while editing the target gene, and thus will further enable subsequent gene editing experiments.

Author contributions

The authors confirm their contributions to the paper as follows: conceived the study and reviewed the manuscript: Li G, Zhang S, Zhang R; performed the experiments: Zeng X; wrote the manuscript: Wei Y; editing the manuscript: Li F, Zhang H, Zhang S, Sun R. All authors reviewed the results and approved the final version of the manuscript.

Data availability

Data sharing is not applicable to this article because no new data were created or analyzed in this study.

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Conflict of interest

The authors declare that they have no conflict of interest

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References

[1] Hu Y, Yan Z. 2025. Comprehensive advances in phytochemical components, bioactive functionality, and processing applications of mustard (*Brassica juncea* (L.) Czern.): a review. *Frontiers in Nutrition* 12:1626333

[2] Rai PK, Yadav P, Kumar A, Sharma A, Kumar V, et al. 2022. *Brassica juncea*: a crop for food and health. In *The Brassica juncea Genome*, eds. Kole C, Mohapatra T. Cham: Springer. pp. 1–13 doi: 10.1007/978-3-030-91507-0_1

[3] Paritosh K, Gupta V, Yadava SK, Singh P, Pradhan AK, et al. 2014. RNA-seq based SNPs for mapping in *Brassica juncea* (AABB): synteny analysis between the two constituent genomes A (from B. *rapa*) and B (from B. *nigra*) shows highly divergent gene block arrangement and unique block fragmentation patterns. *BMC Genomics* 15(1):396

[4] Dai C, Li Y, Li L, Du Z, Lin S, et al. 2020. An efficient *Agrobacterium*-mediated transformation method using hypocotyl as explants for *Brassica napus*. *Molecular Breeding* 40(10):96–96

[5] Gribova TN, Kamionskaya AM, Skryabin KG. 2006. Optimization of the protocol for constructing transgenic plants of the cabbage *Brassica oleracea* var capitata. *Applied Biochemistry and Microbiology* 42(5):519–524

[6] Li X, Li H, Zhao Y, Zong P, Zhan Z, et al. 2021. Establishment of a simple and efficient *Agrobacterium*-mediated genetic transformation system to Chinese cabbage (*Brassica rapa* L. ssp. pekinensis). *Horticultural Plant Journal* 7(2):117–128

[7] Porter JR, Flores H. 1991. Host range and implications of plant infection by *Agrobacterium rhizogenes*. *Critical Reviews in Plant Sciences* 10(4):387–421

[8] Fernández-Piñán S, López J, Armendariz I, Boher P, Figueras M, et al. 2019. *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*-mediated transformation of potato and the promoter activity of a suberin gene by GUS staining. *Journal of Visualized Experiments* 145:e59119

[9] Wang H, Zheng Y, Zhou Q, Li Y, Liu T, et al. 2024. Fast, simple, efficient *Agrobacterium rhizogenes*-mediated transformation system to non-heading Chinese cabbage with transgenic roots. *Horticultural Plant Journal* 10(2):450–460

[10] Wang TZ, Zhang WH. 2020. *Agrobacterium rhizogenes*-mediated transformation of hairy roots in *Medicago ruthenica*. *Acta Agrestia Sinica* 28(1):268–272

[11] Wei M, Huang Y, Zhang H, Liu Y, Zhao Y, et al. 2026. Efficient *Agrobacterium rhizogenes*-mediated transformation of poplar via transgenic hairy root shoot regeneration. *Plant, Cell & Environment* 49:1309–1312

[12] Wang M, Qin YY, Wei NN, Xue HY, Dai WS. 2023. Highly efficient *Agrobacterium rhizogenes*-mediated hairy root transformation in citrus seeds and its application in gene functional analysis. *Frontiers in Plant Science* 14:1293374

[13] Kirienko DR, Luo A, Sylvester AW. 2012. Reliable transient transformation of intact maize leaf cells for functional genomics and experimental study. *Plant Physiology* 159(4):1309–1318

[14] Huai D, Wu J, Xue X, Hu M, Zhi C, et al. 2023. Red fluorescence protein (DsRed2) promotes the screening efficiency in peanut genetic transformation. *Frontiers in Plant Science* 14:1123644

[15] Bai J, Luo Y, Wang X, Li S, Luo M, et al. 2020. A protein-independent fluorescent RNA aptamer reporter system for plant genetic engineering. *Nature Communications* 11(1):3847

[16] Stewart C. 2001. The utility of green fluorescent protein in transgenic plants. *Plant Cell Reports* 20(5):376–382

[17] Azeredo HMC. 2009. Betalains: properties, sources, applications, and stability – a review. *International Journal of Food Science & Technology* 44(12):2365–2376

[18] He Y, Zhang T, Sun H, Zhan H, Zhao Y. 2020. A reporter for noninvasively monitoring gene expression and plant transformation. *Horticulture Research* 7(1):152

[19] Yang H, Zhang Y, Fu Q, Jia X, Zhao T, et al. 2023. The DR5 and E8 reporters are suitable systems for studying the application of the *Ruby* reporter gene in tomato. *Vegetable Research* 3:12

[20] Ge X, Wang P, Wang Y, Wei X, Chen Y, et al. 2023. Development of an eco-friendly pink cotton germplasm by engineering betalain biosynthesis pathway. *Plant Biotechnology Journal* 21(4):674–676

[21] Wang Y, Yang X, Wang W, Wang Y, Chen X, et al. 2025. Efficient genetic transformation and gene editing of Chinese cabbage using *Agrobacterium rhizogenes*. *Plant Physiology* 197:kiae543

[22] Polturak G, Aharoni A. 2018. 'La Vie en Rose': biosynthesis, sources, and applications of betalain pigments. *Molecular Plant* 11(1):7–22

[23] Lee K, Kang M, Ji Q, Grosic S, Wang K. 2023. New T-DNA binary vectors with *NptII* selection and *RUBY* reporter for efficient maize transformation and targeted mutagenesis. *Plant Physiology* 192(4):2598–2603

[24] Deng YJ, Duan AQ, Liu H, Wang YH, Zhang RR, et al. 2023. Generating colorful carrot germplasm through metabolic engineering of betalains pigments. *Horticulture Research* 10(4):uhad024

[25] Li X, Mitchell M, Rolland V, Allen S, MacMillan C, et al. 2023. 'Pink cotton candy' – a new dye-free cotton. *Plant Biotechnology Journal* 21(4):677–679

[26] Khan MI, Polturak G. 2025. Biotechnological production and emerging applications of betalains: a review. *Biotechnology Advances* 81:108576