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Induction and origin of adventitious roots from chimeras of *Brassica juncea* and *Brassica oleracea*

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Abstract Adventitious roots were induced from shoots and leaves of the chimera plant TCC (LI-LII-LIII = TCC; T = Tuber mustard, C = Red Cabbage), previously developed by in vitro grafting of tuber mustard (*Brassica juncea*) and red cabbage (*B. oleracea*). The regeneration frequency of adventitious roots from TCC shoots and leaf sections was markedly higher than that obtained from the parents TTT (tuber mustard) and CCC (red cabbage). Moreover, levels of α-naphthaleneacetic acid in the culture medium had lower effects on rooting efficiency of TCC chimeras compared to those of TTT and CCC. The number and fresh weight of adventitious roots per TCC shoot, 13.11 roots and 0.274 g, respectively, were also significantly higher than those of the parents. This demonstrated that replacing the histogenic LI layer (the outermost apical cell layer) with a different genotype might improve adventitious root induction capability of these vegetative tissues due to likely synergistic effects between LI and the other two histogenic layers, LII and LIII. Following polymerase chain reaction analysis and histological investigation, it was found that these adventitious roots originated from the LIII histogenic layer.

Keywords *Brassica juncea* · *Brassica oleracea* · Chimera · Adventitious root origin · Cell layer

Abbreviation
BA   6-Benzyladenine acid
NAA   α-Naphthaleneacetic acid

MS   Murashige and Skoog medium
g   Gram
PCR   Polymerase chain reaction
L   Layer

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Introduction

Adventitious roots are post-embryonic roots that are different from lateral roots, and they develop from various organs and tissues of the plant, such as stems, leaves, and roots (Zobel 1986; Smart et al. 2003). Induction of adventitious roots is important for successful vegetative propagation of plants and it is influenced by various factors, including genotype, plant growth regulators, other medium components, and environmental conditions (Howard 1994; Calamar and De Klerk 2002). The origin of adventitious root formation in plants has been investigated in different species (Mahlstede and Watson 1952; Belehu et al. 2004; King and Stimart 1998; Falasca et al. 2004; Smart et al. 2003). Calderón Baltierra et al. (2004) studied formation of adventitious roots from basal ends of *Eucalyptus globules* shoots and found that these roots primarily formed from the medulla. Falasca and Altamura (2003) suggested that adventitious roots originated from the pericycle in hypocotyls of three *Arabidopsis thaliana* ecotypes. Whereas, Soh et al. (1998) indicated that adventitious roots developed endogenously from dedifferentiated parenchyma cells around vascular tissues. Therefore, additional studies must be conducted to further elucidate the role of the donor plant on the origin of adventitious roots, and identify the cellular origin of these roots.

Plant chimeras, consisting of genetically different histogenic cell layers (LI, the outermost layer; LII, the middle layer; and LIII, the innermost layer) in shoot apical meristem (SAM), have been useful for tracing the origin of adventitious tissues or organs. In particular, periclinal chimeras, in which a single histogenic cell layer is genetically different from all others, are commonly used to investigate origin of tissues following cellular differentiation. For example, LI was not involved in root formation in periclinal chimera *Hosta* (Zonneveld and Van 2000), whereas, the LIII was found to be involved in adventitious root formation of *Hosta tratt,* following analysis of nuclear DNA content (Zonneveld 2007). However, most of these previous studies have focused on monocots.

In this study, adventitious root formation was induced from shoots and leaves of chimeras of tuber mustard and red cabbage grown in medium containing different levels of α-naphthaleneacetic acid (NAA) and combinations of NAA and 6-benzyladenine (BA). The overall goal of this study was to determine the effects of genetically different histogenic cell layers on rooting ability in *Brassica*. Adventitious roots induced from chimeric tissues were analyzed using morphological, molecular, biological, and histological techniques to assess the cellular origin of these roots.

Materials and methods

Plant materials

Interspecific chimeras between tuber mustard (*B. juncea* Coss. var. *tumida* Tsen et Lee) and the red cabbage cultivar 'Ruby ball' (*B. oleracea* var. *capitata* L.) were artificially developed by in vitro grafting (Chen et al. 2006). This was achieved using the following approach. Six-day-old seedlings of each of tuber mustard and red cabbage were vertically cut in half, and these cut sections were then symmetrically fit in together, and wrapped with parafilm. Developing shoot-tips from grafted plantlets were collected, and grown on Murashige and Skoog (1962) (MS) medium containing 3% sucrose, 0.8% agar (Agar bacteriological grade, Sangon Co. Ltd, Shanghai, China), and supplemented with 0.1 mg L^{-1} BA. Subsequently, some chimeral adventitious shoots developed from these united shoot-tips. These chimeral types were identified following transfer of plantlets to the greenhouse. By convention, histogenic cell layers LI, LII, and LIII of apical shoots of tuber mustard were designated as TTT; while, those of red cabbage were designated as CCC. Therefore, periclinal chimera, identified using transverse leaf sections and phenotypic markers, of the composition LI = tuber mustard and LII and LIII = red cabbage were designated as TCC.

These chimeral shoots were maintained on half-strength MS medium containing 3% sucrose, 0.8% agar, without BA, under a 16 h photoperiod providing 84 μmol m^{-2} s^{-1} light intensity and at 25 ± 2°C.

Adventitious root induction from shoots and leaf sections

Proliferating shoots of TCC chimeras and their parental cultures, TTT and CCC, all grown in vitro, were used sources of explants. Shoots with 3–4 leaves, about 4–5 cm in length, were transferred to glass jars containing fresh medium consisting of half-strength MS medium containing 3% sugar, 0.8% agar, and supplemented with 0.00, 0.05, 0.10, 0.15, or 0.20 mg L^{-1} NAA, pH 5.8. For each treatment, 12 shoots were used and this was replicated three times in a completely randomized design.

Newly-expanded leaves (without petioles) were excised from TCC, TTT, and CCC shoot cultures, cut into 5 × 5 mm^2 sections, and incubated in glass jar containing MS medium, 3% sucrose, 0.8% agar, and supplemented with different combinations of NAA (0.5, 1.0, 2.0 and 3.0 mg L^{-1}) and BA (0.5 mg L^{-1}). A total of 24 leaf sections were used per treatment, and this was replicated three times in a completely randomized design.

All cultures were gown under a 16 h photoperiod providing 84 μmol m^{-2} s^{-1}, and at 25 ± 2°C. Adventitious roots were harvested after 15 days, and data were recorded on root number and length of each shoot; while fresh weight of all roots from each shoot was determined after 20 days of culture. There were three replicates and nine shoots for each replicate.

Characterization of adventitious roots

Adventitious roots derived from both shoots and leaf explants were characterized by morphological observations and molecular analysis. Total DNA was extracted from fresh tissues of TCC, CCC, and TTT using the CTAB method with minor modifications (Dellaporta et al. 1983). Primers for the mitochondria-specific gene *atpA*, encoding ATP synthase subunit 1, including 5′-GCTGCTTACAGGAGTTAGCC-3′ and 5′-GTCCAATCGCTACATAGACA-3′ were used. The PCR reaction was conducted in 25 μl reaction consisting of 25 ng DNA template, 12.5 μM of each of forward and reverse primer, 200 μM dNTPs, 2.0 mM MgCl$_2$, and 0.6 U *Taq* polymerase (Takara, Japan). The PCR protocol was performed with 2 min initial denaturation at 94°C, followed by 35 cycles each consisting of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C, followed by a final extension for 10 min at 72°C. Three biological replicates were used each of the tissues.

Histological analysis

In order to observe the process of adventitious root origin and development, the stem bases (about 3–4 mm long) and the margins of leaf sections (about 2–3 mm wide) of TCC shoots were collected over different time points, including 0, 24, 48, 72, 96, 120, 144, 168, and 192 h following culture. These were fixed in FAA (ethanol: acetic acid: formaldehyde = 90: 5: 5), dehydrated in a gradient ethanol series (30–100%), and embedded in paraffin. Transverse and longitudinal sections were cut with a sliding microtome (RM2126, Leica, Germany), stained in 0.5% hematoxylin, and then observed under a optical microscope (BX51, Olympus, Japan), and photographed using a digital camera (SP-350, Olympus, Japan).

Statistical analysis

Data were subjected to analysis of variance (ANOVA), and means were compared using least significant difference (LSD) test at 0.05 level of probability using the SPSS statistical package (SPSS Inc, Chicago, IL, USA).

Results

Adventitious root induction from shoots and leaf sections

Adventitious roots were initiated from shoots of TCC, TTT, and CCC after 6 days. The frequencies of TTT shoots developing adventitious roots decreased with increasing concentrations of NAA, and there were markedly different frequency of CCC shoots in the different levels of NAA (Table 1). However, the frequency of TCC shoots developing adventitious roots were not significant different between the different levels of NAA (Table 1). Moreover, TCC shoots had higher rooting efficiency than their parents in each level of NAA. In addition, the mean number and fresh weight of adventitious roots from each TCC shoot was substantially higher than those of both TTT and CCC, while the mean length of adventitious roots from TCC shoots was intermediate between the two parents (Table 2).

Leaf explants were also used for the induction of adventitious roots. Hair-like outgrowths appeared first. After 8 days of culture, adventitious roots gradually grew out from the margins of the leaf sections. Meanwhile, a small amount of calli formed in some leaf sections' margins as well, but the calli did not produced adventitious roots, adventitious roots originated directly from leaf sections. In order to suppress the formation of hair-like outgrowths, BA was added to the media as detailed in our previous study (Chen et al. 2001), and effectively suppressed them. However, adventitious root formation was also depressed, as shown in Table 3. The percentage of TTT leaf sections developing adventitious roots was most severely suppressed, and that of CCC was also decreased, but there were no significantly different frequencies of TCC leaf explants developing adventitious roots among the combinations of BA and NAA. BA strongly inhibited the adventitious root development in the leaf sections of TTT and CCC, but imposed less of an effect on TCC, which was probably more tolerant to the inhibition of BA. Therefore, the percentages of TCC leaf sections producing roots were always highest in every combination of NAA and BA compared to its parents, although the rooting efficiencies of

Table 1 Effects of NAA on frequency of shoots developing adventitious root in TCC, TTT and CCC

NAA concentrations (mg L^{-1})	TTT (%)	TCC (%)	CCC (%)
0	92 ± 0.08 a	92 ± 0.08 a	75 ± 0.14 a
0.05	42 ± 0.08 b	100 ± 0.00 a	33 ± 0.08 bc
0.1	17 ± 0.17 bc	67 ± 0.08 b	42 ± 0.08 bc
0.15	33 ± 0.08 bc	92 ± 0.08 a	58 ± 0.08 ab
0.2	8 ± 0.08 c	92 ± 0.08 a	25 ± 0.00 c

Means with different letters within a column are significant different using LSD at $P = 0.05$

The frequency means percent shoots developing adventitious roots

Table 2 Number, length, and fresh weight of adventitious roots formed from TCC, TTT, and CCC shoots grown on MS medium without NAA

Root	TTT	TCC	CCC
Mean number per shoot	9.05 ± 0.45 b	13.11 ± 0.63 a	8.54 ± 1.03 b
Mean length ± SD (cm)	4.60 ± 0.36 a	3.76 ± 0.44 ab	2.67 ± 0.39 b
Fresh weight (g/per shoot)	0.099 ± 0.05 c	0.274 ± 0.04 a	0.127 ± 0.01 b

Means with different letters within a column are significant different using LSD at $P = 0.05$

Table 3 Effects of different combinations of NAA and BA on the frequency of leaf sections developing adventitious roots in TCC, TTT and CCC

Combination of NAA and BA (mg L^{-1})	TTT (%)	TCC (%)	CCC (%)
0.5 NAA + 0.0 BA	92 ± 0.08 a	100 ± 0.00 a	83 ± 0.08 ab
0.5 NAA + 0.5 BA	0 ± 0.00 c	100 ± 0.00 a	42 ± 0.08 c
1.0 NAA + 0.5 BA	8 ± 0.83 bc	100 ± 0.00 a	67 ± 0.17 abc
2.0 NAA + 0.5 BA	0 ± 0.00 c	75 ± 0.43 a	92 ± 0.08 ab
3.0 NAA + 0.5 BA	50 ± 0.29 ab	75 ± 0.00 a	58 ± 0.08 c

Means with different letters within a column are significant different using LSD at $P = 0.05$

The frequency means percent leaf sections developing adventitious roots

TTT and CCC were enhanced when NAA increased to 3.0 mg L^{-1} for TTT and 1.0 mg L^{-1} for CCC.

Morphological and molecular characterization of adventitious roots

The appearances of adventitious roots from TTT and CCC shoots were very different (Fig. 1a, c). Adventitious roots of TTT were very thin, while those of CCC were thick. TCC adventitious roots were same with those of CCC (Fig. 1b). On the other hand, TTT leaf sections readily produced too many white hair-like outgrowths and few adventitious roots. The leaf sections of CCC produced thicker and shorter adventitious roots than those of TTT, although many hair-like outgrowths also appeared. Adventitious roots from TCC leaf sections were visibly longer than those of CCC (Fig. 1e).

DNA was extracted from adventitious roots coming from either shoot or leaf sections, and then subjected to PCR analysis, using primer pairs designed to amplify fragments of the *atpA* gene with excepted sizes of 1,000 bp for TTT and 1,500 bp for CCC. Fragments of the excepted size can not only differentiate TTT from CCC, but can also identify the component cell lineages of the organs and tissues of TCC chimeras. For example, the leaves of TCC chimeras generated PCR fragments of both sizes. However, adventitious roots from both TCC shoots and leaf sections had only the CCC-specific band (Fig. 2a, b). This indicated that adventitious roots originated from LII and/or LIII cell layer(s), and the epidermis (LI) did not directly participate in the adventitious root origin, since the LII and LIII of TCC shoot apex were formed by CCC cells, while LI was from TTT.

Histological analysis

In order to further confirm adventitious roots originated from which tissue, histological analysis was carried out in
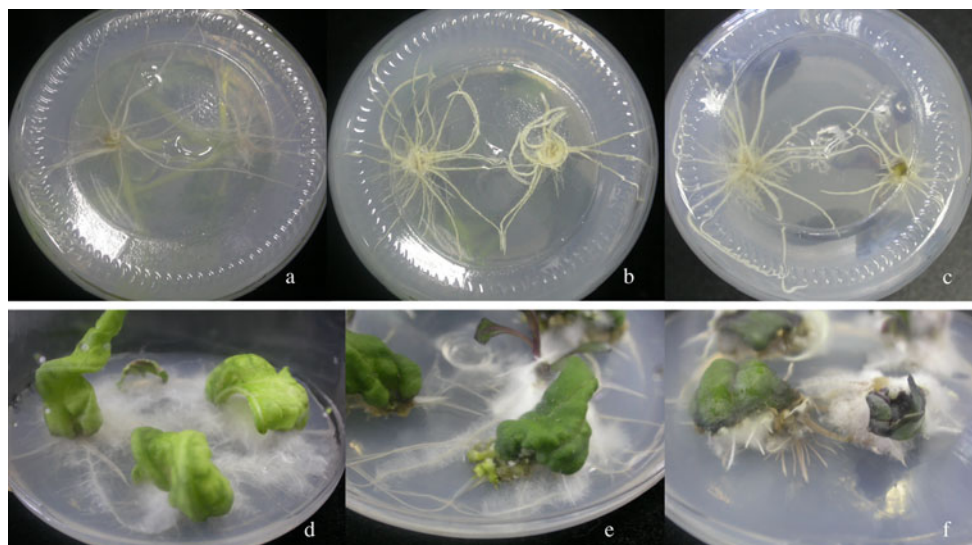

Fig. 1 The morphological characteristics of adventitious roots from shoots and leaf sections after culturing for 20 days. **a–c** The adventitious roots from shoots in medium without NAA; **d–f** the adventitious roots from leaf sections in medium with 0.5 mg L^{-1} NAA; **a, d** adventitious roots from TTT; **b, e** adventitious roots from TCC; **c, f** adventitious roots from CCC
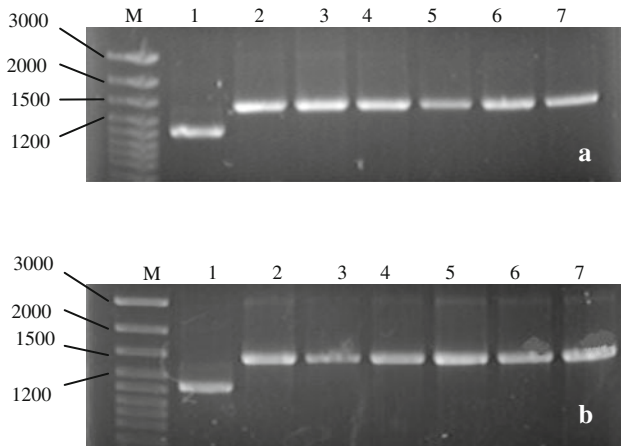
Fig. 2 PCR analysis of adventitious roots from shoots (**a**) and leaf sections (**b**). **a** *M* mark DNA; *1* TTT adventitious roots; *2* CCC adventitious roots; *3–7* adventitious roots from TCC shoots. **b** *M* mark DNA; *1* TTT adventitious roots; *2* CCC adventitious roots; *3–7* adventitious roots from TCC leaf sections

shoots and leaf sections. When TCC shoots were cultured in the medium for 2 days, pith rays disappeared, so vascular bundles did not separate from each other in stem bases of the shoots (Fig. 3a). The fascicular cambium formed a continuous cambium ring around the pith, because of periclinal division of the cambium cells on the 3rd day (Fig. 3b). Periclinal and anticlinal division of the cambium cells resulted in adventitious root meristemoid formation at day 4 (Fig. 3c). Adventitious root primordia formed at day 5 (Fig. 3d, e), and at day 6 adventitious roots had protruded through the cortex and epidermis of stem bases of TCC shoots (Fig. 3f). After 7 days, adventitious roots could be seen by naked eyes. The results showed that adventitious roots originated from cambium cells in stems of shoots.

In leaf sections, there were no obvious changes in the transverse section observations during the first 3 days of culture (Fig. 3g). On day 4 (Fig. 3h), the parenchyma cells adjacent to the veins and vascular bundles divided. On day 5, adventitious root meristemoid formation, with densely stained cells, was initiated by periclinal and anticlinal division of the dedifferentiated parenchyma cells (Fig. 3i). On day 6, the adventitious root primordia came into being and interpenetrated with veins (Fig. 3j, k). Adventitious roots protruded out of the leaf epidermis after day 7 (Fig. 3l). From these observations, it was clear that adventitious roots originated from parenchyma cells adjacent to the vascular tissue in leaf sections.

From both shoot and leaf sections, the histological observations of adventitious root origin suggested that adventitious root initiation site was closely adjacent to the vascular tissue, which was derived from the LIII apical cell layer.

Discussion

The ability of plants to regenerate roots varies among species and cultivars, and many studies have shown that root regeneration is mainly affected by plant growth regulators. Thus, treating shoots with plant hormones in vitro or in vivo has been employed to improve rooting efficiency. In this study, the rooting efficiency of TTT and CCC shoots declined with increasing NAA. However, TCC chimera shoots displayed higher adventitious root regeneration frequencies in each concentration of NAA than did either parent TTT or CCC. In addition, the mean number and fresh weight of adventitious roots per TCC shoot were also greater than those of TTT and CCC individuals. BA suppressed adventitious root development much less in TCC leaf sections than in TTT and CCC ones. The frequency of TCC leaf sections developing adventitious roots was also higher than that of both TTT and CCC individuals. These results indicated that TCC chimeras showed greater rooting capacities than both parents under the same external conditions. The internal difference of the TCC chimera from its parents is the composition and arrangement of its apical meristem cell layers, which consist of two genetically different cell lineages from both TTT and CCC. Morphogenetic and physiological interactions between the genetically different cell layers in the plant chimeras have been detected previously (Szymkowiak and Irish 1999; Zhang et al. 2007). It is possible that interactions between the heterogeneous epidermis and inner tissues influenced rooting in the TCC chimeras. There have been reports that the 'exogenous' epidermis (LI) can exert strong effects on many characteristics of chimeras (Stewart et al. 1972; Kaddoura and Mantell 1991), and it can impose effects on quantitative characteristics derived from or determined by the inner tissue (Zhou et al. 2002). In the present study, the frequency, mean number and fresh weight of adventitious roots from TCC shoots and leaf sections were markedly higher than those of both parents, and the length of adventitious roots from TCC shoots was intermediate between those of both parents (Table 2). These suggested that two genetically different cells in the chimera could coordinate well with each other, and LI imposed large effects on the characteristics of adventitious roots. Although the outermost cell layer (LI) was not involved in the origin of adventitious roots (Zonneveld and Van 2000), it might function to provide 'nursing cells' during adventitious root development, just as many papers report that nursing cells can stimulate the regeneration of nursed cells by nurse culture methods (Chen et al. 2004; Hargreaves et al. 2002). A TCC chimera, whose epidermis (LI) has a genotype different from the inner tissues (LII and LIII), and may be a special 'nurse culture' system, in which the endogenous hormones may undergo changes
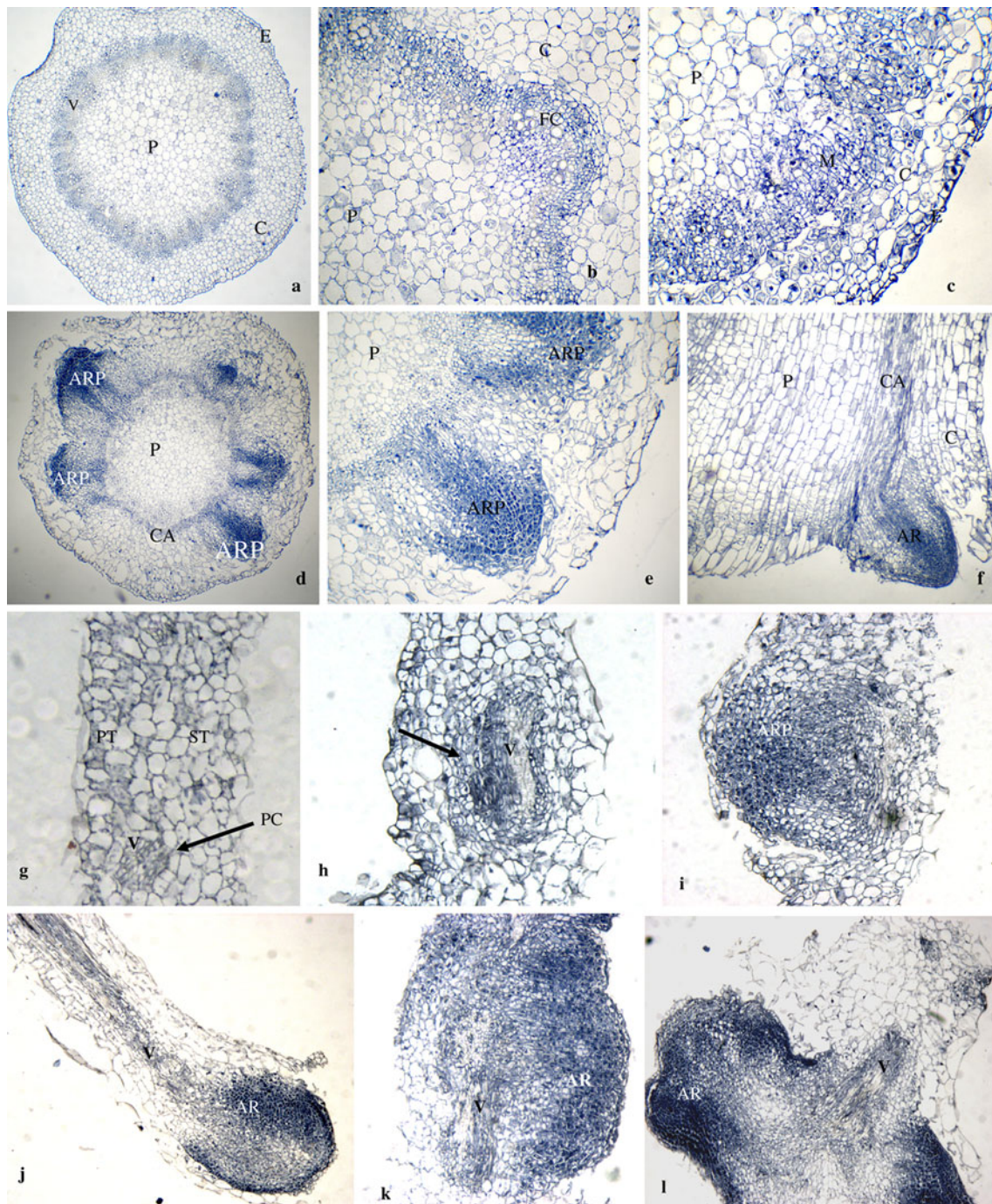
Fig. 3 Observation of adventitious root origin and development by histology. **a** Transverse section of a stem base of TCC shoot induced for 2 days (×100); **b** transverse section of a stem base of TCC shoot induced for 3 days (×400); **c** transverse section of a stem base of TCC shoot induced for 4 days (×400); **d** transverse section of a stem base of TCC shoot induced for 5 days (×100); **e** transverse section of a stem base of TCC shoot induced for 5 days (×400); **f** longitudinal section of a stem base of TCC shoot induced for 6 days (×200); **g** transverse section of a TCC leaf section induced for 3 days (×400); **h** transverse section of a TCC leaf section induced for 4 days (×400); **i** transverse section of a TCC leaf section induced for 5 days (×400); **j**, **k** transverse section of a TCC leaf section induced for 6 days (×200); **l** transverse section of a TCC leaf section induced for 7 days (×200). *AR* adventitious root, *ARP* adventitious root primordial, *C* cortex, *CA* cambium, *E* epidermis, *FC* fascicular cambium, *M* meristemoid of adventitious root, *P* pith, *V* vascular bundles, *PC* parenchyma cells, *PT* palisade tissue, *ST* spongy tissue

(Labunskaya et al. 2007). It is possible that the altered status of endogenous hormones caused the TCC chimeras to show distinct responses to NAA and BA compared to TTT and CCC.

Yang et al. (2005) reported that specific bands were amplified from a cytoplasmic male-sterile line of *B. juncea* and its maintainer by using PCR primer pairs targeting *atpA*. Our previous study (Zhu et al. 2007) found that *atpA*-derived fragments of different size could be used to distinguish TTT from CCC by PCR analysis using the same pair of primers, and that the origin of adventitious shoots regenerated from explants of TCC chimeras were precisely identified by this molecular marker. In the present study, we showed by PCR analysis that the adventitious roots from both shoot and leaf sections of TCC chimeras only had fragments that match the expected size of CCC (Fig. 2), regardless of the original site of explants. This suggested that adventitious roots of TCC chimeras were not chimeric, and confirmed that LI (epidermis) was not involved in the origin of adventitious roots, but LII and/or LIII cell layer(s) were. This result supported the viewpoint of Zonneveld and Van (2000). Furthermore, the histological observation demonstrated that adventitious roots originally developed from the fascicular cambium of the stem bases of shoots and from the parenchyma cells close around the veins and fascicular tissues of the leaf sections. In other words, the formation site of adventitious roots was close to the vascular tissues.

In the apple tree, Naija et al. (2008) recently found that some cells in the phloem regions near the cambium divided and produced a meristemoid which signaled the initiation of adventitious roots in microcuttings. Their study also suggested that the adventitious roots were initiated in the vascular tissues. As Hantke et al. (1995) concluded, LIII gives rise to the internal and vascular tissues of plants. The result from histological observation in this study indicated that adventitious roots originated only from the LIII apical cell layer regardless of the source of the explant. This finding also indicated that the adventitious root apical meristem in dicotyledons was only composed of a single apical cell layer (LIII), unlike its SAM consisting of three layers (LI, LII and LIII). This result is consistent with the report by Zonneveld (2007) that LIII is the only apical or "germ" layer contributing to adventitious roots in monocotyledons.

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