Developmental Plasticity of Glandular Trichomes into Somatic Embryogenesis in *Tilia amurensis*

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- **Background and Aims** In *Tilia amurensis*, two types of trichomes (hairy and glandular) develop from epidermal surfaces of cotyledons and hypocotyls of zygotic embryos soon after germination. Here, it is demonstrated that glandular trichome initials develop directly into somatic embryos when treated *in vitro* with 2,4-dichlorophenoxyacetic acid (2,4-D).
- **Methods** Zygotic embryos of *Tilia amurensis* were cultured on Murashige and Skoog medium with 3% sucrose and various concentrations (0, 2.2, 4.4 and 8.8 μM) of 2,4-D. Morphological development of trichomes and somatic embryos was analysed by scanning electron microscope and light microscope after histological sectioning.
- **Key Results** In zygotic embryos cultured on medium with 4.4 μM 2,4-D, formation of hairy trichomes was completely suppressed but formation of glandular trichome initials increased. That some filamentous trichome initials developed directly into somatic embryos was confirmed by histological and scanning electron microscope observation. When explants with different stages of trichome initials (two-, four- and eight-celled filamentous and fully mature trichomes) were temporally pre-treated with 4.4 μM 2,4-D for 24 h and transferred into hormone-free medium, two-celled and four-celled filamentous trichome initials were the effective stage of trichomes for somatic embryo induction.
- **Conclusions** It is suggested that early developing filamentous trichome initials have developmental plasticity and that with 2,4-D treatment these trichome initials develop directly into somatic embryos.

**Key words:** *Tilia amurensis*, glandular trichome, hairy trichome, somatic embryo, filamentous trichome initials, developmental plasticity.

INTRODUCTION

*Tilia* species are large deciduous trees, typically 20–40 m tall, belonging to the family *Tiliaceae*, which are native throughout most of the temperate northern hemisphere, in Asia, Europe and eastern North America (Hickok and Anway, 1972). The timber of *Tilia* trees is soft and easily worked and the wood is popular for carving and making musical instruments. Beekeepers value *Tilia* species as their nectar produces a very pale but richly flavoured monofloral honey. The nectar and scent are produced from trichomes in the flowers (Naef et al., 2004).

Plants have a developmental plasticity to change their growth and development enabling them to adapt and endure extreme environmental stimuli. One of the representative phenomena of developmental plasticity is that whole plants can be regenerated from specifically differentiated cells, tissues or organs. Under appropriate *in vivo* or *in vitro* conditions, certain somatic plant cells have the capacity to initiate embryogenesis (somatic embryogenesis). Somatic embryogenesis provides a unique experimental model to understand the molecular and cellular bases of developmental plasticity and totipotency in plants.

Competency for somatic embryo development in plant cells largely depends upon the developmental stage of plant tissues (Green and Phillips, 1975; Pence et al., 1980; Gingas and Lineberger, 1989; Choi et al., 1998).

Somatic embryos develop directly from cultured explants or indirectly from differentiated embryogenic cells or calluses. In direct somatic embryogenesis, cultured tissues have predetermined embryogenic competency (Pence et al., 1980; Sharp et al., 1980). Groups of epidermal cells and/or subepidermal cells or single epidermal cells contribute to somatic embryo development (Williams and Maheswaran, 1986; Choi et al., 1998).

Trichomes are derived from various kinds of epidermal outgrowths on leaf or stem surfaces (Wagner, 1991; Marks, 1997; Glover, 2000; Liakopoulos et al., 2006). Epidermal tissues give rise to various types of trichomes, such as spines, hairs or glands that give plant leaves or stems distinctive textures. Trichomes have various functions and protect against insect or herbivore attack (Levin, 1973; Agrawal, 1998; Traw and Dawson, 2002). One of the trichome defence mechanisms found in many crops is spatial hindrance by physical protection against herbivores. Another important trichome defence mechanism is physical entrapment. Glandular trichomes are known to secrete a variety of chemicals toxic to insects and cause allergic and irritant responses to herbivores (Hare and Smith, 2005). Glandular trichomes on flowers produce volatile fragrances that attract pollinators (Pichersky and Gershenzon, 2002; Naef et al., 2004).

In this study it was found that somatic embryos develop from the glandular trichome initials when zygotic embryos are cultured on a medium with 2,4-dichlorophenoxyacetic acid.

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acid (2,4-D), thus somatic embryo and trichome development share a common origin in the initial stages. It was demonstrated that early filamentous trichome initials developed into somatic embryos in the presence of 2,4-D in *Tilia amurensis*.

**MATERIALS AND METHODS**

**Somatic embryo induction from the culture of zygotic embryos**

Mature seeds of *Tilia amurensis* were collected at the Forest Seed Research Center, Korea Forest Research Institute during September. Seeds were sterilized in 70% ethanol for 1 min, disinfected in 2% sodium hypochlorite solution for 8 min, and rinsed five times in sterile distilled water. After zygotic embryos were dissected out from the seeds they were placed on culture medium.

Zygotic embryos before germination were cultured on MS (Murashige and Skoog, 1962) solid (0.25% gelrite) medium with 3% sucrose, with or without 4-4 μM 2,4-D. The medium was adjusted to pH 5.8 before autoclaving at 121 °C for 20 min. The culture room was maintained with a 16/8 h (light/dark) light regime using white fluorescent tubes at 35 μmol s⁻¹ m⁻² and kept at 25 ± 1 °C.

Hypocotyls with radicles were cut from cotyledonary zygotic embryos before germination and were cultured on MS solid (0.25% gelrite) medium with 3% sucrose and various concentrations (0, 2.2, 4.4 and 8.8 μM) of 2,4-D. After 5 weeks of culture, frequency of hairy and glandular trichomes, and somatic embryo formation were examined. Five explants were cultured in each Petri dish. Each experiment was performed five times.

To investigate the developmental fate of epidermal cells for trichome and somatic embryo formation, hypocotyl segments of zygotic embryos were pre-cultured on MS solid (0.25% gelrite) medium with 3% sucrose and various concentrations (0, 2.2, 4.4 and 8.8 μM) of 2,4-D. Thereafter, these explants with different developmental stages of trichome initials (before trichome development, two-cell, four-cell and eight-celled filamentous stage, and fully developed trichome) were transferred onto MS medium lacking 2,4-D. The number of somatic embryos per explant was analysed after 5 weeks of culture. Five explants were cultured in each Petri dish. Each experiment was performed five times.

To study the cellular origin of trichome and somatic embryos, samples were fixed at 4°C for 24 h in 1:5% glutaraldehyde and 1:6% paraformaldehyde, buffered with 0.05 M phosphate buffer, pH 6.8, and post-fixed in 2% OsO₄ for 2 h. They were dehydrated in an ethanol series (30, 50, 60, 70, 80, 90, 95 and 100%) and embedded in epoxy resin. The samples were semi-thin (3 μm) or ultrathin sectioned using an autocut microtome (Leica RM 2165, Germany). Semi-thin sections were stained with 0.05% toluidine blue O in 0.1 M phosphate buffer at pH 6.8, and thin sections with 1% uranyl acetate and lead citrate (Reynolds, 1963). The sections were observed with a light (Olympus CX31) or transmission electron microscope (LEO 912AB; Carl Zeiss, Oberkochen, Germany).

**RESULTS AND DISCUSSION**

**Trichome development in Tilia**

Zygotic embryos were cultured on hormone-free MS medium with 3% sucrose. Two types of trichomes started to develop on the surfaces of leaves, cotyledons, epicotyls and hypocotyls soon after germination. One was a non-branched unicellular hairy trichome formed by the protuberance of single epidermal cells (Fig. 1A, B). The second was a glandular trichome with a multicellular head (Fig. 1C–F). Hairy trichomes preferentially develop on the surface of leaf veins and leaf margins (Fig. 1B). Both glandular and hairy trichomes develop on similar parts of the petiole, stem and hypocotyl (Fig. 1C). When glandular trichomes were fully developed, they were covered with mucilaginous liquid exudates (Figs. 1E, F, arrows) and the head cells of the trichomes were coloured with brown or red pigments (Fig. 1D, E).

**Somatic embryo development from filamentous trichome initial-like cellular structure**

When zygotic embryos were cultured on medium with 4.4 μM 2,4-D, hairy trichome development was strongly suppressed (Fig. 2). However, formation of filamentous trichome initials, which are an early stage of development in glandular trichomes, significantly increased (Fig. 2).
Interestingly, these filamentous trichome initials participated in somatic embryogenesis (Fig. 2). In the presence of 4.4 $\mu$M 2,4-D, about 45.3% of trichome initials developed into somatic embryos (Fig. 2) and the others developed normally into glandular trichomes. The optimal concentration of 2,4-D for somatic embryo formation from zygotic embryos was 4.4 $\mu$M 2,4-D with a decline again with 8.8 $\mu$M 2,4-D treatment (Fig. 2). The mean number of somatic embryos per explant was 89.7 ± s.e. with 4.4 $\mu$M 2,4-D (Fig. 2). With other auxins, naphthaleneacetic acid (NAA) and indole-3-butyric acid (IBA), somatic embryos were formed, but the frequency of somatic embryo formation was lower than with 2,4-D (data not shown). Cytokinin treatment alone suppressed somatic embryo induction (data not shown). In Arabidopsis, gibberellin and jasmonic acid had a synergistic effect on the induction of trichomes (non-glandular) (Traw and Bergelson, 2003). In the culture of zygotic embryos of Tilia on medium with 2,4-D, non-glandular hairy trichome formation was strongly suppressed, while glandular trichome formation increased. This result indicates that the hormonal requirement for signalling the development of the two types of trichomes might be different. A detailed analysis of how development of both trichomes is regulated has yet to be performed.

When trichome initials develop on all surfaces of hypocotyls, as shown in Fig. 3A and B, somatic embryos were located near the radicle region (Fig. 3B, C), probably due to competition for nutrients for further growth in somatic embryos. In a few explants, all trichome initials participated in somatic embryogenesis (Fig. 3C). However, in the

![Fig. 1. Hairy- and glandular trichome development in Tilia seedling.](image1)

![Fig. 2. Effect of 2,4-D concentration on hairy and glandular trichomes, and somatic embryo formation from hypocotyl segments of zygotic embryos after 5 weeks of culture.](image2)
majority of explants, both somatic embryos and glandular trichomes formed (Figs 2 and 3D, E). Radical tips of somatic embryos were interconnected with the epidermal surfaces due to remnant filamentous initials (Fig. 3E). Thus, somatic embryos were easily separated from maternal explants (Fig. 3F). Without intermittent filamentous trichome initial development, somatic embryos never formed.

Scanning electron microscope investigations revealed that glandular trichome development was initiated from the protrusion of single epidermal cells, then they transversely divided two or three times (Fig. 4A) and eventually took on a filamentous shape with eight to ten cells in a uniserate layer (Fig. 4B). At the filamentous stage (Fig. 4A, B), it is very difficult to discriminate between the somatic embryos and glandular trichomes. Somatic embryo development started from the intercalary positioned cells of filamentous trichome initials (Fig. 4C, arrow), and developed into globular (Fig. 4D) and subsequently into heart-shaped and torpedo-stage embryos (Fig. 4E).

Histological observation revealed that trichome initials (Fig. 5A, arrows) developed from single epidermal cells after 5 d of culture (Fig. 5B, C). Numerous trichome initials (Fig. 5C, arrows) developed at the surface of hypocotyls and cotyledons after 10 d of culture. These trichome initials developed into glandular trichomes (Fig. 5E, F) or into somatic embryos (Fig. 5G, H).

It is very exceptional for the developmental fate of a glandular trichome to be changed to somatic embryogenesis by auxin treatment. Direct somatic embryogenesis from epidermal cells is extensively reported in many plant species. In this case, plant cells and tissues have predetermined embryogenic competency and the growth regulators or

Fig. 3. Direct secondary somatic embryogenesis from the surfaces of somatic embryos on medium with 4-4 μM 2,4-D: (A) trichome initial development on the surfaces of hypocotyls of somatic embryos after 10 d of culture (arrow indicates the enlarged view); (B) glandular trichome (T) and somatic embryo (SE) formation after 2 weeks of culture; (C) various stages of somatic embryos after 4 weeks of culture; (D) somatic embryos at globular stages; (E) somatic embryos at heart-shape stages, arrows indicate interconnection between epidermal surface and radicle of somatic embryos; (F) somatic embryos at torpedo stages; (G) somatic embryos at cotyledonary stages. Scale bars: A = 1200 μm; B, C = 800 μm; D, E, F = 500 μm; G = 1500 μm.
other stress treatments play a triggering role in somatic embryo development (Pence et al., 1980; Sharp et al., 1980). In the culture of *Tilia* zygotic embryos, somatic embryogenesis always starts at an early stage from filamentous trichome initials. Without an intervening filamentous cellular stage, somatic embryos never formed. These results indicate that the filamentous trichome initials have high developmental plasticity and these trichome initials can change their developmental fate into somatic embryogenesis with 2,4-D treatment. This is the first report that early developmental stages of glandular trichomes have developmental plasticity for somatic embryogenesis.

It is widely accepted that the early developmental pattern of somatic embryos is random and does not correspond to zygotic embryogenesis, which has a unique pattern of cellular structures depending on the plant species (Halperin, 1966; Haccius and Bhandari, 1975). Most somatic embryos appear to lack a suspensor during somatic embryo development (Halperin, 1966). Haccius and Bhandari (1975) suggest that the suspensor-like figure of pro-embryos in cell suspension cultures is nothing more than a rudimentary pro-embryonal cell complex. Williams and Maheswaran (1986) suggested that the subtending embryonic tissue fulfills the role of a suspensor. On the contrary, in zygotic embryogenesis, pro-embryos have a unique pattern of cellular structures depending on the plant species (Dodeman et al., 1997). In many species, the early pro-embryo stage of zygotic embryos has a filamentous cellular structure with long suspensor cells (Dodeman et al., 1997). In *Tilia*, although all stages of embryos have clear suspensor-like appendages between explants and the root pole of somatic embryos, the suspensor-like structure of somatic embryos may simply be due to the remnant filamentous trichome initials.

**Stage-dependent embryogenic competency of trichome initials**

It is still unclear at what stage trichome initials take part in the developmental plasticity and competency of somatic embryo formation. To solve this question, hypocotyl segments with trichomes at various stages of development after being cultured on MS medium with 4-4 μM 2,4-D were transferred to hormone-free MS medium (Fig. 6). Transferring explants without filamentous trichome initials was not effective for somatic embryo development. Frequency of somatic embryo formation increased as the explants were exposed for longer time to 2,4-D (Fig. 6).

Explants with different stages of trichomes (before initiation and two-celled, four-celled, eight-celled and fully developed stages) on hormone-free medium were pulse-treated with 4-4 μM 2,4-D for 24 h and then transferred to hormone-free medium. Pulse treatment of filamentous initials with 2,4-D at two- and four-celled stages was most effective at inducing somatic embryogenesis, although the frequency of somatic embryo formation was less effective than continuous treatment with 2,4-D (Fig. 7). Mature trichomes were not effective for somatic embryo induction by pulse treatment of 2,4-D (Fig. 7). This result indicates that developmental plasticity and embryogenic competency...
**Fig. 5.** Histological observation of trichome and somatic embryo formation: (A) filamentous trichome initials (arrows) on the epidermal surfaces of zygotic embryos; (B) ultra-thin section of trichome initials; (C) filamentous trichomes (arrows) on the surfaces of cotyledons; (D, E) mature glandular trichomes (the arrow in E indicates thickened cell wall); (F) globular somatic embryos; (G) heart-shaped somatic embryos. Scale bars: A = 180 μm; B = 18 μm; C = 320 μm; D and E = 80 μm; F = 160 μm; G = 200 μm.

**Fig. 6.** Somatic embryo formation from different stages of filamentous trichome initials. Different stages of filamentous trichomes were induced from hypocotyl segments on medium with 4.4 μM 2,4-D and the explants were transferred onto hormone-free medium. Vertical bars represent the mean values ± s.e. of three independent experiments. Means followed by a different letter are significantly different (Duncan’s multiple range test, \( P < 0.05 \)).

**Fig. 7.** Effect of 2,4-D pulse treatment on somatic embryogenesis from different stages of filamentous trichome initials. The different stages of filamentous trichomes were induced from hypocotyl segments on hormone-free medium and the explants were pulse-treated with 4.4 μM 2,4-D for 24 h and transferred onto hormone-free medium. Vertical bars represent the mean values ± s.e. of three independent experiments. Means followed by a different letter are significantly different (Duncan’s multiple range test, \( P < 0.05 \)).
are maximal early on at the initial stage of filamentous trichome growth.

There are some reports on somatic embryogenesis in Tilia cordata (Chalupa, 1990; Kärkönen, 2000) and Tilia amurensis (Kim et al., 1988). By culturing immature zygotic embryos of Tilia cordata, Kärkönen (2000) demonstrated direct somatic embryogenesis from the surfaces of cotyledons of immature zygotic embryos, but did not describe the role of trichomes in somatic embryo formation. Kim et al. (1988) reported somatic embryogenesis from embryogenic callus in Tilia amurensis.

In conclusion, in Tilia, glandular trichome initials have high developmental plasticity for somatic embryogenesis, which is induced by 2,4-D treatment. High frequency somatic embryogenesis from epidermal single cells in Tilia indicates that this system may encourage rapid plant regeneration and efficient genetic transformation.

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LITERATURE CITED


