

High frequency in vitro regeneration from embryonic tip of Wucai (*Brassica campestris* L.)

Danni Yang

Anhui Agricultural University

Na Liao

Anhui Agricultural University

Ting Li

Anhui Agricultural University

Shiyong Zhang

Anhui Agricultural University

Qian Yin

Anhui Agricultural University

Xiaoyan Tang

Anhui Agricultural University

Wenjie Wang

Anhui Agricultural University

Lingyun Yuan

Anhui Agricultural University

Chenggang Wang

Anhui Agricultural University

Guohu Chen

cgh@ahau.edu.cn

Anhui Agricultural University <https://orcid.org/0000-0002-6996-425X>

Research Article

Keywords: cotyledonary node, embryonic tip, hypocotyl, regeneration, Wucai

Posted Date: September 25th, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-7615740/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at Plant Cell, Tissue and Organ Culture (PCTOC) on May 21st, 2026. See the published version at <https://doi.org/10.1007/s11240-026-03491-2>.

Abstract

An effective and efficient regeneration system is essential for the successful implementation of plant genetic transformation. Establishing a high-frequency regeneration system requires appropriate explants and a differentiation medium conducive to explant growth. In this study, the efficiency of three regeneration protocols were investigated for multiple shoot induction in Wucai (*Brassica campestris* syn. *B. rapa* L.). Explants including embryonic tip (derived from mature seeds), hypocotyl, and cotyledonary node explants were tested, and various factors were optimized under *in vitro* conditions. Following optimization using the 'Huiwu 20' material, embryonic tips from mature seeds germinated for 60 hours achieved the highest regeneration frequency (87.10%) and produced the maximum number of shoots per explant (8.17). In contrast, cotyledonary node and hypocotyl explants from 4-day-old seedlings displayed lower regeneration frequencies (66.47% and 45.73%, respectively) and fewer shoots per explant (5.07 and 3.23, respectively). Healthy, rooted plantlets were successfully obtained from all types of explants. However, regenerated shoots (approximately 2 cm in height) derived from embryonic tip explants exhibited the most rapid development, requiring approximately 20 days. These findings highlight the potential of the embryonic tip regeneration system for an efficient and reproducible method for *in vitro* shoot regeneration, providing a foundation for genetic transformation in Wucai.

Introduction

Chinese cabbage vegetables are classified into two main categories: heading Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) and non-heading Chinese cabbage (*B. campestris* syn. *B. rapa* L. ssp. *chinensis*), both originating from China. Wucai (ssp. *chinensis* var. *rosularis* Tsen et Lee), a subspecies variant of non-heading Chinese cabbage, has gained increasing attention and popularity worldwide due to its appealing morphology, abundant health benefits, and rich content of vitamins and minerals (Chen et al., 2020; Chen et al., 2019; Liu et al., 2025; Liu et al., 2024). The substantial demand for both quality and quantity requires accelerated genetic improvement of Wucai. Although several genetic transformation methods have been developed for Chinese cabbage crops (Li et al., 2021; Liu et al., 2018; Sivanandhan et al., 2021), limited research has focused on tissue regeneration and genetic transformation in Wucai, such as extended regeneration time and lower genetic transformation efficiency (Chen et al., 2019; Chen et al., 2017). Developing an efficient plant tissue culture protocol with a high regeneration frequency is crucial for most genetic transformation approaches. Therefore, establishing a stable and highly efficient regeneration protocol for Wucai is essential for preserving and propagating valuable germplasm, as well as for facilitating genetic transformation.

In general, plant *in vitro* regeneration encompasses two distinct processes: somatic embryogenesis and shoot morphogenesis. The latter predominantly drives regeneration in Chinese cabbage crops, utilizing various explant types, including root (Neumann et al., 2020), leaf (Abbasi et al., 2011; Liu et al., 2018; Memon et al., 2009b), cotyledon (Cogbill et al., 2010; Fang et al., 2023; Sivanandhan et al., 2023), cotyledonary node (Toma et al., 2022), and hypocotyl (Sivanandhan et al., 2019; Toma et al., 2022). Additionally, microspores and anthers have been utilized to induce embryos for the production of double

haploid plants (Gao et al., 2020; Jia et al., 2019a; Jia et al., 2019b; Lu et al., 2016; Niu et al., 2019). Despite successful *in vitro* regeneration from these explants in certain genotypes, *B. campestris* remains one of the most challenging species for regeneration due to its A genome (Gao et al., 2020).

The process of shoot regeneration in Chinese cabbage crops is influenced by various factors, including genotype, explant type, seedling age, plant growth regulators, and additional chemicals such as AgNO₃ and cysteine (Gao et al., 2020; Sivanandhan et al., 2019; Sivanandhan et al., 2021). To date, hypocotyls, cotyledons, cotyledonary nodes, and leaves have been the primary explant types used for adventitious shoot regeneration in *in vitro* tissue culture, although their efficiency is often genotype-dependent (Jeong et al., 2019; Li et al., 2021; Liu et al., 2018; Ma et al., 2017; Sivanandhan et al., 2021). Moreover, significant variation in shoot regeneration efficiency has been observed among *B. campestris* crops (Liu et al., 2018; Memon et al., 2009b). Therefore, identifying the most suitable explant type is imperative for successful adventitious shoot regeneration.

In this study, an embryonic tip regeneration system derived from mature seeds was first provided, assessing the impacts of seedling age, pre-culture, and plant growth regulators concentrations in culture mediums on shoot regeneration. These results demonstrated several advantages of this system over previously reported methods, including higher regeneration frequency and faster adventitious bud formation. Overall, these findings indicated that this novel embryonic tip regeneration system may represent a promising advancement over current techniques for transforming Chinese cabbage crops.

Materials and methods

Plant material and explant preparation

Mature seeds of Wucai (cv. 'Huiwu 20', 'Huiwu No.1', and 'Huangxinwu') were used as the plant material in this study. The seeds were obtained from the Vegetable Genetics and Breeding Laboratory of Anhui Agricultural University. Sterilization was performed by treating the seeds with 70% for 1 min, followed by 2% NaOCl for 10 min, as described in a previous protocol (Liu et al., 2018). The sterilized seeds were germinated on a germination medium (GM; MS + 3% sucrose + 0.8% agar, pH = 5.8), incubated at 24 ± 1°C under dark conditions.

The preparation of explants is illustrated in Fig. 1. Embryonic tip explants were obtained from mature seeds germinated with different time by carefully removing the seed coat, radicle, cotyledons, and all primary leaves, leaving approximately 3–5 mm of embryonic tips containing meristems (Fig. 1A). Cotyledonary node explants were prepared by horizontally slicing the region approximately 1–2 mm below the hypocotyl-cotyledon junction, followed by the vertical removal of the embryonic axis and primary leaves (Fig. 1B). Hypocotyl explants were obtained by excising the upper segment of the hypocotyl, approximately 3–5 mm below the cotyledons, ensuring the complete removal of meristematic tissue (Fig. 1C). All explants were cultured under a 16 h light/8 h dark photoperiod in incubators maintained at 24 ± 1°C, with a light intensity of 50 μmol·m⁻²·s⁻¹.

Effect of seedling ages on multiple shoot induction

To determine the optimal seedling age for multiple shoot induction, embryonic tip explants were collected from mature seeds germinated for 24, 36, 48, 60, and 72 hours. Cotyledonary node and hypocotyl explants were collected from seedlings aged 2, 3, 4, 5, and 6 days, respectively. All explants were initially cultured on a pre-culture medium [PCM; MS + 2.0 mg·L⁻¹ 6-benzyl adenine (BAP) + 3% sucrose + 0.8% agar, pH = 5.8] for 2 days. Subsequently, embryonic tip explants were transferred to a shoot induction medium [SIM; MS + 0.3 mg·L⁻¹ BAP + 0.1 mg·L⁻¹ 1-naphthaleneacetic acid (NAA) + 3% sucrose + 0.8% agar, pH = 5.8], while cotyledonary node and hypocotyl explants were transferred to an alternate SIM (MS + 2.0 mg·L⁻¹ BAP + 0.5 mg·L⁻¹ NAA + 3% sucrose + 0.8% agar, pH = 5.8). After 20–40 days of culture, the following parameters were recorded: regeneration frequency, number of multiple shoots per explant, and average number of shoots per responsive explant.

Effect of pre-culture on multiple shoot induction

To optimize pre-culture duration, embryonic tip explants from 60-hour-old seedlings were inoculated on PCM containing different concentrations of BAP (0, 1.0, 1.5, 2.0, or 2.5 mg·L⁻¹) for 0, 12, 24, 36, or 48 hours. Cotyledonary node and hypocotyl explants from 4-day-old seedlings were subjected to PCM containing varying BAP concentrations (2.0, 4.0, or 6.0 mg·L⁻¹) for 24, 36, or 48 hours. After pre-culture, the explants were transferred to their respective SIMs. Following 20–40 days of culture, the regeneration frequency, number of multiple shoots, and the average number of shoots per responsive explant were recorded. All pre-culture treatment were conducted under a 16 h light/8 h dark photoperiod at 24 ± 1°C, with a light intensity of 50 μmol·m⁻²·s⁻¹.

Effect of BAP and NAA concentrations on multiple shoot induction

To determine the optimal SIM composition, explants pre-cultured for 36 hours were transferred to SIMs containing various concentrations of BAP and NAA. For embryonic tip explants: SIM formulations included 0, 0.3, 0.5, 0.8, or 1.0 mg·L⁻¹ BAP combined with 0, 0.1, 0.2, 0.3, or 0.4 mg·L⁻¹ NAA. For cotyledonary node and hypocotyl SIM formulations included 0, 0.5, or 1.0 mg·L⁻¹ BAP combined with 0, 0.5, or 1.0 mg·L⁻¹ NAA. After 20–40 days, the regeneration frequency, number of multiple shoots, and average number of shoots per responsive explant were assessed.

Effect of IBA concentration on rooting

To optimize the rooting medium (RM), regenerated shoots were transferred to RM containing different concentrations of indole-3-butyric acid (IBA; 0.5, 1.0, or 1.5 mg·L⁻¹). After 14–20 days of culture, the rooting frequency of the plantlets was assessed.

Statistical analyses

Each treatment consisted of 30 explants and was replicated three times. Regeneration effectiveness was evaluated using the following parameters: adventitious shoot regeneration rate (%) = (number of explants with adventitious shoot/total number of explants) × 100%, average number of regenerated adventitious shoots per explant = (total number of adventitious shoots/number of explants with adventitious shoot) × 100%, and adventitious shoot induction time = days required for adventitious shoot to reach approximately 2 cm, rooting rate (%) = (number of rooted shoots/total number of inoculated shoots) × 100%. Statistical analyses were conducted using analysis of variance (ANOVA) with SPSS 20 (SPSS Inc., Chicago, USA). Significant differences were determined using Duncan's multiple range test at $P < 0.05$.

Results

Influence of seedling age on adventitious shoot regeneration

To investigate the effect of seedling age on adventitious shoots regeneration, embryonic tips were extracted from mature seeds at different germination stages (Fig. 2). Cotyledonary nodes and hypocotyl explants were also collected from seedlings aged 2, 3, 4, 5, and 6 days. The results revealed that seedling age significantly influenced shoot regeneration across all explant types (Fig. 2). Embryonic tips from 60-hour-old seedlings exhibited the highest shoot differentiation frequency (47.33%), and produced an average of 4.40 shoots per responsive explant (Fig. 3A and 3B). Similarly, cotyledonary nodes and hypocotyls showed maximum shoot regeneration frequencies (47.61% and 31.96%, respectively) at 4-day-old seedlings, producing an average of 3.981 and 2.61 shoots per explant (Fig. 3C-3F). Based on these results, embryonic tips from mature seeds germinated for 60 hours, as well as cotyledonary nodes and hypocotyls from 4-day-old seedlings, were selected for further optimization.

Influence of pre-culture on adventitious shoot regeneration

The effect of pre-culture duration and BAP concentration on adventitious shoot regeneration was investigated using embryonic tip, cotyledonary node, and hypocotyl explants. The results indicated that pre-culture conditions significantly affected shoot differentiation frequencies (Tables 1–2). Embryonic tips exhibited optimal regeneration (70.30%) and the highest shoot number per explant (6.23) when pre-cultured for 36 hours on a medium containing 2.0 mg·L⁻¹ BAP (Table 1). For cotyledonary nodes and hypocotyls, the highest regeneration frequencies (53.73% and 39.13%, respectively) and shoot numbers per explant (4.27 and 3.0, respectively) were achieved with pre-culture on a medium containing 4.0 mg·L⁻¹ BAP for 36 hours (Table 2). After pre-culture, explants appeared visibly enlarged (Fig. 4), indicating robust differentiation and division capacity. Therefore, pre-culture durations of 36 hours and the respective BAP concentrations were chosen for further optimization.

Table 1
Effect of pre-culture on shoot regeneration from embryonic tips

BAP concentration (mg·L⁻¹)	Pre-culture time (h)	Shoot regeneration (%)	Number of shoots per responsive explant
0.0	0	36.03 ± 0.37 ij	3.27 ± 0.13 efg
0.0	12	30.70 ± 0.89 k	3.07 ± 0.09 fgh
0.0	24	21.33 ± 1.27 l	2.50 ± 0.15 h
0.0	36	33.23 ± 3.01 jk	2.50 ± 0.10 h
0.0	48	19.60 ± 1.95 l	2.70 ± 0.20 gh
1.0	0	36.03 ± 0.37 ij	3.27 ± 0.13 efg
1.0	12	40.50 ± 2.63 fghi	3.77 ± 0.09 e
1.0	24	39.77 ± 1.57 ghi	3.67 ± 0.12 ef
1.0	36	42.97 ± 0.57 fgh	3.13 ± 0.13 fg
1.0	48	44.97 ± 1.79 ef	3.63 ± 0.27 ef
1.5	0	36.03 ± 0.37 ij	3.13 ± 0.13 fg
1.5	12	43.03 ± 1.33 fgh	4.33 ± 0.20 d
1.5	24	48.50 ± 0.06 e	5.00 ± 0.10 bc
1.5	36	54.30 ± 2.95 d	5.10 ± 0.12 bc
1.5	48	57.27 ± 1.37 cd	4.80 ± 0.40 cd
2.0	0	36.03 ± 0.37 ij	3.27 ± 0.13 efg
2.0	12	61.20 ± 2.21 bc	5.57 ± 0.27 b
2.0	24	62.37 ± 1.81 b	5.03 ± 0.15 bc
2.0	36	70.30 ± 1.48 a	6.23 ± 0.12 a
2.0	48	52.97 ± 1.47 d	5.50 ± 0.21 b
2.5	0	36.03 ± 0.37 ij	3.27 ± 0.13 efg
2.5	12	44.60 ± 1.76 efg	4.77 ± 0.39 cd
2.5	24	39.00 ± 1.40 hi	5.13 ± 0.09 bc
2.5	36	36.03 ± 0.37 ij	4.07 ± 0.15 bc
2.5	48	40.80 ± 0.87 fghi	4.87 ± 0.24 de

Data are mean values $\pm$ S.E. of three replicates. Different letters within the same column indicate significant differences according to Duncan's multiple range test at $p < 0.05$.

Table 2
Effect of pre-culture on shoot regeneration from cotyledonary nodes and hypocotyls

BAP concentration (mg·L ⁻¹)	Pre-culture time (h)	cotyledonary nodes		hypocotyls	
		Shoot regeneration (%)	Number of shoots per responsive explant	Shoot regeneration (%)	Number of shoots per responsive explant
2	24	29.47 $\pm$ 0.95 e	1.80 $\pm$ 0.25 d	5.43 $\pm$ 1.07 e	1.30 $\pm$ 0.06 c
2	36	28.07 $\pm$ 3.32 e	2.03 $\pm$ 0.32 d	8.80 $\pm$ 1.20 e	2.13 $\pm$ 0.20 b
2	48	42.30 $\pm$ 0.78 bc	2.73 $\pm$ 0.20 c	17.87 $\pm$ 2.73 d	2.33 $\pm$ 0.19 b
4	24	43.67 $\pm$ 4.17 bc	2.17 $\pm$ 0.12 d	22.77 $\pm$ 1.68 c	2.37 $\pm$ 0.12 b
4	36	53.73 $\pm$ 2.36 a	4.27 $\pm$ 0.09 a	39.13 $\pm$ 0.90 a	3.00 $\pm$ 0.10 a
4	48	47.97 $\pm$ 1.32 ab	3.13 $\pm$ 0.13 bc	32.40 $\pm$ 1.99 b	2.10 $\pm$ 0.12 b
6	24	39.17 $\pm$ 0.90 cd	3.53 $\pm$ 0.09 b	27.07 $\pm$ 0.67 c	2.37 $\pm$ 0.13 b
6	36	38.33 $\pm$ 1.47 cd	3.20 $\pm$ 0.17 bc	24.27 $\pm$ 1.60 c	2.17 $\pm$ 0.07 b
6	48	33.90 $\pm$ 1.70 de	2.90 $\pm$ 0.10 c	17.47 $\pm$ 0.75 d	1.57 $\pm$ 0.12 c

Evaluation of BAP and NAA concentrations on shoot induction

To optimize shoot induction, explants were cultured on shoot induction media (SIM) containing different concentrations of BAP and NAA. The results demonstrated a significant effect of these regulators on shoot regeneration across all explants (Tables 3–4). For embryonic tips, the highest regeneration frequency (87.10%) and shoot number per explant (8.17) were achieved with 0.8 mg·L⁻¹ BAP and 0.2 mg·L⁻¹ NAA (Table 3). Cotyledonary nodes and hypocotyl explants exhibited optimal regeneration (66.47% and 45.73%, respectively) and shoot numbers per explant (5.07 and 3.23, respectively) when cultured with 1.0 mg·L⁻¹ BAP and 0.5 mg·L⁻¹ NAA (Table 4). These combinations were selected for further studies.

Table 3
Effect of BAP and NAA concentrations on shoot regeneration from embryonic tips

BAP concentration (mg·L⁻¹)	NAA concentration (mg·L⁻¹)	Shoot regeneration (%)	Number of shoots per responsive explant
0.0	0.0	13.27 ± 0.97 k	5.03 ± 0.18 bc
0.0	0.1	10.27 ± 0.76 kl	5.03 ± 0.23 bc
0.0	0.2	10.53 ± 0.57 kl	4.97 ± 0.19 bc
0.0	0.3	8.07 ± 1.13 kl	5.07 ± 0.20 bc
0.0	0.4	4.90 ± 2.59 l	4.93 ± 0.32 bc
0.3	0.0	22.47 ± 1.31 j	5.43 ± 0.32 bc
0.3	0.1	31.87 ± 1.43 hi	4.63 ± 0.2 3c
0.3	0.2	27.07 ± 2.16 ij	4.87 ± 0.14 bc
0.3	0.3	24.93 ± 2.63 ij	5.43 ± 0.08 bc
0.3	0.4	22.53 ± 1.44 j	5.60 ± 0.15 b
0.5	0.0	37.87 ± 2.99 gh	4.93 ± 0.33 bc
0.5	0.1	41.63 ± 2.06 fg	4.80 ± 0.05 bc
0.5	0.2	61.27 ± 7.15 c	4.97 ± 0.28 bc
0.5	0.3	48.97 ± 2.96 ef	5.13 ± 0.20 bc
0.5	0.4	40.17 ± 1.43 g	5.43 ± 0.13 bc
0.8	0.0	53.33 ± 3.47 de	5.10 ± 0.15 bc
0.8	0.1	76.03 ± 0.52 b	5.50 ± 0.12 bc
0.8	0.2	87.10 ± 0.23 a	8.17 ± 0.73 a
0.8	0.3	79.97 ± 0.95 b	5.33 ± 0.07 bc
0.8	0.4	73.37 ± 1.21b	5.36 ± 0.23 bc
1.0	0.0	38.10 ± 3.93 gh	5.33 ± 0.39 bc
1.0	0.1	52.60 ± 1.90 de	5.30 ± 0.15 bc
1.0	0.2	59.03 ± 0.87 cd	5.43 ± 0.13 bc
1.0	0.3	45.63 ± 2.25 efg	5.50 ± 0.32 bc
1.0	0.4	48.50 ± 3.27 ef	5.13 ± 0.20 bc

Data are mean values $\pm$ S.E. of three replicates. Different letters within the same column indicate significant differences according to Duncan's multiple range test at $p < 0.05$.

Table 4

Effect of BAP and NAA concentrations on shoot regeneration from cotyledonary nodes and hypocotyls

BAP concentration (mg·L ⁻¹)	NAA Concentration (mg·L ⁻¹)	Cotyledonary nodes		Hypocotyls	
		Shoot regeneration (%)	Number of shoots per responsive explant	Shoot regeneration (%)	Number of shoots per responsive explant
0.0	0.0	10.43 $\pm$ 0.64 f	2.73 $\pm$ 0.43 c	5.43 $\pm$ 1.07 g	1.30 $\pm$ 0.06 e
0.0	0.5	22.27 $\pm$ 1.46 e	2.37 $\pm$ 0.17 c	8.80 $\pm$ 1.20 f	2.13 $\pm$ 0.20 d
0.0	1.0	25.27 $\pm$ 1.02 e	2.57 $\pm$ 0.29 c	14.53 $\pm$ 0.67 e	2.67 $\pm$ 0.31 bc
0.5	0.0	45.73 $\pm$ 1.22 c	4.07 $\pm$ 0.47 b	20.60 $\pm$ 0.97 d	2.93 $\pm$ 0.08 ab
0.5	0.5	31.93 $\pm$ 3.21 d	3.77 $\pm$ 0.15 b	17.46 $\pm$ 0.75 de	1.57 $\pm$ 0.12 e
0.5	1.0	52.63 $\pm$ 1.89 b	2.40 $\pm$ 0.40 c	24.26 $\pm$ 1.60 c	2.17 $\pm$ 0.06 cd
1.0	0.0	57.23 $\pm$ 1.23 b	4.23 $\pm$ 0.22 ab	39.13 $\pm$ 0.89 b	2.37 $\pm$ 0.13 cd
1.0	0.5	66.47 $\pm$ 3.43 a	5.07 $\pm$ 0.20 a	45.73 $\pm$ 1.22 a	3.23 $\pm$ 0.20 a
1.0	1.0	57.13 $\pm$ 2.07 b	3.70 $\pm$ 0.32 b	41.73 $\pm$ 0.96 b	2.10 $\pm$ 0.11 d

Data are mean values $\pm$ S.E. of three replicates. Different letters within the same column indicate significant differences according to Duncan's multiple range test at $p < 0.05$.

Effect of IBA concentration on rooting

The influence of IBA concentration on rooting was assessed using regenerated shoots. Rooting was most effective with 1.0 mg·L⁻¹ IBA, achieving the highest rooting rates for embryonic tip-derived shoots (94.73%), cotyledonary node-derived shoots (92.63%), and hypocotyl-derived shoots (95.83%) (Fig. 5). No significant differences in rooting efficiency were observed among the explant types.

Stability of shoot regeneration systems

Based on the optimized protocols, stable shoot regeneration systems were established for all explant types. Embryonic tips from mature seeds germinated for 60 hours (Figs. 6A and 6B) were pre-cultured

for 36 h on PCM1 (MS + 2.0 mg·L⁻¹ BAP + 3% sucrose + 0.8% agar, pH = 5.8) (Fig. 6C), and transferred to SIM1 (MS + 0.8 mg·L⁻¹ BAP + 0.2 mg·L⁻¹ NAA + 3% sucrose + 0.8% agar, pH = 5.8) for adventitious shoot induction. Regeneration shoots emerged on the explants in approximately 7 days (Fig. 6D), and multiple adventitious shoots were observed after approximately 3 weeks of cultivation (Fig. 6E).

Similarly, cotyledonary nodes and hypocotyls from 4-day-old seedlings (Figs. 7A and 7B, 8A and 8B) were pre-cultured for 36 h (Figs. 7C and 8C) on PCM2 (MS + 4.0 mg·L⁻¹ BAP + 3% sucrose + 0.8% agar, pH = 5.8), followed by transfer to SIM2 (MS + 1.0 mg·L⁻¹ BAP + 0.5 mg·L⁻¹ NAA + 3% sucrose + 0.8% agar, pH = 5.8). After approximately 2 weeks of cultivation, macroscopic callus colonies became visible (Fig. Figures 7D and 8D), with adventitious shoots regenerated in 4–5 weeks (Figs. 7E and 8E). Subsequently, the regeneration shoots from all three types of explants were rooted on RM (MS + 1.0 mg·L⁻¹ IBA + 3% sucrose + 0.8% agar, pH = 5.8) (Figs. 6F, 7F, and 8F). After approximately 2 weeks, the rooted plantlets (Figs. 6G, 7G, and 8G) were transplanted and acclimatized in the greenhouse (Figs. 6H, 7H, and 8H), which all roots facilitating the recovery of regenerated plantlets during acclimation.

Independent repeated tests confirmed that embryonic tips exhibited the highest shoot regeneration frequency, the maximum number of shoots per explant, and the shortest regeneration time (Figs. 9A-9C). Furthermore, the regeneration efficiency and shoot number per responsive explant remained consistent across different Wucai genotypes (Figs. 9D-9E). These results underscore the stability and potential of the embryonic tip shoot regeneration protocol for future genetic transformation experiments in Wucai.

Discussion

Several stable and efficient regeneration systems have been established for various Chinese cabbage cultivars. Efficient *in vitro* plant regeneration depends on the optimal explant type. Previous studies have shown that different varieties exhibit varying optimal explants, and even within the same variety, different explant types display differences in regeneration efficiency (Li et al., 2021; Sivanandhan et al., 2019; Zhao et al., 2021). Commonly used explants for genotype-dependent shoot regeneration in Chinese cabbage include hypocotyls, cotyledons, and cotyledons with petioles (Baskar et al., 2016). Significant variations in regeneration frequency among different *Brassica rapa* cultivars have been reported. For example, the double haploid line 'CX001' demonstrated robust regeneration potential using cotyledons (Zhao et al., 2021). Similarly, assessment of different explant types (hypocotyl, root, or cotyledon with petiole) from the same variety has shown that the cotyledon with petiole of 'C-24' cultivar and both hypocotyls and cotyledons of the 'Kenshin' cultivar exhibit higher regeneration frequencies (Li et al., 2021; Sivanandhan et al., 2019). Additionally, alternative explant types such as stem tips, roots, and leaves have been used successfully in certain varieties, achieving notable regeneration frequencies (Abbasi et al., 2011; Liu et al., 2018; Memon et al., 2009a; Memon et al., 2009b; Neumann et al., 2020).

In this study, key factors influencing tissue regeneration of embryonic tips, cotyledonary nodes, and hypocotyl segments were optimized in Wucai (Fig. 3 and Tables 1,2,3,4). These results demonstrated that embryonic tips exhibited higher regeneration frequencies and number of regenerated shoots per

explant compared to hypocotyls and cotyledonary nodes (Figs. 9A-9B). The embryonic tip regeneration system, well established in other crops like soybean (*Glycine max*) (Dang and Wei, 2007;Liu et al., 2004;Liu et al., 2016) and maize (*Zea mays*) (Huang and Wei, 2004), exhibits distinct advantages, including higher regeneration frequency, stronger meristematic ability, faster formation and elongation of adventitious shoots, and better repeatability (Liu et al., 2004;Liu et al., 2016). Similar features were observed in this study and may be attributed to the greater number of meristematic cells and enhanced division potential in the promeristem and procambium regions of embryonic tips (Fig. 2). Furthermore, embryonic tips exhibited a shorter growth cycle compared to cotyledonary nodes and hypocotyls (Fig. 9C), likely due to the direct regeneration of shoots from embryonic tips without an intervening callus stage (Fig. 6). In contrast, cotyledonary nodes and hypocotyls required callus formation before adventitious shoot development (Figs. 7 and 8).

Seedling age significantly affects *in vitro* plant tissue culture outcomes and explant quality (Zhao et al., 2021). For Chinese cabbage, explants from young seedlings are commonly employed due to their relatively straightforward regeneration process. Optimal shoot regeneration responses are often observed in explants from 4- to 5-day-old seedlings in systems involving hypocotyls and cotyledons (Baskar et al., 2016;Li et al., 2021;Sivanandhan et al., 2019;Sivanandhan et al., 2023;Zhao et al., 2021). These observations align with our findings, where hypocotyls and cotyledonary nodes of Wucai exhibited the highest regeneration frequencies using 4-day-old seedlings (Figs. 7 and 8). Interestingly, the embryonic tip regeneration system exhibited an even younger optimal seedling age. The highest shoot regeneration frequency and the greatest number of shoots per explant were achieved with 60-hour-old germinated seeds (Fig. 3). However, soybean embryonic tip explants require only 20- to 24-hour-old germinated seeds (Chen et al., 2011;Dang and Wei, 2007;Liu et al., 2004;Liu et al., 2016), a significantly shorter duration than observed in this study. This disparity could arise from the challenges of effectively obtaining embryonic tip explants from the small seeds of Wucai (Fig. 2), and the difficulty in inducing adventitious shoots from rapidly germinated seedlings (Fig. 3).

Plant growth regulators are critical for *in vitro* plant regeneration. During the shoot induction stage, the combination of BAP and NAA has been shown to be highly effective in Chinese cabbage regeneration systems (Abbasi et al., 2011;Baskar et al., 2016;Cui et al., 2017;Li et al., 2021;Sivanandhan et al., 2019;Sivanandhan et al., 2023). Higher concentrations of BAP ($2-4 \text{ mg}\cdot\text{L}^{-1}$) and lower concentrations of NAA ($0.5-1.0 \text{ mg}\cdot\text{L}^{-1}$) have proven effective for hypocotyl and cotyledon explants. However, for embryonic tips, cotyledonary nodes, and hypocotyls, optimal regeneration outcomes were achieved using relatively lower concentrations of both BAP and NAA in SIMs (Tables 3–4). This difference may be attributed to the pre-culture strategy employed in this study, where the PCMs were supplemented with relatively higher concentrations of BAP and NAA (Tables 1–2). Pre-culture has been demonstrated to enhance cell division and transformation by mitigating the hypersensitive response of explants and stimulating cell division (Li et al., 2021;Ma et al., 2017). In this study, pre-culture with high BAP concentration for 36 hours activated the physiological and biochemical state of explants, enhancing cell

differentiation and division during pre-culture (Fig. 4), ultimately leading to significantly improved regeneration efficiencies (Table 1).

Conclusions

In conclusion, an efficient plant regeneration protocol for Wucai was successfully developed by systematically optimizing explant type, seedling age, pre-culture conditions, and plant growth regulator combinations. Among the tested systems, the embryonic tip regeneration system emerged as the most effective, providing a reliable and efficient *in vitro* propagation method. This protocol holds significant potential for advancing genetic transformation efforts and developing novel Wucai cultivars.

Declarations

Author Contributions

D.Y., N.L., Q.Y., and G.C. wrote the original manuscript. N.L. and W.W. reviewed and edited the manuscript. X.T. and G.C. supervised. K.W. and T. L. Visualized. Q.Y. data curated. L.Y. Formal analyzed. C.W. and G.C. Funding acquiesced. All authors read and approved the manuscript.

Funding

This work was supported by the Anhui Provincial Natural Science Foundation (2308085MC96); Hefei Natural Science Foundation (202343); Anhui Provincial Program on Major Basic Research Project (2023z04020005); Excellent Young Talents Fund Program of Higher Education Institutions of Anhui Province (2024AH030017).

Data Availability

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors agree with the submission of this manuscript to Plant Methods.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Abbasi B H, Khan M, Guo B, Bokhari S A, Khan M A (2011) Efficient regeneration and antioxidative enzyme activities in *Brassica rapa* var. *turnip*. Plant Cell, Tissue and Organ Culture 105:337-344
2. Baskar V, Gangadhar B H, Park S W, Nile S H (2016) A simple and efficient *Agrobacterium tumefaciens*-mediated plant transformation of *Brassica rapa* ssp. *pekinensis*. 3 Biotech 6:88
3. Chen G, Wang J, Wang H, Wang C, Tang X, Li J, Zhang L, Song J, Hou J, Yuan L (2020) Genome-wide analysis of proline-rich extension-like receptor protein kinase (PERK) in *Brassica rapa* and its association with the pollen development. BMC Genomics 21:401
4. Chen G, Zeng F, Wang J, Ye X, Zhu S, Yuan L, Hou J, Wang C (2019) Transgenic Wucai (*Brassica campestris* L.) produced via *Agrobacterium*-mediated anther transformation in planta. Plant Cell Reports 38(5):577-586
5. Chen G, Zhang S, Yuan L, Zhu S, Liu S, Zhang H, Wang C (2017) Establishment of effective regeneration system of Wucai *in vitro*. Molecular Plant Breeding 15(4):1466-1472
6. Chen W, Song K, Cai Y, Li W, Liu B, Liu L (2011) Genetic modification of soybean with a novel grafting technique: downregulating the *FAD2-1* gene increases oleic acid content. Plant Molecular Biology Reporter 29:866-874
7. Cogbill S, Faulcon T, Jones G, McDaniel M, Harmon G, Blackmon R, Young M (2010) Adventitious shoot regeneration from cotyledonary explants of rapid-cycling fast plants of *Brassica rapa* L. Plant Cell, Tissue and Organ Culture 101:127-133
8. Cui J, Li M, Qiu L, Cao J, Huang L (2017) Stable expression of exogenous imported sporamin in transgenic Chinese cabbage enhances resistance against insects. Plant Growth Regulation 81:543-552
9. Dang W, Wei Z (2007) An optimized *Agrobacterium*-mediated transformation for soybean for expression of binary insect resistance genes. Plant Science 173(4):381-389
10. Fang S, Ma Y, Liu Z, Feng H, Zhang Y (2023) Successful application of microspore culture in pakchoi (*Brassica campestris* L. ssp. *chinensis* Makino var. *communis* Tsen et Lee) for hybrid breeding. Protoplasma 260:545-555
11. Gao Y, Jia J, Cong J, Ma Y, Feng H, Zhang Y (2020) Non-ionic surfactants improved microspore embryogenesis and plant regeneration of recalcitrant purple flowering stalk (*Brassica campestris* ssp. *chinensis* var. *purpurea* Bailey). In Vitro Cellular & Developmental Biology-Plant 56:207-214
12. Huang X, Wei Z (2004) High-frequency plant regeneration through callus initiation from mature embryos of maize (*Zea mays* L.). Plant Cell Reports 22:793-800
13. Jeong S Y, Ahn H, Ryu J, Oh Y, Sivanandhan G, Won K H, Park Y D, Kim J S, Kim H, Lim Y P, Kim S G (2019) Generation of early-flowering Chinese cabbage (*Brassica rapa* spp. *pekinensis*) through CRISPR/Cas9-mediated genome editing. Plant Biotechnology Reports 13:491-499
14. Jia J, Zhang Y, Cui L, Feng H (2019a) Effect of thidiazuron on microspore embryogenesis and plantlet regeneration in Chinese flowering cabbage (*Brassica rapa*. var. *parachinenis*). Plant Breeding 138:916-924

15. Jia J, Zhang Y, Feng H (2019b) Effects of brassinolide on microspore embryogenesis and plantlet regeneration in pakchoi (*Brassica rapa* var. *multiceps*). *Scientia Horticulturae* 252:354-362
16. Li X, Li H, Zhao Y, Zong P, Zhan Z, Piao Z (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
17. Liu H K, Yang C, Wei Z M (2004) Efficient *Agrobacterium tumefaciens*-mediated transformation of soybeans using an embryonic tip regeneration system. *Planta* 219(6):1042-1049
18. Liu S, Li G, Qu J, Guan S, Yao D, Wang P, Liu S, Jia Y, Guan P, Wang L (2016) Establishment of an embryonic tip regeneration system of soybean. *Brazilian Archives of Biology and Technology* 59:e16160548
19. Liu W, Yang Y, Liu Q (2018) Establishment of an efficient regeneration system using heading leaves of Chinese cabbage (*Brassica rapa* L.) and its application in genetic transformation. *Horticulture, Environment, and Biotechnology* 59:583-596
20. Liu X, Liao N, Tang X, Wang K, Wang W, Khan A, Wang C, Yuan L, Chen G (2025) TMT-label comparative proteomics reveals the vernalization mechanism in Wucai (*Brassica campestris* L.). *Journal of Proteomics* 314:105398
21. Liu X, Wang Y, Tang X, Wang W, Khan A, Pang X, Wang Y, Wang C, Yuan L, Hou J, Chen G (2024) Genome-wide identification, transcript profiling and functional analyses of *PCP* gene family in Wucai (*Brassica campestris*). *Scientific Reports* 14:28236
22. Lu Y, Dai S, Gu A, Liu M, Wang Y, Luo S, Zhao Y, Wang S, Xuan S, Chen X (2016) Microspore induced doubled haploids production from ethyl methanesulfonate (EMS) soaked flower buds is an efficient strategy for mutagenesis in Chinese cabbage. *Frontiers in Plant Science* 7:1780
23. Ma X, Zhu Z, Li Y, Yang G, Pei Y (2017) Expressing a modified cowpea trypsin inhibitor gene to increase insect tolerance against *Pieris rapae* in Chinese cabbage. *Horticulture, Environment, and Biotechnology* 58:195-202
24. Memon S A, Hou X, Wang L, Li Y (2009a) Promotive effect of 5-aminolevulinic acid on chlorophyll, antioxidative enzymes and photosynthesis of Pakchoi (*Brassica campestris* ssp. *chinensis* var. *communis* Tsen et Lee). *Acta Physiologiae Plantarum* 31(1):51-57
25. Memon S A, Hou X, Zhu B, Wolukau J (2009b) High-frequency adventitious shoots regeneration from leaf of non-heading Chinese cabbage (*Brassica campestris* ssp. *chinensis*) cultured *in vitro*. *Acta Physiologiae Plantarum* 31:1191-1196
26. Neumann M, Prah S, Caputi L, Hill L, Kular B, Walter A, Patallo E P, Milbredt D, Aires A, Schöpe M (2020) Hairy root transformation of *Brassica rapa* with bacterial halogenase genes and regeneration to adult plants to modify production of indolic compounds. *Phytochemistry* 175:112371
27. Niu L, Shi F, Feng H, Zhang Y (2019) Efficient doubled haploid production in microspore culture of Zengcheng flowering Chinese cabbage (*Brassica campestris* L. ssp. *chinensis* [L.] Makino var. *utilis* Tsen et Lee). *Scientia Horticulturae* 245:57-64

28. Sivanandhan G, Choi S B, Moon J, Choi S R, Kim S G, Park Y D, Lim Y P (2019) High frequency in vitro regeneration of Chinese cabbage (cv. *Kenshin*) from hypocotyl and cotyledon explants. Horticultural Science and Technology 37(5):640-650
29. Sivanandhan G, Kapildev G, Selvaraj N, Lim Y P (2023) The effect of chitosan and β -cyclodextrin on glucosinolate biosynthesis in *Brassica rapa* ssp. *pekinensis* (Chinese cabbage) shoot culture. Plant Physiology and Biochemistry 194:570-575
30. Sivanandhan G, Moon J, Sung C, Bae S, Yang Z H, Jeong S Y, Choi S R, Kim S G, Lim Y P (2021) L-Cysteine increases the transformation efficiency of Chinese cabbage (*Brassica rapa* ssp. *pekinensis*). Frontiers in Plant Science 12:767140
31. Toma T I, Biswas A, Akhter S, Islam T, Hoque M I, Sarker R H (2022) Establishment of an efficient *in vitro* regeneration protocol for *Brassica campestris* L. var. Tori-7. Plant Tissue Culture and Biotechnology 32(2):237-246
32. Zhao Y, Huang S, Zhang Y, Shi F, Liu X, Du S, Feng H (2021) Establishment of an efficient shoot regeneration system in vitro in *Brassica rapa*. In Vitro Cellular & Developmental Biology-Plant 57:977-986

Figures

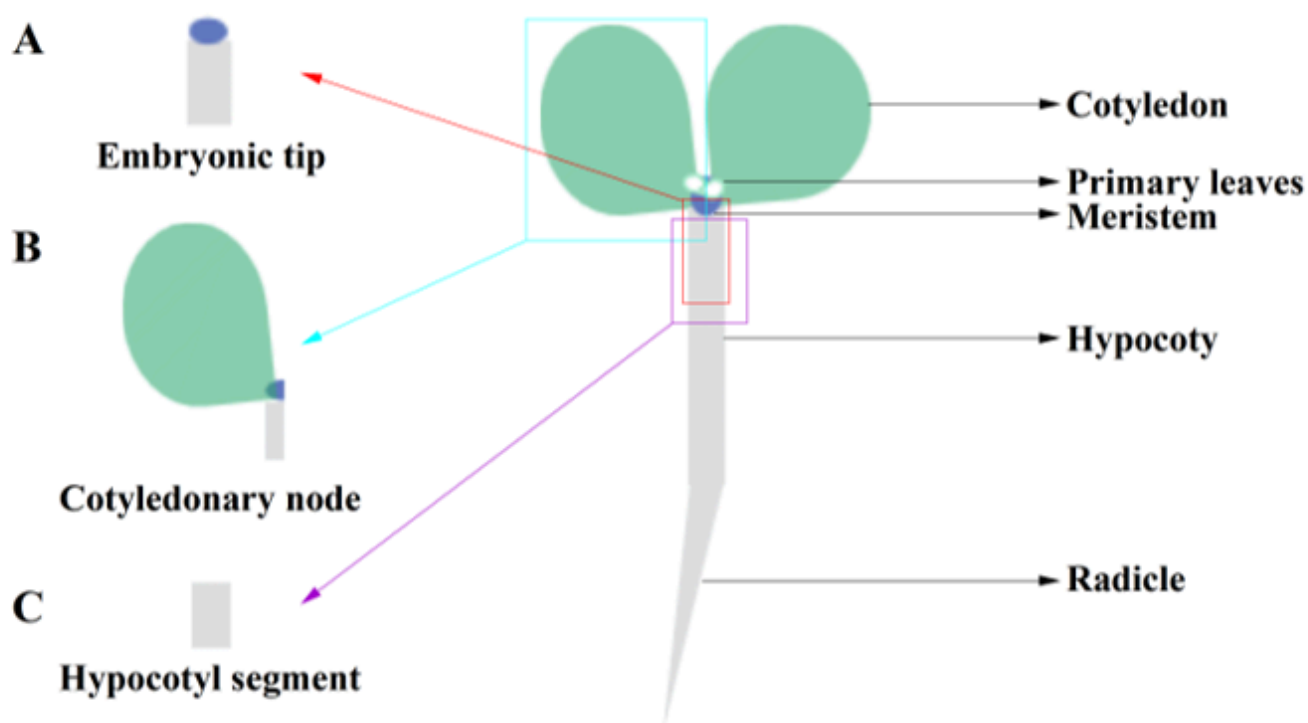


Figure 1

Preparation of different explants. (A) Embryonic tip. (B) Cotyledonary node. (C) Hypocotyl segment.

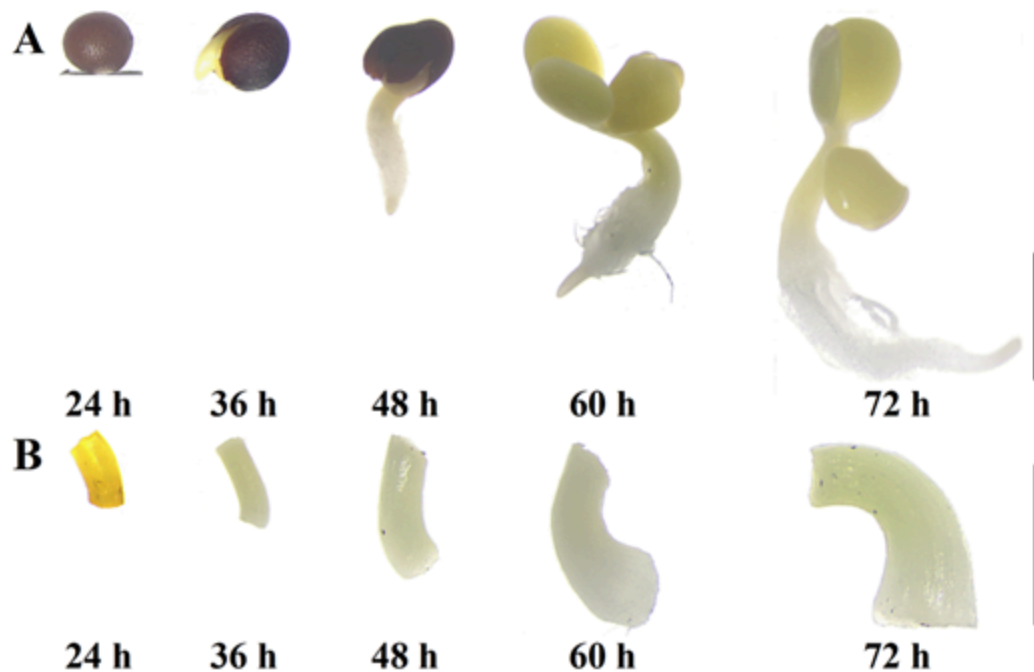


Figure 2

Morphology of embryonic tip explants from mature seeds germinated for different durations. (A) Germinated seeds of varying times. (B) Embryonic tips from germinated seeds of varying times. Scale bar = 3 mm.

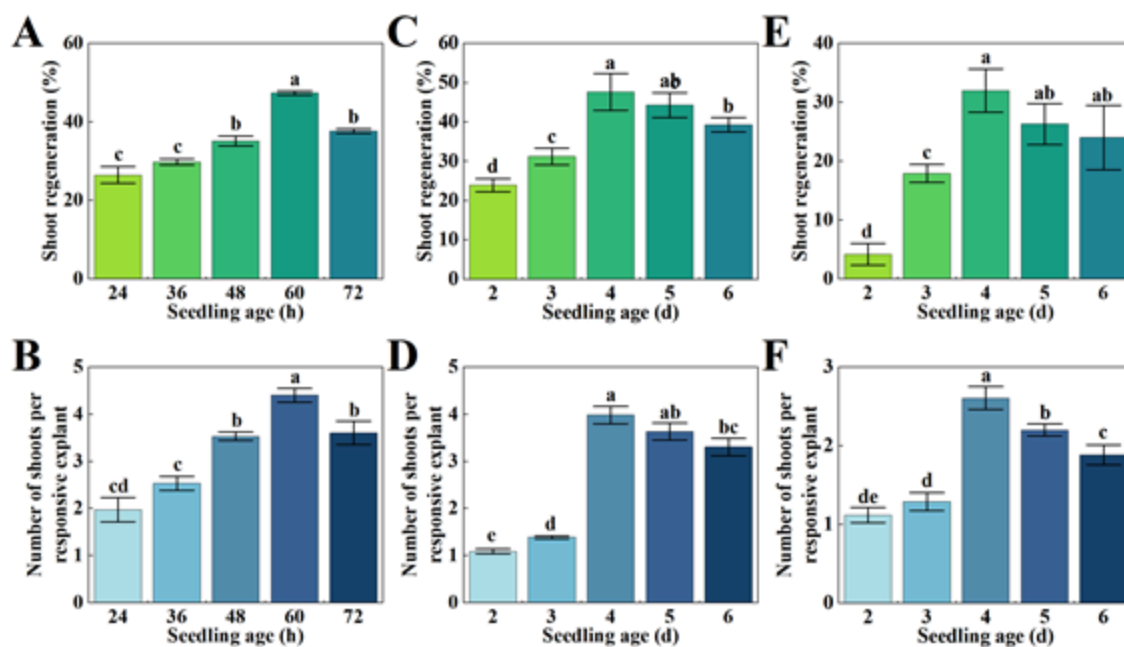


Figure 3

Effects of seedling age on shoot regeneration from embryonic tips (A and B), cotyledonary nodes (C and D), and hypocotyls (E and F). Data are mean values $\pm$ S.E. of three replicates. Different letters above bars indicate significant differences according to Duncan's new multiple range at $P < 0.05$.

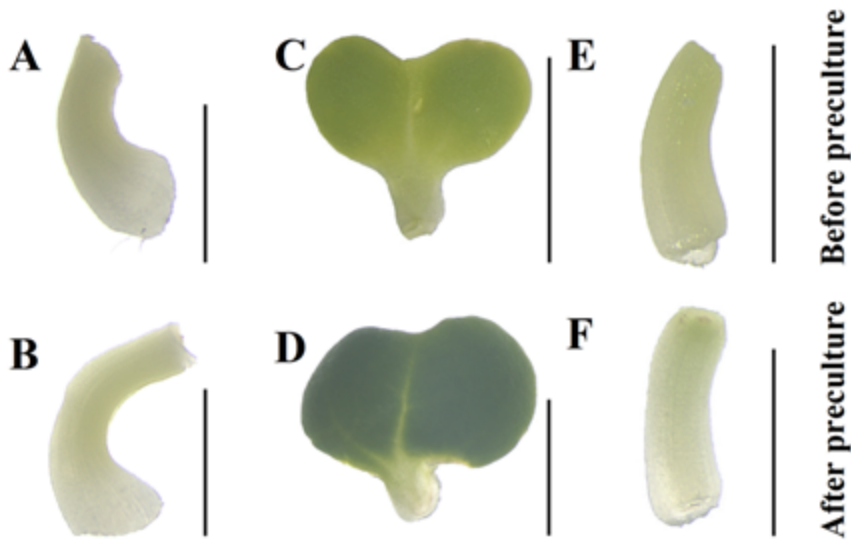


Figure 4

Comparison of explants before and after pre-culture. (A and B) Embryonic tips. (C and D) Cotyledonary nodes. (E and F) Hypocotyls. Scale bar = 3 mm.

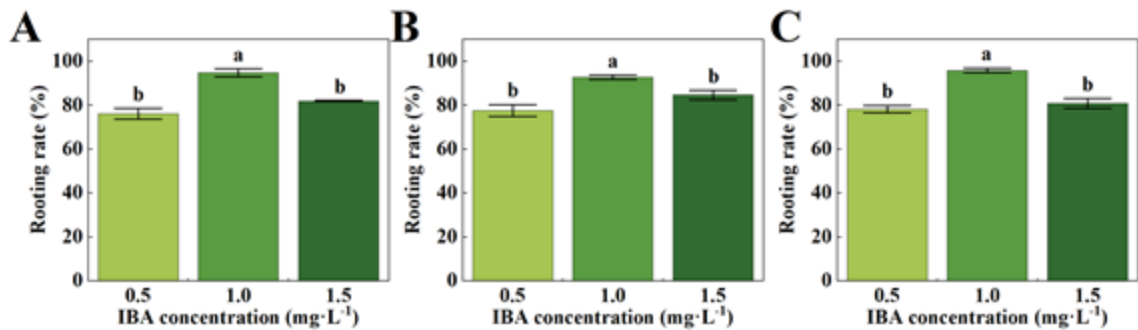


Figure 5

Effect of IBA concentrations on rooting. (A) Embryonic tip. (B) Cotyledonary node. (C) Hypocotyl. Data are mean values $\pm$ S.E. of three replicates. Different letters above bars indicate significant differences according to Duncan's new multiple range at $P < 0.05$.

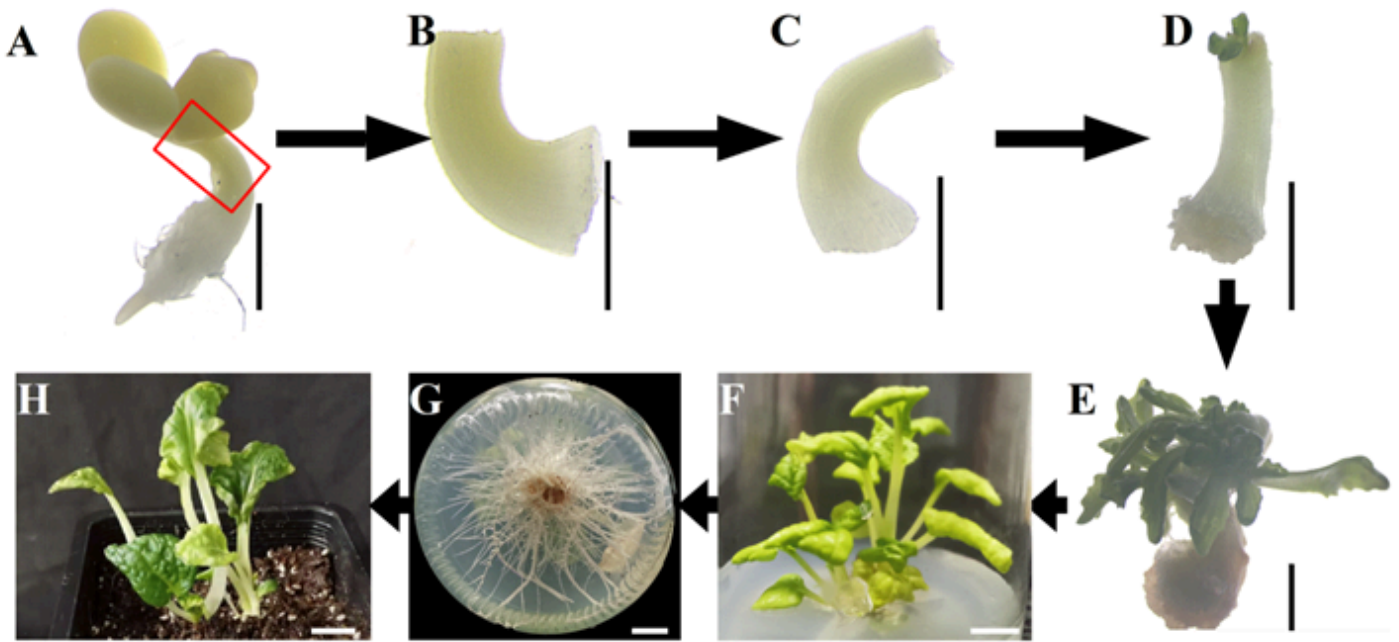


Figure 6

Embryonic tip regeneration systems from Wucan. (A) 60-hour-old seedling. (B) Embryonic tip explant. (C) Embryonic tip after 36 h of pre-culture. (D) Adventitious shoots induced for 7 days. (E) Multiple adventitious shoots after approximately 20 d of culture. (F) Regenerated shoot. (G) Rooting. (H) Regenerated plant transplanted in the greenhouse. Scale *bar* = 3 mm in A-E; scale *bar* = 1 cm in F-H.

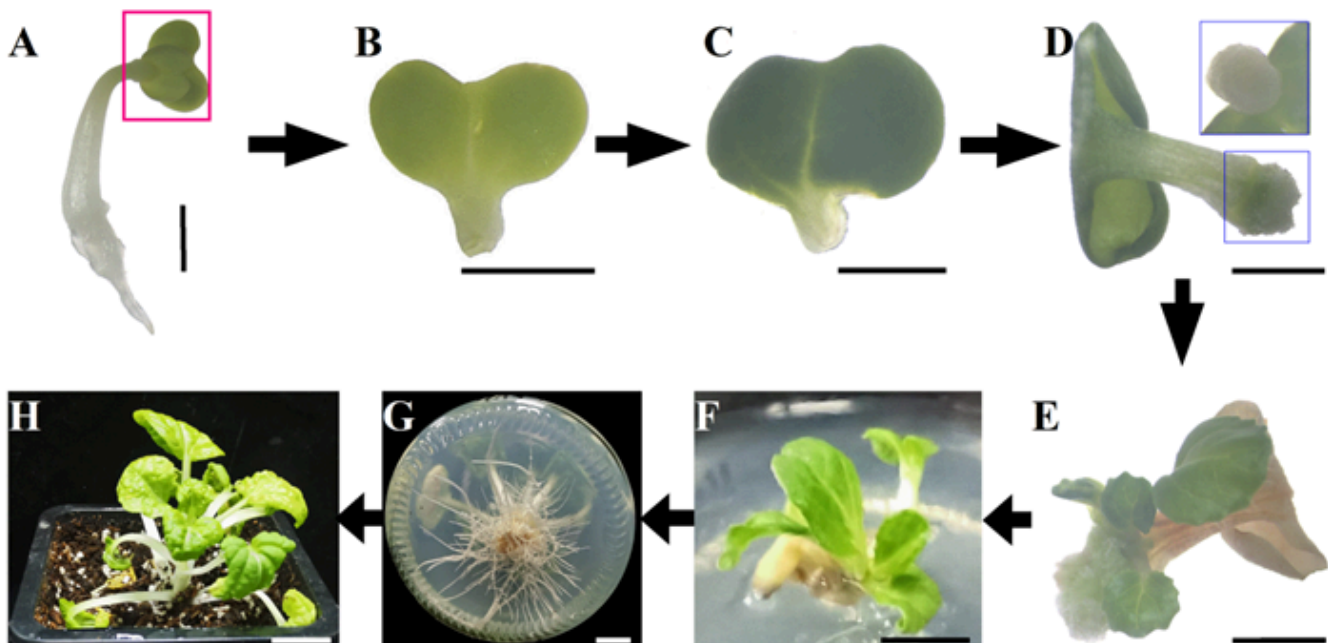


Figure 7

Cotyledonary node regeneration systems from Wucai. (A) 4-day-old seedling. (B) Cotyledonary node explant. (C) Cotyledonary node after 36 h of pre-culture. (D) Callus induced for 2 weeks. (E) Multiple adventitious shoots after approximately 30 d of culture. (F) Regenerated shoot. (G) Rooting. (H) Regenerated plant transplanted in the greenhouse. Scale *bar* = 3 mm in A-E; scale *bar* = 1 cm in F-H.

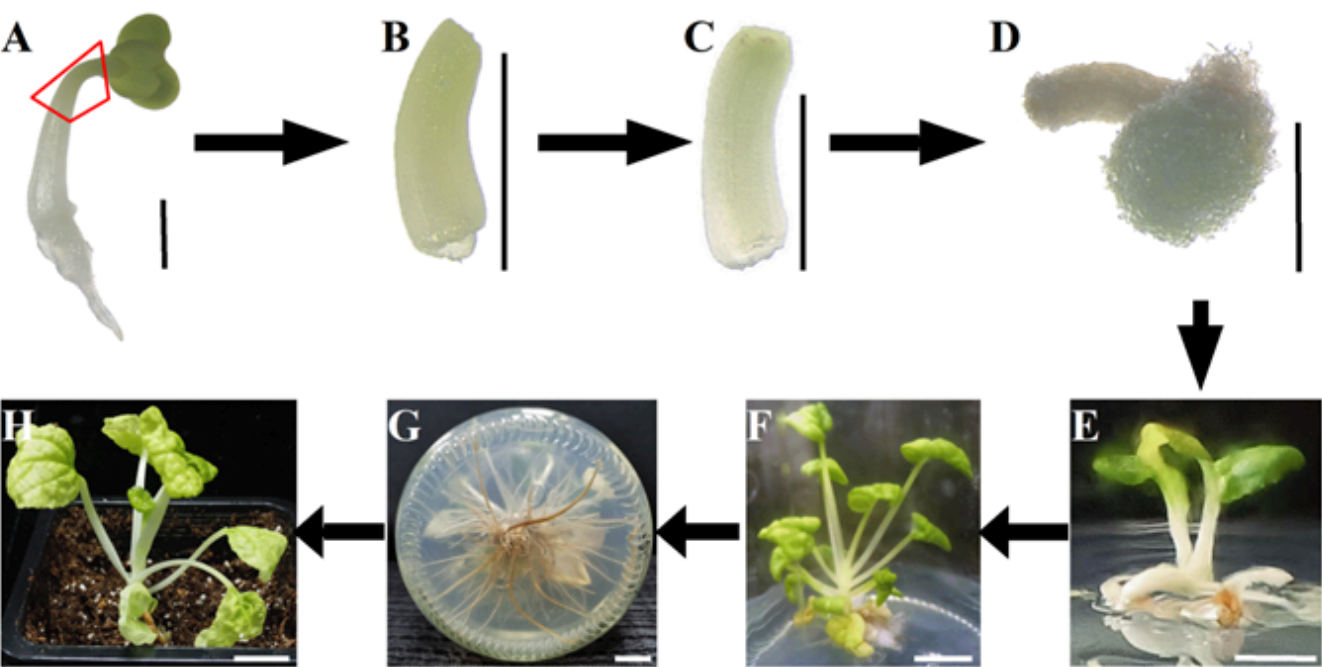


Figure 8

Hypocotyl segment regeneration systems from Wucai. (A) 4-day-old seedling. (B) Hypocotyl segment explant. (C) Hypocotyl segment after 36 h of pre-culture. (D) Callus induced for 2 weeks. (E) Adventitious shoot after approximately 35 d of culture. (F) Regenerated shoot. (G) Rooting; (H) Regenerated plant transplanted in the greenhouse. Scale *bar* = 3 mm in A-E; scale *bar* = 1 cm in F-H.

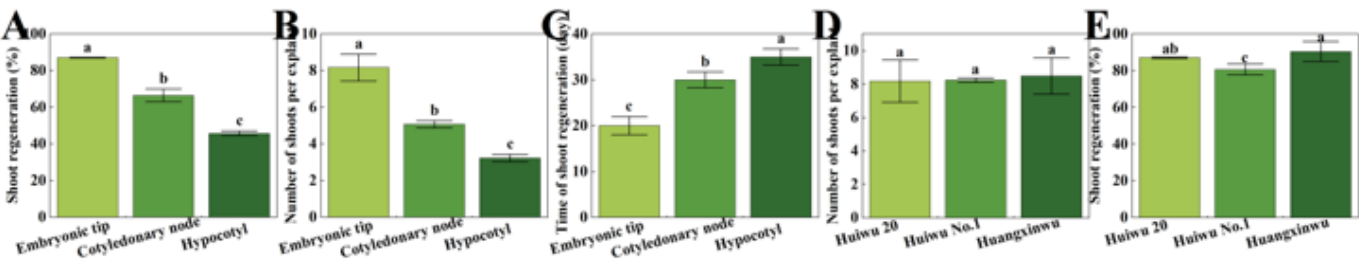


Figure 9

Comparison of different explant regeneration systems of Wucan. (A) Shoot regeneration frequency. (B) Number of shoots per responsive explant. (C) Shoot regeneration time. (D) Shoot number per explant for different Wucan genotypes. (E) Shoot regeneration frequency for different Wucan genotypes. Data are mean values $\pm$ S.E. of three replicates. Different letters above bars indicate significant differences according to Duncan's new multiple range at $P < 0.05$.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [TableS.docx](#)