Plant Biotechnology 40, 83–86 (2023)
DOI: 10.5511/plantbiotechnology.22.1124a

Short Communication

Transformation of *Nicotiana paniculata* L., a recalcitrant species, using a T-DNA construct carrying two *WUSCHEL-related homeobox* genes

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Received October 18, 2022; accepted November 24, 2022 (Edited by M. Nakano)

Abstract A binary vector carrying two *WUSCHEL-related homeobox* (*WOX*) genes, *WOX2* and *WOX8*, under the control of a chemical-inducible expression system, worked in the transformation in *N. paniculata*, a recalcitrant species of *Nicotiana*. The resulting transformants exhibited improved culture performance in regeneration from leaf segments and suspended cells. Multicellular masses generated from freely suspended cells showed a specific cell division pattern similar to that of somatic embryo, likely owing to the function of the two *WOX* genes.

Key words: *Nicotiana paniculata*, *WOX2*, *WOX8*, *WUSCHEL-related homeobox*, XVE system.

Since the classical tissue culture experiments in *Nicotiana tabacum* (Skoog and Miller 1957), it is well known that shoot primordia are induced by cytokinin. However, the empirical rules do not apply to all plant species. Without a means to induce regeneration, it is difficult to produce transgenic plants, and molecular breeding techniques cannot be applied. Therefore, improving the regeneration performance by introducing a specific gene into the genome of such recalcitrant species is an effective approach. Previous studies have reported many regeneration-promoting genes (reviewed by Gordon-Kamm et al. 2019). However, only a few genes have been successfully applied in different species. Thus, it is desirable to identify universal regeneration-promoting genes that are applicable to recalcitrant species as well.

WOX genes were defined in *Arabidopsis thaliana*, of which *WOX2*, *WOX8* and *WOX9* show spatiotemporal expression patterns and play important roles in early embryogenesis (Breuninger et al. 2008; Haecker et al. 2004). We established *N. tabacum* transgenic lines possessing one of the *Arabidopsis* genes under the control of a chemical-inducible expression system (XVE system, Zuo et al. 2000), and found that hybrids between transgenic lines possessing the *WOX2* or *WOX8* showed higher performance of regeneration from leaf tissue depending on the transgene expression than the parental lines (Kyo et al. 2018). We designed a binary vector carrying the two *Arabidopsis* genes (referred to as pER8::*WOX2*+*WOX8*, Figure 1A) and used it to transform *Rhizobium radiobacter* AGL1 for *N. tabacum* transformation (Kyo et al. 2023). In the present study, we applied the transformation method to *N. paniculata*, which is one of the most recalcitrant five among 53 *Nicotiana* species (115 accessions), as reported in previous culture experiments (Li et al. 2003). We also cultured wild type *N. paniculata* according to the manner in the present study, and had the impression that regeneration would not be easily induced (Supplementary Figure S1D).

We used mMS as the basal medium for tissue culture and two stock solutions of 17-β-estradiol—the expression inducer for the XVE system—and 3′,5′-dimethoxy-4′-hydroxyacetophenone (acetosyringone)—the bacterial vir gene activator—prepared as described previously (Kyo et al. 2018). The culture temperature was maintained at 25±1°C. To solidify the medium, agar was added at the concentration of 0.8%. *N. paniculata* (wild type) was seeded on vermiculite and grown indoors at 25°C under fluorescent lighting. The transformation protocol comprised three periods; i) co-culture period, ii) induction period, and iii) regeneration period.

Co-culture period: Leaves were harvested from 1-month-old *N. paniculata* plants and surface-sterilized with 10-fold dilution of commercial bleach (Kitchen haiter, Kao Corp., Tokyo). After rinsing well, the leaves were partly injured using a scalpel and soaked in 3 ml liquid mMS with 1 μM 2,4-D and 100 μM acetosyringone. Thirty microliter aliquots of overnight culture (LB

Abbreviations: BA, 6-benzylaminopurine; mMS, modified Murashige and Skoog medium; 2,4-D, 2,4-dichlorophenoxyacetic acid; *WOX*, *WUSCHEL-related homeobox*; XVE, LexA-VP16-estrogen receptor chimeric transcription activator.
Published online February 9, 2023

medium with 100 mg l^{-1} spectinomycin) of *R. radiobacter* AGL1 possessing pER8::*WOX2*+*WOX8* were added to the cultures (Figure 1B). After 3 h incubation in the dark, after blotting off the excess liquid on a filter paper, the leaves were placed on solid mMS with 1 μM 2,4-D and kept in the dark for 4 days; the medium was replaced with fresh medium depending on the extent of bacterial proliferation. Another experimental result, including a control experiment using LB medium without bacteria, was shown in Supplementary Figure S1A.

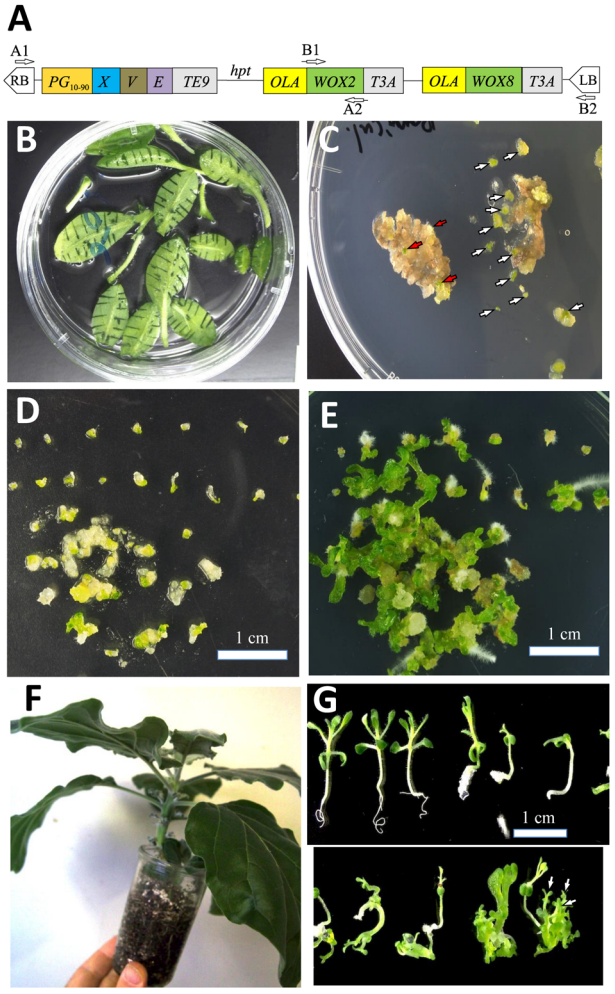

Figure 1. Regeneration process of *N. paniculata* leaves infected with *R. radiobacter* AGL1 with pER8::*WOX2*+*WOX8*. (A) Construct of the T-DNA region. *OLA*: operator LexA, *T3A*: 3A terminator, *XVE*: coding region for LexA-VP16-estrogen receptor chimeric transcription factor, *hpt*: hygromycin resistance gene, RB: right border sequence, LB: left border sequence. For other abbreviations, see Zuo et al. (2000). Arrows indicate the position of the primers. (B) Injured leaves at the beginning of the co-culture period. The diameter of the dish was 6 cm. (C) Appearance of green spots and clumps at the end of the induction period. Red arrows: those in the original positions, white arrows: isolated from the tissue segment. The diameter of the dish was 9 cm. (D, E) Morphological aspects at the beginning (D) and on day 14 (E) of the regeneration period. (F) Regenerants cultivated in a bottle. (G) The morphology of seedlings was classified into normal (left three in the top panel) and abnormal, and the latter was further classified into mild (right three in the top panel) and severe (bottom panel). The shoots indicated by arrows were subcultured as donor plants for tissue and cell culture.

Induction period: On day 5, leaves were rinsed with liquid mMS with 1 μM 2,4-D and 100 mg l^{-1} meropenem (inhibitor of bacterial proliferation) and placed on solid mMS containing 1 μM 2,4-D, 50 mg l^{-1} meropenem, and 10 μM β-estradiol in the dark for 2 weeks; the medium was replaced with fresh medium once at the midterm. Then, the leaf tissues were placed on a medium with the same composition except for the additional 30 mg l^{-1} hygromycin B—a selection compound for transformants with the T-DNA—and cultured in light conditions for 10 days (Figure 1C). As indicated by red arrows, green or light-colored parts were observed on the brown tissue in several places. By teasing the tissue with forceps, several callus clumps were isolated, as indicated by white arrows. In the three culture experiments of similar scale, the average frequency of clumps per leaf was five. However, the frequency seemed to change depending on the timing of medium exchange and the concentration of auxin and expression inducer. Supplementary Figure S1A shows the case where hygromycin was not added to the medium. In this case, green clumps or small shoots appeared together with actively growing callus. Such regenerative responses were observed only in cultures infected with the bacteria.

Regeneration period: The isolated callus clumps were placed on solid mMS with 50 mg l^{-1} meropenem and cultured in light conditions (Figure 1D). After 14 days, shoots appeared in many clumps (Figure 1E). These shoots were cut and placed on fresh medium to facilitate the development of normal-shaped plantlets, as reported in a previous study (Kyo et al. 2023). The resulting plantlets were placed on sterilized soil in bottles. As the plants grew, the lids of the bottles were removed, and the plants were allowed to grow outward (Figure 1F); then, they were transplanted into pots for maturity and allowed to bloom. Selfed seeds were aseptically sown on solid half-strength mMS (1/2 mMS). Immediately after germination, they were placed on solid 1/2 mMS with 1 μM β-estradiol. After 1 month, the seedlings showed abnormal morphology, especially for the root tip parts. One line that clearly segregated into normal and abnormal morphology—the latter showing mild and severe aberrations likely corresponding to heterozygous and homozygous transgenics, respectively—was selected as the material for subsequent experiments (Figure 1G). The transgene was detected from seedlings showing abnormal morphology but not from normal morphology (Supplementary Figure S1C). From the plants showing severe aberrations, several adventitious shoots (as indicated by arrows in Figure 1G) were cut and placed on solid mMS and grown aseptically (Figure 2A) to serve as a donor for harvesting leaves (Figure 2B) for expression analysis and culture experiments of leaf segments and suspended cells.

Expression analysis: The leaf blades were excised, cut

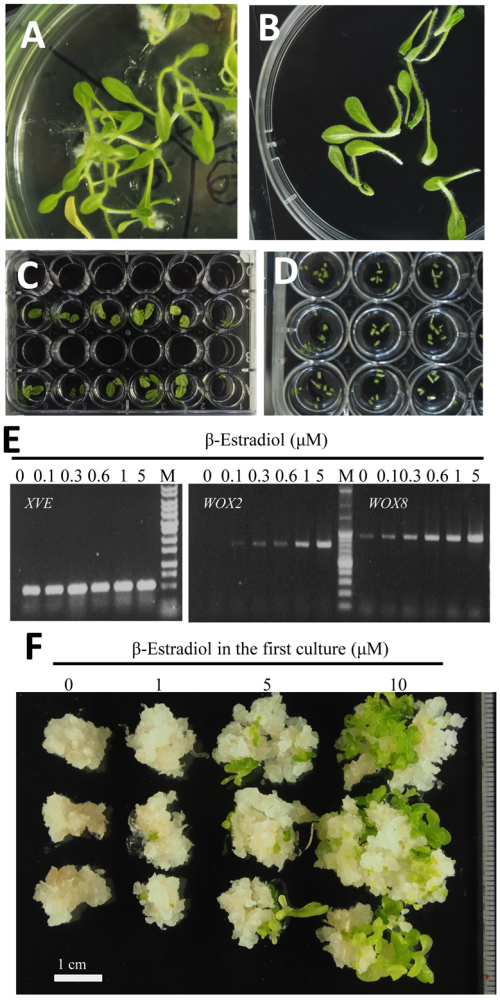

Figure 2. *N. paniculata* leaf tissue culture and expression analysis. (A) Plants derived from the shoots generated on seedlings treated with *β*-estradiol. The diameter of the dish was 10 cm. (B) Leaves isolated from the donors shown in (A). (C) Leaf blades treated with *β*-estradiol for expression analysis. The diameter of the wells was 15 mm. (D) Petioles treated with *β*-estradiol for tissue culture. (E) Expression analysis. The size of amplified products for *XVE*, *WOX2*, and *WOX8* was calculated to be 527, 806, and 1,002 bp, respectively, and a band corresponding to each size was observed. (F) Effect of *β*-estradiol in the first culture on regeneration from leaf segments of the transformants.

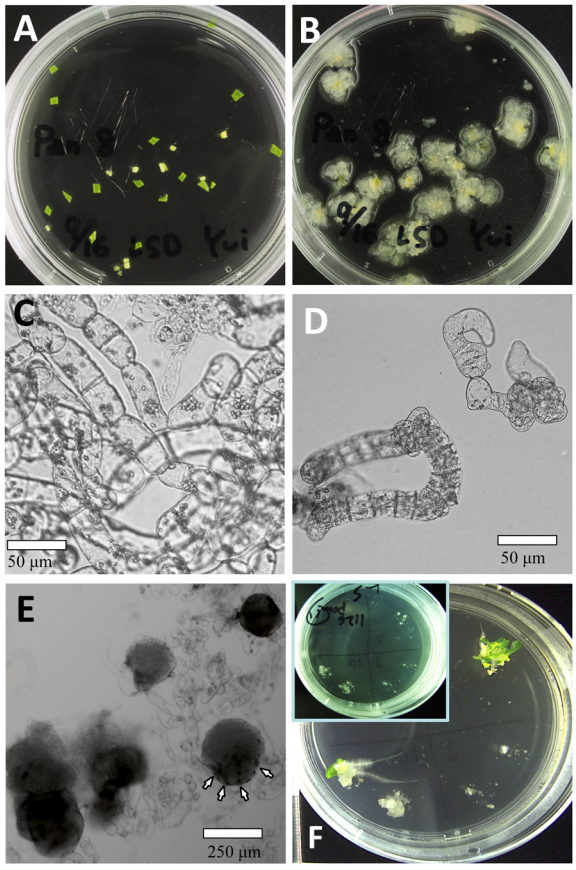

Figure 3. *N. paniculata* suspended cell culture. (A, B) Morphological aspects of leaf and petiole segments culture at the beginning (A) and after 3 weeks (B). The diameter of the dish was 6 cm. (C) Suspended cells released from the callus shown in panel B. (D) Multicellular masses generated in the conditioned medium supplemented with 1 *μ*M *β*-estradiol. (E) Embryo-like structures developed from freely suspended cells. Arrows indicate the early signs of browning. (F) Development of plantlets on solid mMS. Aspects at the beginning (inset) and after 3 weeks. The diameter of the dish was 7 cm.

with a scalpel, and placed in liquid mMS containing *β*-estradiol at concentrations ranging from 0 to 5 *μ*M (Figure 2C). After 48 h, total RNA was extracted from the segments and used for RT-PCR analysis as described previously (Kyo et al. 2018; Rashid et al. 2007). While the expression level of *XVE* was constitutive, that of *WOX2* and *WOX8* increased depending on the concentration of the inducer (Figure 2E). The results suggest that the donor plant was a reliable material for the two subsequent experiments although low expression of *WOX8* was detected in the absence of the inducer.

Petiole segment culture: We examined the effect of the expression inducer on the regeneration performance in petiole segment culture. The petioles were cut into 2–3 mm segments and placed in mMS with 1 *μ*M 2,4-D and *β*-estradiol (0, 1, 5 and 10 *μ*M) for 10 days in the dark (first culture) using multi-well plates (Figure 2D). After rinsing, the segments were placed on solid mMS and kept for 3 weeks in a light condition (second culture). Appearance of shoots, depending on the concentration of the inducer, was observed together with vigorous growth of white calli (Figure 2F). A control experiment using wild type was shown in Supplementary Figure S1E.

Suspended cell culture: Approximately 2–3 mm square sections of the leaves and petioles were placed in 3 ml of mMS with 1 *μ*M 2,4-D and kept without agitation in the dark for 3 weeks (Figure 3A). In response to auxin, cells elongated and divided to break the tissues (Figure 3B, C). Using gentle agitation, cells were released from the callus into the medium; the aliquots of the cell suspension were placed into 3.5 cm diameter Petri dishes, and concentrated *β*-estradiol in DMSO or DMSO was added to the culture (1 or 0 *μ*M). After culturing without agitation in the dark for 1 week, specific multicellular masses appeared in the presence of the inducer (Figure

3D). The morphological trait of the masses seemed to be the result of the manner of cell division with suppression of elongation. After one more week, they developed into somatic embryo-like structures with a smooth body surface (Figure 3E). As shown in Supplementary Figure S1B, neither multicellular masses nor embryo-like structures appeared in the control culture without the inducer. In addition, suspension cells were also obtained using explants from wild type plants but showed no response to the inducer (data not shown).

The embryo-like structures were placed on solid mMS as several droplets with their conditioned medium. After 3 weeks, although the regeneration frequency was not high, a few plantlets developed from the cell population in the droplets (Figure 3F). Therefore, the structures observed in Figure 3E and 3F can be regarded as regenerative structures. The structures showed a tendency of browning over a longer period of culture; the early signs are observed in Figure 3E. Reduced concentrations of the inducer and auxin seem to be necessary for their development; thus, we had previously developed similar embryo-like structures by gradually adding fresh mMS to *N. tabacum* culture (Kyo et al. 2018). However, the optimal conditions and methods for their development remain unclear.

In conclusion, we demonstrated that the T-DNA carrying *WOX2* and *WOX8* under the control of the XVE system worked as an inducer for regeneration in leaf tissue culture of a recalcitrant species, *N. paniculata*. Considering the putative conserved function of the *WOX* genes, these demonstrative experiments may be broadly applicable to species other than *Nicotiana* species. Additionally, the successful methods for inducing embryo-like structures provide an experimental system to investigate which part of the transgenes causes freely suspended cells to develop such structures.

Acknowledgements

We would like to acknowledge the technical expertise of the members of the DNA core facility of the Gene Research Center, Kagawa University. We would like to thank the former laboratory members M. Nagata and Y. Kawanishi.

Author contribution

M.K. designed the research; M.H., M.T. and A.M. performed the experiments; and M.K. wrote the manuscript.

Conflict of interest

None declared.

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