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Note

Production of chrysanthemum periclinal chimeras through shoot regeneration from leaf explants

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Abstract Periclinal chimeras play important roles in vegetatively propagated plants such as chrysanthemum (*Chrysanthemum morifolium*). For example, periclinal chimerism causes flower color variation in chrysanthemums. In this study, a method for periclinal chimera production in chrysanthemum was examined. A wild-type plant of chrysanthemum 'Taihei' and its transgenic plant carrying a yellowish-green fluorescent protein gene from the marine plankton *Chiridius poppei* (*CpYGFP*) were used as plant materials. The cut faces of the leaf explants of both materials were partially attached and then were detached for further culture. Mosaic calli consisted of transgenic and wild-type cells formed on the detached faces of the explants. We examined 996 regenerated shoots from 4,120 explants and found only a single chimeric shoot that appeared to show mericlinal chimerism. Repeated axillary bud elongation from the nodes of the mericlinal chimera produced one L1-fluorescent and one L3-fluorescent chimeric plant. The L1 chimera showed fluorescence in the epidermal cells and trichomes of leaf and stem. The L3 chimera showed fluorescence in the cells of the central parts of stem and leaf, as well as in the whole root tissues. In summary, we obtained chrysanthemum periclinal chimeras through regeneration from leaf explants using the fluorescent protein transgene as a selection marker.

Key words: Asteraceae (Compositae), *Chrysanthemum morifolium*, CpYGFP, periclinal chimera, shoot regeneration.

The shoot apical meristems of plants commonly consist of three layers: the outermost layer (L1), the second outer layer (L2), and the inner layer (L3). L1 forms the epidermis, L2 forms the sub-epidermal tissues including gametes, and L3 forms the central tissues including the pith and roots (Geier 2012). Sectorial chimeras, having genetically different cell sectors forming in all three layers, represent an unstable chimeric condition that tends to disappear during vegetative growth. In contrast, a periclinal chimeric plant, having different cell layers can maintain its chimerism by vegetative propagation because cells of a shoot apex with a periclinal chimeric structure may elongate to form an entire plant of the same chimeric structure (Geier 2012). A plant with periclinal chimeric structure in some sectors is called a mericlinal chimera, which can be considered as an incomplete periclinal chimera. Periclinal chimerism plays an important role in vegetatively propagated plants including chrysanthemum (*Chrysanthemum morifolium*), one of the most important ornamental plants. In chrysanthemum sport cultivars, flower color differences are caused by periclinal chimerism (Shibata and Kawata 1986; Stewart and Dermen 1970). The development of methods for periclinal chimera production in chrysanthemum will be useful for its breeding in the future.

Periclinal chimeras spontaneously appear in nature by natural mutation. The artificial production of periclinal chimeras by grafting was first reported in the early 20th century, and the method has been adapted to plant species in the family Solanaceae and in *Brassica* species, as reviewed by Burge et al. (2002). Recently, cassava periclinal chimeras have also been developed by grafting (Nassar and Bomfim 2013). Another method of periclinal chimera production through shoot regeneration from in vitro-cultured cells has also been reported in the family Solanaceae (Binding et al. 1987; Carlson and Chaleff 1974; Marcotrigiano and Gouin 1984). We decided to use the latter method in this study, considering that the use of in vitro explants allows easy preparation of ample material. To identify the chimeric structure, phenotypic characteristics or ploidy levels of

Abbreviations: BA, 6-benzyladenine; GFP, green fluorescent protein; MS, Murashige and Skoog; NAA, 1-naphthaleneacetic acid; PPFD, photosynthetic photon flux density; YGFP, yellowish-green fluorescent protein.

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cells have usually been used for analysis. The observation of color differences in cells was an efficient method for chimera identification in plastid mutant (albino) lines or pigment-accumulating lines, as reviewed by Burge et al. (2002). We conducted this experiment with a fluorescent protein gene as an optical selection marker for efficient screening for chimeras. This report describes a method for producing periclinal chimeras of chrysanthemum through regeneration from leaf explants with a fluorescent protein transgene as a selection marker.

The wild-type chrysanthemum 'Taihei' and its transgenic plant carrying a yellowish-green fluorescent protein gene from the marine plankton *Chiridius poppei* (*CpYGFP*; Masuda et al. 2006) were used as plant materials. The fluorescence activity of the CpYGFP protein is stable over a wide pH range in higher plants (Masuda et al. 2006). 'Taihei' was transformed with a highly improved *CpYGFP* expression vector containing three tandem *CpYGFPs* controlled by cauliflower mosaic virus 35S promoter with optimized translational enhancer and terminator (3×*CpYGFP*; Sasaki et al. 2014). The 3×*CpYGFP* expression vector can stably accumulate high amounts of the fluorescent protein CpYGFP in plant tissues (Sasaki et al. 2014) which permit efficient visualization for observing chimerism. We used explants from in vitro leaves of the wild type and the *CpYGFP*-carrying transformant to construct periclinal chimeric plants consisting of material from both plants. Suitable methods for combining the two materials and efficient regeneration conditions from the mixed cells were examined. Regenerated shoots were observed under a fluorescence detection microscope (MZ16FA with GFP2 filter set, Leica Microsystems, Wetzlar, Germany) to examine their chimeric structures.

We used approximately 2×8 mm leaf explants from in vitro-maintained chrysanthemum plants. Explants from wild-type plants were first placed on solid Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 1.0 mg l^{-1} 6-benzyladenine (BA) and 0.5 mg l^{-1} 1-naphthaleneacetic acid (NAA). The explants from *CpYGFP*-carrying transformants were then placed against the long sides of each wild-type explant and cultured at 25°C under a 16-h photoperiod with a photosynthetic photon flux density (PPFD) of 70 μmol m^{-2} s^{-1} [Figure 1A (left)]. Figure 1A (right boxes) shows partially attached explants 5 days after placement. After 7 days of culture, partially attached explants were separated using forceps and only wild-type explants were used for further experimentation. We observed wild-type explants scattered with fluorescent cells along the partially attached face (Figure 1B). These wild-type explants with scattered transgenic cells were cultured on solid MS medium containing 1.0 mg l^{-1} BA, 0.5 mg l^{-1} NAA, and 50 mg l^{-1} paromomycin at 20°C under a 16-h photoperiod with low light intensity (PPFD, 7 μmol m^{-2} s^{-1}). The transgenic cells were resistant to paromomycin. Mosaic calli consisted of transgenic and wild-type cells formed on the explants approximately 1 week after detachment (Figure 1C). The culture was maintained for 2–3 months with transfer of the explants to new medium every 2 weeks until shoot regeneration (Figure 1D). We examined 996 regenerated shoots from 4,120 explants. Most of the shoots were non-chimeric except for a single shoot which appeared to be sectorial chimeric and unstably periclinal chimeric, namely mericlinal chimeric (Figure 1E, F). We tried to construct stable periclinal chimeric plants from the mericlinal chimeric shoot by repeated elongation of axillary buds, as suggested by Tilney-Bassett (1986).

Three repeated axillary bud elongations from nodes (Figure 1G) produced two plants with putative periclinal chimerism. One plant was a putative L1-chimeric plant that showed fluorescence in the epidermal cells and trichomes of leaf and stem; however, no fluorescence was observed in the inner cells of leaf or stem as well as in the whole root (Figure 2C). The other was a putative L3-chimeric plant that showed fluorescence in the central tissues of the leaf and stem, particularly at veins and vascular bundles, and whole root tissues; however, no fluorescence was observed in the epidermal cells of leaf or stem (Figure 2D). The wild-type plants never showed green fluorescence under the observation conditions (Figure 2A) and a solid *CpYGFP*-carrying transformant showed fluorescence in the whole tissues of leaf, stem, and root (Figure 2B).The epidermal cells and cells of the sub-epidermal tissues of leaves and stems are derived from the L1 and L2, layers, respectively, whereas cells of central tissues of leaves and stems and whole root tissues are derived from the L3 layer (Geier 2012). We, therefore, considered these two L1- and L3-chimeric plants as periclinal chimeras carrying *CpYGFP* transgenes only in the L1 and L3 layer, respectively.

The two periclinal chimeric plants were vegetatively propagated in vitro, and each appeared to maintain its characteristic chimeric structure. Axillary shoots from these putative periclinal chimeras showed a fluorescence pattern similar to that of the base plants (Figure 1H). These two plants appeared to be stable periclinal chimeric structures. Thus, periclinal chimeric chrysanthemum plants can be produced through partial combination and detachment of leaf explants with different genetic backgrounds followed by shoot regeneration from explants under appropriate selection pressure.

In this study, we obtained only a single chimeric shoot from 996 regenerated shoots (about 0.1%). In former reports with *Nicotiana* plants, Carlson and Chaleff (1974) obtained 28 chimeras from approximately 7000 regenerated shoots (about 0.4%) and Marcotrigiano and Gouin (1984) obtained four chimeras from 1321

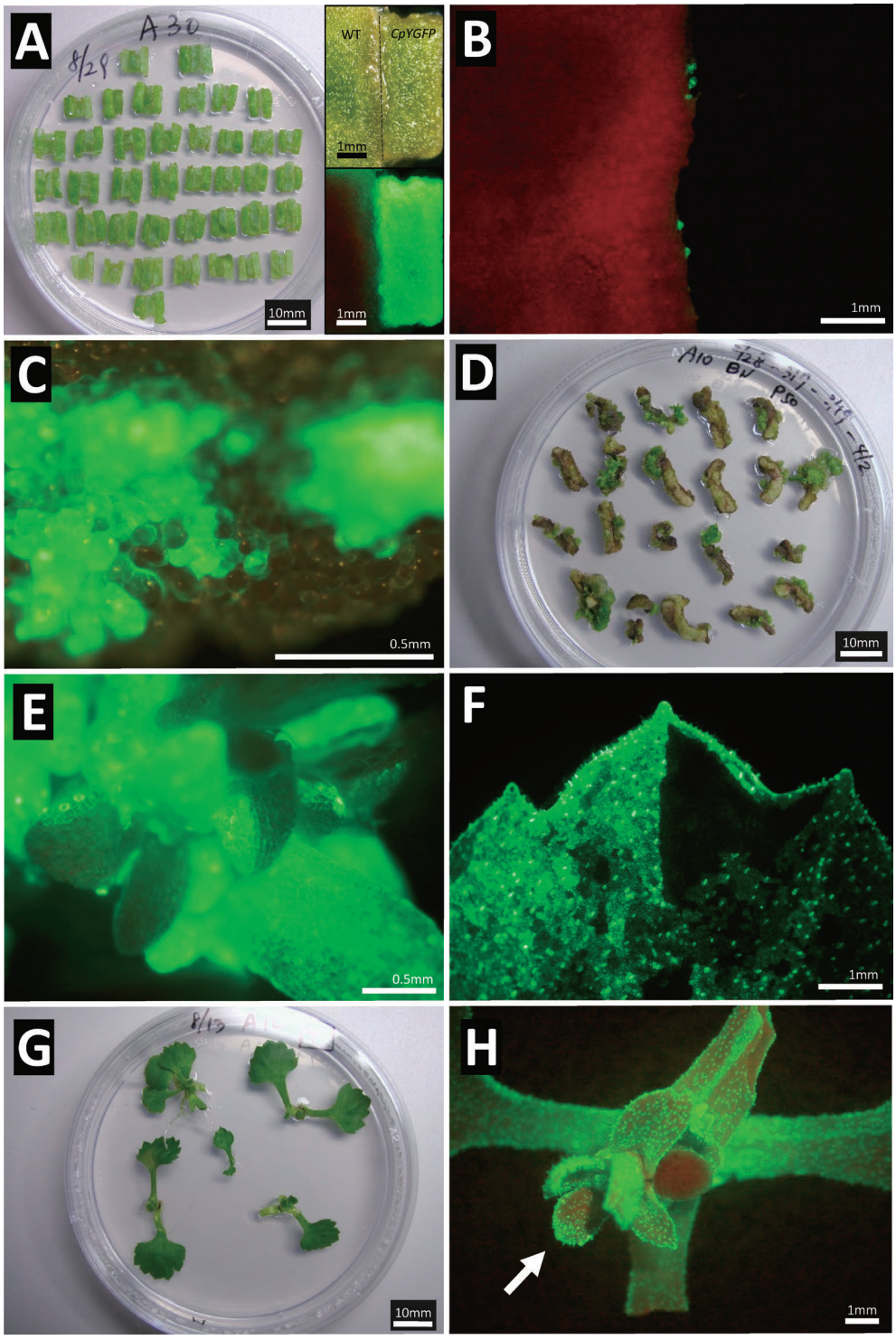

Figure 1. Production of periclinal chimeras in chrysanthemum. A: Two leaf explants of *CpYGFP*-carrying transformants were placed on both sides of a wild-type explant (left picture). Images in right boxes show partially attached leaf explants of the wild-type plant (left) and a *CpYGFP*-carrying transformant (right) 5 days after placement under visible light (up) and excitation light (down). B: Some *CpYGFP*-carrying fluorescent cells remaining on the attached face of a wild-type explants after separating from the transgenic explant. C: Formed mosaic calli consisted of fluorescent transgenic cells and non-fluorescent wild-type cells. D: Regenerating shoots from wild-type explants with mosaic calli. E: A regenerated shoot that appears to show mericlinal chimerism. F: Part of a leaf of the elongated mericlinal chimera showing mosaic fluorescence. G: Nodes of the mericlinal chimera line were repeatedly cultured to elongate axillary shoots for obtaining stable periclinal chimeras. H: An axillary shoot (arrow) of a putative L1-fluorescent chimeric plant showing the same fluorescent pattern as the base plant.

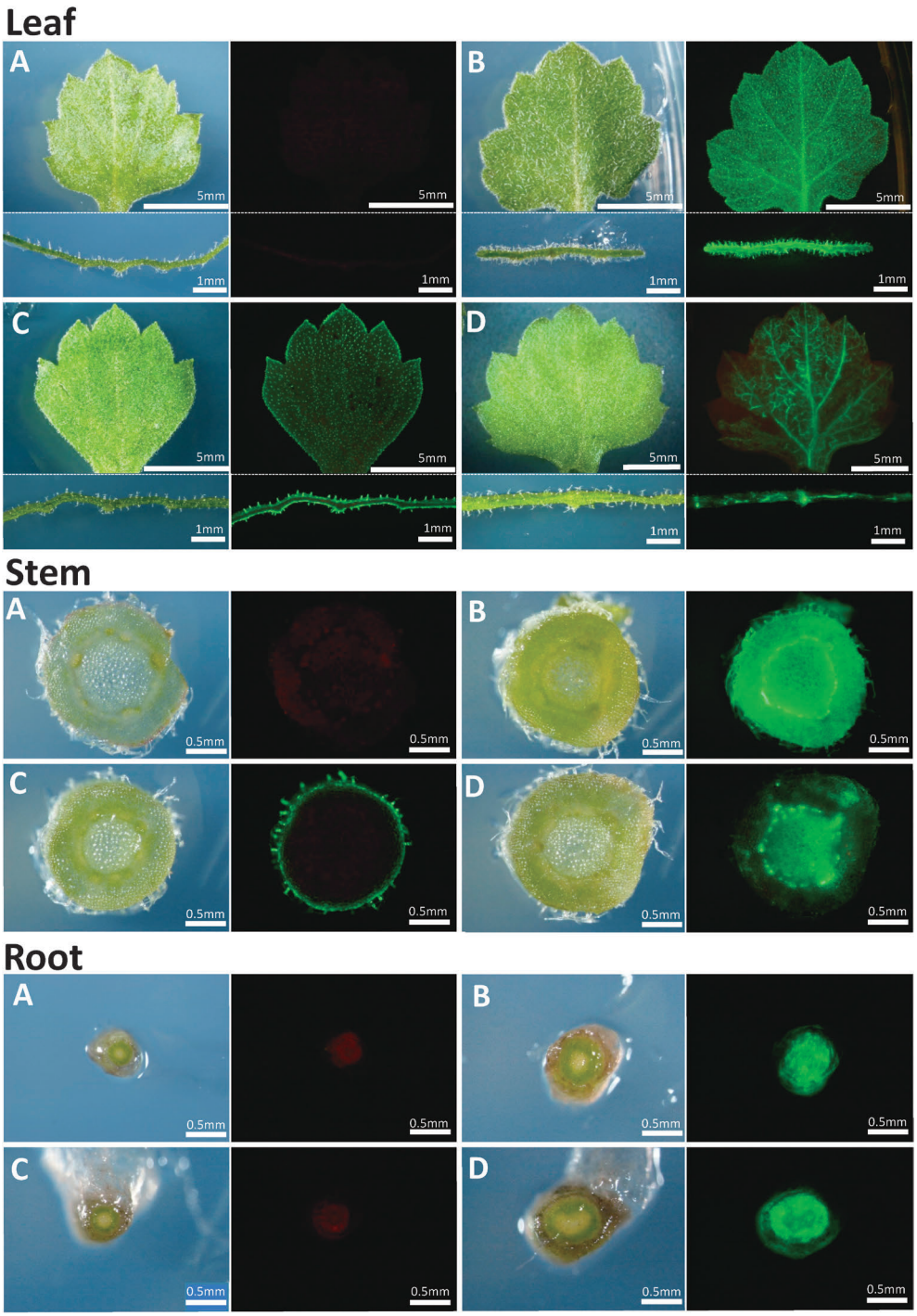

Figure 2. Fluorescence distribution in chrysanthemum plants. A: wild type plant, B: solid *CpYGFP*-carrying transformant, C: L1-fluorescent chimeric plant, D: L3-fluorescent chimeric plant. Leaf: The adaxial face and horizontal cut face of each plant is shown under visible light (left) and excitation light (right). Stem and root: The horizontal cut face of each plant is shown under visible light (left) and excitation light (right). The wild-type plant showed slight reddish self-emitted fluorescence but no green fluorescence under the observation conditions. The solid *CpYGFP*-carrying transformant showed fluorescence in whole tissues of leaf, stem, and root. The L1-chimeric plant showed fluorescence in epidermal cells and trichomes of leaf and stem, but none in the inner cells of the leaf or stem, or whole root tissue. The L3-chimeric plant showed fluorescence in the cells of the central part of the leaf and stem including vascular bundles and whole root tissue, but none in the epidermis of leaf or stem.

regenerated shoots (about 0.3%). Binding et al. (1987) produced 17 chimeras with *Solanum* plants, but they do not indicate the number of total regenerated shoots. Reported efficiency of chimeric shoot production by in vitro regeneration method has been low until now. It seems that at least a thousand regenerated shoots should be examined for producing chrysanthemum chimeric plant. Revising protocol would improve the efficiency in the future.

Plants with periclinal chimeric structures caused by

natural or artificial mutations, such as flower color sports in chrysanthemum, have been used as cultivars (Shibata and Kawata 1986; Stewart and Dermen 1970). Synthetic production of periclinal chimeras is a potential future breeding method. For example, L1 layer replacement with a different anthocyanin background can change the flower color, given that anthocyanin pigment is present only in the L1-derived epidermal cells of petals. In contrast, germ cells are normally derived from the L2 layer (Satina 1945; Satina and Blakeslee 1941), so that transgenes in the L1 layer are rarely transmitted to progeny. The risk of transgene flow from transgenic chrysanthemum to wild relatives would be reduced by L1-specific gene modification. However, because there are several reports in tobacco of the presence of L1 or L3 layer-derived genes in progenies (Burk et al. 1964; Marcotrigiano and Bernatzky 1995; Schmülling and Schell 1993; Stewart and Burk 1970), we plan to investigate the frequency of inheritance from the L1 layer in chrysanthemum.

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