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BRIEF COMMUNICATION

The effect of 2,4-D and kinetin on dedifferentiation of petiole cells in *Arabidopsis thaliana*

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Abstract

Phytohormones are indispensable factors regulating plant cell dedifferentiation. In this paper, different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (KIN) were incorporated in the culture medium and the anatomy of dedifferentiated cells prior to callus formation from *Arabidopsis thaliana* petiole explants was examined. The results indicated that the cytoplasm of parenchyma cells in the vascular bundle gradually became denser with time of culture only if 2,4-D was included in the medium. The *WUSCHEL* (*WUS*) gene was expressed in derivative cells of the vascular bundle after culture for 24 h in the presence of 2,4-D and there was no obvious signal in these cells of cultured petioles with KIN alone. These results suggest that 2,4-D plays an important role in the process of dedifferentiation of vascular bundle cells in *Arabidopsis* petioles and KIN has no obvious effect on it.

Additional key words: auxin, cytokinin, *WUS* gene.

Explants of different plant tissues can be induced to produce regenerated plants. The technique often requires for cultured cells to return from a mature, differentiated state to embryonic state, a process termed dedifferentiation (Feher *et al*. 2003). Auxins can induce the formation of callus by initiating cell dedifferentiation process, and cytokinin can induce regeneration of plumules by promoting cell division and differentiation (Skoog and Miller 1957). Accordingly, dedifferentiation or redifferentiation is usually initiated by adjusting the ratios of these two plant hormone groups (Skoog and Miller 1957, Centeno *et al*. 1996). Before callus formation, some ultrastructural characteristics of dedifferentiated cells have been reported, *e.g*., the cytoplasm becomes dense, the large central vacuole becomes reduced in size, small vacuoles form, and the number of organelles such as amyloplasts, mitochondria, Golgi apparatus and endoplasmic reticulum increases (Yeoman and Evans 1967, Yu *et al*. 2010). However, the role of auxin or cytokinin on cell dedifferentiation is mainly deduced based on the effect of these hormones on callus induction, and their effects on cytological features of dedifferentiated cells prior to callus formation is poorly understood.

In this study, the roles of auxin and cytokinin in the process of dedifferentiation of parenchyma cells were studied by observing the structure of these cells in *Arabidopsis thaliana* petioles cultured on medium containing different concentration ratios of 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (KIN). To further validate the dedifferentiation of parenchyma cells, expression of *WUSCHEL* (*WUS*), a marker gene exclusively expressed in embryogenic cells (Endrizzi *et al*. 1996, Atta *et al*. 2009), was monitored in transverse section of cultured petioles using RNA *in situ* hybridization..

The seeds of *Arabidopsis thaliana* L. ecotype Columbia were sown on Murashige and Skoog (1962; MS) medium supplemented with 3 % sucrose and 0.8 % agar, chilled at 4 °C for 2 d, and then germinated in a growth chamber (12-h photoperiod, irradiance of 120 to 130 μmol m^{-2} s^{-1}, day/night temperature of 24/20 °C and

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; KIN - kinetin; *WUS* - *WUSCHEL*.

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relative humidity of 70 %. After three weeks, mature petioles were excised and 2 - 3 mm^3 explants were cultured on B5 medium (Gamborg *et al*. 1968) supplemented with 3 % sucrose, 0.8 % agar, and three different combinations of 2,4-D and KIN (1 mg dm^{-3} 2,4-D + 0 mg dm^{-3} KIN; 1 mg dm^{-3} 2,4-D + 0.1 mg dm^{-3} KIN and 0 mg dm^{-3} 2,4-D + 0.1 mg dm^{-3} KIN) under the same growth conditions as for seed germination.

Sample preparation was performed as described by Feder and Obrien (1968) with some modifications. The explants cultured for 6, 12, 18, 24, 30, 36 or 48 h, and the non-cultured petioles used as controls, were fixed in 1 % glutaraldehyde 1 h before postfixing in 1 % osmium tetroxide for 1 h. The samples were dehydrated in an acetone gradient series before being embedded in *Epon 812* resin. Semithin transverse sections (900 nm) were cut with a *Leica* (Vienna, Austria) *EM UC6* microtome, stained with toluidine blue, and examined with a *Nikon* (Tokyo, Japan) *Eclipse 80i* microscope.

RNA *in situ* hybridization was performed according to the method of Brewer *et al*. (2006) with some modifications. The petiole explants cultured on B5 medium containing 1 mg dm^{-3} 2,4-D or 0.1 mg dm^{-3} KIN for 24 h were fixed and embedded in paraffin wax, and 8 μm thick sections were cut. Non-cultured petioles were used as the control. To identify the position where dedifferentiation occurred, *WUS* expression was detected in cross-sections of petioles. Labeled *WUS* sense and antisense probes were generated from a *WUS* cDNA fragment using a digoxigenin RNA-labeling kit (*Roche*, **location?**) according to the manufacturer's instructions. The primers used to amplify the cDNA fragment were P1 (ACAAGCCATATCCCAGCTTCA) and P2 (CCACCG TTGATGTGATCTTCA). After slide pretreatment, hybridization and washing, *WUS* transcripts were detected and photographed under a light microscope.

In transverse section, the vascular bundle of non-cultured control petioles consisted of xylem and phloem tissues (Fig. 1*A*). The xylem, on the adaxial side of the vascular bundle, contained vessels and highly vacuolated parenchyma cells distributed between the series of vessels. The phloem, on the abaxial side of the vascular bundle, contained sieve elements, companion cells and parenchyma cells. Three cell layers adjacent to the phloem were present between the xylem and phloem. One of these cell layers was the preserved protocambium and the other two layers of paired of parenchyma cells with a larger vacuole were named the derivative cells which were derived from the protocambium (Fig. 1*A*).

In contrast, in petioles cultured for 18 h on medium containing either 1 mg dm^{-3} 2,4-D + 0.1 mg dm^{-3} KIN or 1 mg dm^{-3} 2,4-D alone, the cytoplasm of the vacuolated parenchyma cells (derivative cells) between the xylem and phloem started to become more dense (Fig. 1*B*). After 24 h, the cytoplasm of parenchyma cells between the vessels also increased in density. After 36 h, most parenchyma cells in the vascular bundle were full of cytoplasm and the nucleus was obvious (Fig. 1*B*). In contrast, in petioles cultured for 18 h or 24 h in the medium containing KIN alone, the cytoplasm of the derivative parenchyma cells between the xylem and phloem did not become denser and remained highly vacuolated and after 36 h the derivative cells were destroyed (Fig. 1*B*). These results demonstrated that the derivative cells between the xylem and phloem first started to dedifferentiate after 18 h culture in the presence of 2,4-D, and subsequently other parenchyma cells in the vascular bundle entered a dedifferentiated state. In the presence of KIN alone, the parenchyma cells in the vascular bundle did not dedifferentiate, and some destroyed cells were observed after 36 h culture. Our data confirmed the previous supposition that only auxins and not cytokinins induce callogenesis by initiating explant cell dedifferentiation (George and Eapen 1994, Yuan *et al*. 2009, Huang *et al*. 2010). Similarly, Centeno *et al*. (1996) reported that the embryogenic callus was easily induced in explants with higher concentration of endogenous auxins and lower concentration of cytokinins.

The synthetic auxin 2,4-D was applied to initiate dedifferentiation of explant cells in many plants (Mitsuoka *et al*. 1994, Litz *et al*. 1998, Sandal *et al*. 2005). Here, we observed that derivative cells first dedifferentiated followed by the parenchyma cells between the vessels in the petiole explants cultured in the 2,4-D-containing culture medium, whereas no dedifferentiation was seen in the same cells of petioles cultured on medium containing only KIN. The timing of the initiation of dedifferentiation of the parenchyma cells was not affected by the concentration of KIN, but influenced by the concentration of 2,4-D in the medium (data not shown). These results indicated that 2,4-D was a key hormone for dedifferentiation of parenchyma cells in the vascular bundle of petioles. It was reported that many Aux/IAA genes were up-regulated during the course of callus formation in *Arabidopsis* root explants cultured on auxin-rich medium (Che *et al*. 2006). Our gene expression analysis also indicated that the auxin-responsive gene *At*1g59500, a member of the GH3 family, increased 11-fold in petioles cultured for 26 h on medium containing 2,4-D (data not shown), which was coincident with a period of prominent dedifferentiation of vascular bundle parenchyma cells. Accordingly, *GH3*, the auxin-responsive gene, is suggested to play key roles in the process of dedifferentiation of vascular bundle parenchyma cells. At the same time of prominent dedifferentiation of vascular bundle parenchyma cells, four A-type *ARR* genes of *Arabidopsis*, as exogenous cytokinin-responsive factors (To *et al*. 2004), had no obvious change (data not shown). This suggests that KIN had no obvious effect on cell dedifferentiation. In addition, our work also indicated that KIN promoted protocambium cell division, subsequently, a mass of newly formed meristematic cells with denser cytoplasm

developed between the xylem and phloem in petioles cultured for 7 d in the medium containing 0.1 mg dm^{-3} KIN alone, which was produced by division of the procambium cells, and callus-like concentric circles of cells was observed in cross-sections of the petioles (data not shown), which is consistent with the previous report that exogenous cytokinin promotes cell division during tissue culture (Miller *et al*. 1956). Consequently, promoting cell division of meristem tissue appeared to be the principal role of KIN. Besides its action on meristematic cell division, some evidence also indicates that KIN promotes cell differentiation of *Arabidopsis* root meristematic tissue (Dello Ioio *et al*. 2008). In our study, similar results were observed. The macroscopic differentiated buds originating from the procambium cells, had formed after culture for 14 d on medium containing KIN alone. In contrast, the petioles cultured after 7 d became swollen and callus formed on medium containing either 1 mg dm^{-3} 2,4-D + 0.1 mg dm^{-3} KIN or 1 mg dm^{-3} 2,4-D alone, and the radial arrangement of callus was centered on parenchyma cells between vessels in the vascular bundle, which originated mainly from parenchyma cell dedifferentiation in the petioles cultured only in the presence of 2,4-D (data not shown).

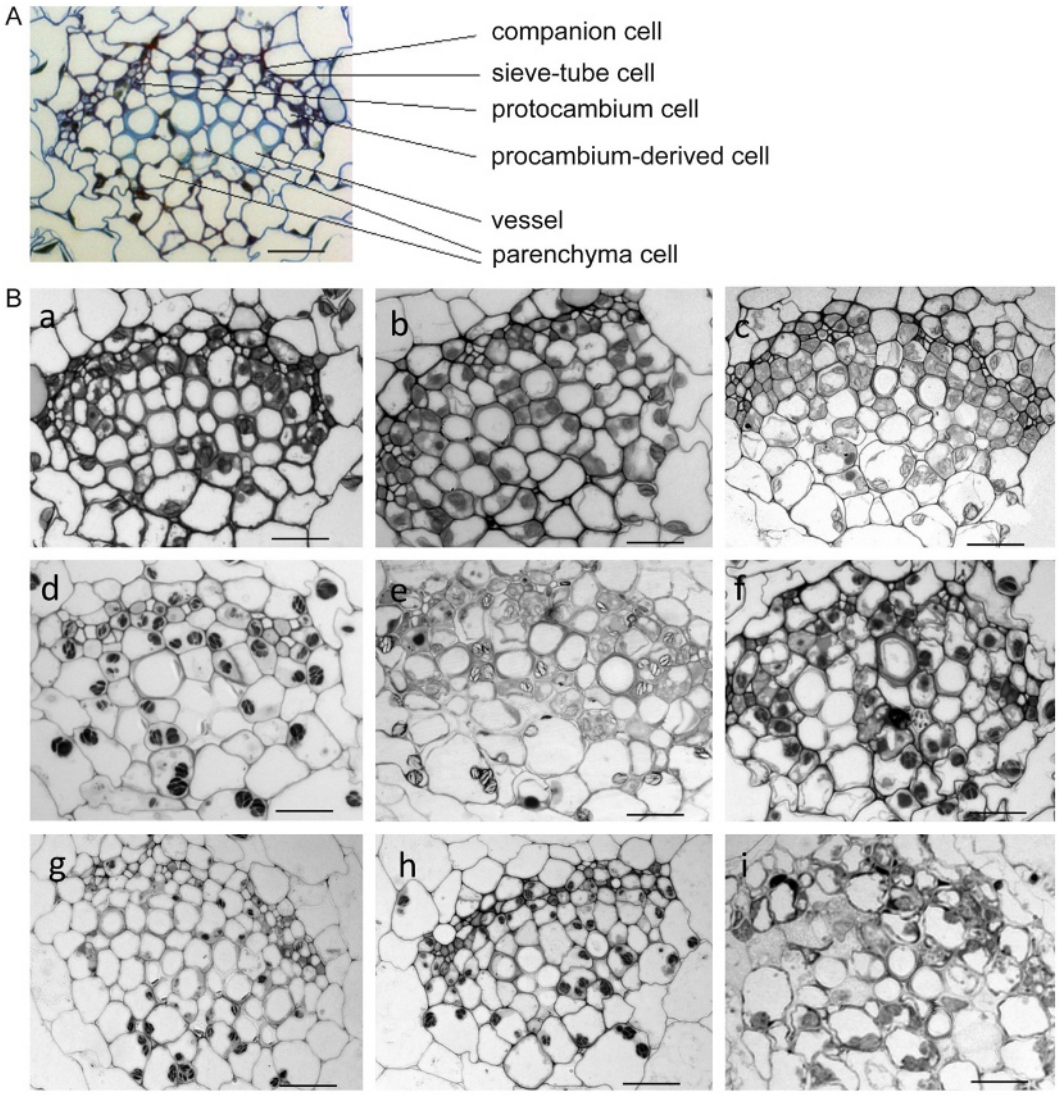

Fig. 1. Microstructural changes in the vascular bundle of *Arabidopsis* petiole explants under different culture conditions. *A* - Structure of the vascular bundle of non-cultured *Arabidopsis* petiole. *B* - Structure of the vascular bundle in the *Arabidopsis* petiole cultured on the medium containing both 1 mg dm^{-3} 2,4-D and 0.1 mg dm^{-3} KIN for 18 h (*a*), 24 h (*b*) and 36 h (*c*); on the medium containing 1 mg dm^{-3} 2,4-D alone for 18 h (*d*), 24 h (*e*) and 36 h (*f*); on the medium containing 0.1 mg dm^{-3} KIN alone for 18 h (*g*), 24 h (*h*) and 36 h (*i*); (*bar* = 50 μm).

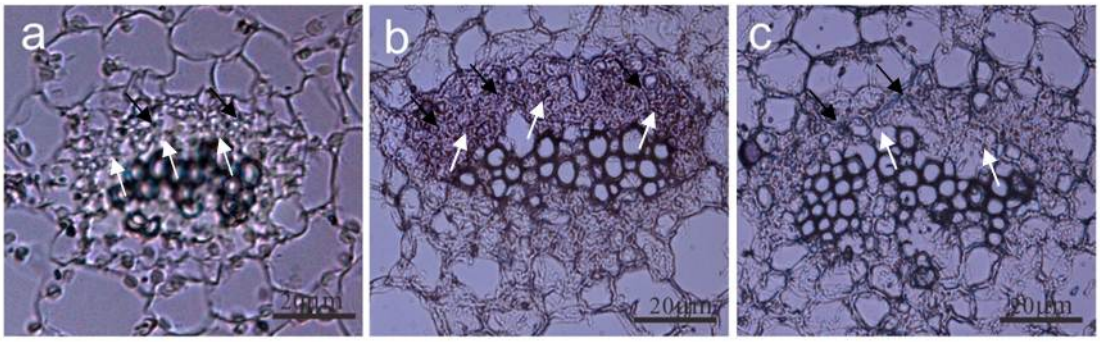

Fig. 2. *WUS* expression in the vascular bundle of *Arabidopsis* non-cultured petiole (*a*), petiole cultured in the medium containing 1 mg dm^{-3} 2,4-D (*b*) or 0.1 mg dm^{-3} KIN (*c*) for 24 h (*bar* = 20 μm). The *black* and *white arrows* indicated the location of protocambium cells and the derivative parenchyma cells, respectively. There is no *WUS* expression in the area indicated by *black* and *white arrows* in (*a*) and positive *WUS* expression only in the area indicated by *black* and *white arrows* in (*b*), and no obvious signal in the area indicated by *white arrows* in (*c*).

To further confirm the role of 2,4-D and KIN during the dedifferentiation process, mRNA of the *WUSCHEL* (*WUS*) gene was detected by *in situ* hybridization in tissues of petioles cultured on medium containing 2,4-D or KIN. The result indicated that *WUS* transcripts were detected in both protocambium cells and derivative parenchyma cells in the vascular bundle of petioles cultured for 24 h in the medium containing only 1 mg dm^{-3} 2,4-D (Fig. 2*b*). In contrast, *WUS* mRNA was only present in protocambium cells in the vascular bundle when petioles were cultured for 24 h in the medium containing only 0.1 mg dm^{-3} KIN, and there was no obvious mRNA signal in other cells (Fig. 2*c*). *WUS* mRNA was not detected in the non-cultured control petioles (Fig. 2*a*). The expression analysis not only indicated that the derivative parenchyma cells had turned from differentiated parenchyma cells into embryogenic cells in petioles cultured using 2,4-D, as the *WUS* gene is expressed only in embryogenic cells (Endrizzi *et al*. 1996), and illustrated that 2,4-D could induce dedifferentiation of derivative cells in the vascular bundle to turn into embryogenic cells, which coincided with cytoplasmic changes in the vascular bundle parenchyma cells of petioles cultured in the presence of 2,4-D. In addition, *WUS* gene expression only in the procambium cells of petioles cultured with KIN alone suggested that KIN had no significant role in dedifferentiation of these cells, because the preserved protocambium cells between the xylem and phloem retain characteristics of embryogenic cells with dense cytoplasm.

In conclusion, the present study demonstrated that the auxin 2,4-D played a crucial role in the dedifferentiation of vascular bundle parenchyma cells in the petioles, whereas cytokinin had no obvious effect on cell dedifferentiation, but promoted division and differentiation of embryogenic cells.

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