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TECHNICAL NOTE

Quantification of pollen tube attraction in response to guidance by female gametophyte tissue using artificial microscale pathway

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We developed two types of artificial platforms, T-junction and crossroad microchannel devices, and obtained guidance response ratio of pollen tubes to the female tissue as 56–57%. The crossroad device was also able to collect the attracted pollen tubes with high purity, which is useful for future omics analysis.

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[**Key words:** Plant reproduction; Pollen tube; Ovule; Pollen tube guidance; *Torenia fournieri*; Purification; Polydimethylsiloxane; Microchip; Microchannel; Micro-electro-mechanical system]

Pollen tube guidance plays an essential role in plant reproduction because fertilization and subsequent seed development cannot be achieved without pollen tubes (PTs) delivering two sperm cells to an egg cell and to a central cell in the female gametophyte, respectively. While signals from female gametophyte tissue are believed to guide the PTs to the embryo sac, interaction between the PT and female gametophyte tissue is essential for successful fertilization in plants. Therefore, precise quantification by means of a PT guidance assay is an important objective to gain an improved understanding of the mechanism of PT guidance. An *in vitro* guidance assay has been performed on an agarose medium with ovules of *Torenia fournieri* and the guidance ratio has been defined using the total number of ovules as the population (1–3). However, not all germinated PTs show capability for the guidance response to an attractant released from ovules and at least germination of pollen grains on the stigma and elongation of PTs through the cut style are essential for acquisition of the guidance response capacity (1). Therefore, measurement of the guidance response ratio (GRR) of PTs, defined as the proportion of guided PTs in the total population of PTs, is important for exploration of the mechanism of PT guidance. In the previous studies described above, however, the GRR of PTs was not measured probably because it was difficult to determine whether or not PTs were guided during variable growth of PTs under the bulk condition on an agarose medium in a petri dish.

To allow access to measure the GRR of PTs, miniaturized microdevices, represented by micro-electro-mechanical systems (MEMS), show various advantages in functionality compared with their macroscopic counterparts with decreasing scale (4). In general, the volume effect is reduced more than the surface effect in down-scaling. Taking advantage of the physical phenomena of a microscale platform, various novel types of biological experiments have been realized using microdevices (5), such as a microheater (6), microchamber array (7), and microfluidic devices (8), frequently in combination with micro/nano manipulation techniques (9). Microchannel devices or microfluidic systems have been adopted, in the past few years, to perform PT growth assays efficiently and quantitatively. The on-chip method has considerable potential as a future platform for studies of plant reproduction because it is expected to allow not only precise handling of PTs but also to create and control the chemical environment at micrometer-scale resolution. Such technology can be used to develop a microenvironment mimicking that within the pistil during *in vivo* PT growth. Given these advantages, PT growth rate measurement has become possible under conditions that closely simulate the *in planta* environment (10). A microsystem-based assay has been successfully adopted to develop a microfluidic network to examine the cellular penetrative forces (11) and the device was further modified for experimentation and phenotyping of PTs (12). An on-chip guidance assay was first performed in a relatively large microchannel that held a pistil of *Arabidopsis thaliana* and allowed the PTs to grow in the channel filled with a gel-based pollen growth medium (13). An advanced on-chip guidance assay was realized by adopting *Torenia fournieri*, whose pollen tubes grow faster and longer than those of *A. thaliana*. Consequently, PT growth orientation at the T-junction showed left or right distinction, which made the guidance assay more efficient for further screening of materials that may be involved in PT attraction (14). However, in these assays, the GRR is unavoidably overestimated because, in addition to the attracted PTs, whether non-attracted PTs grow towards or away from the female gametophyte tissues is assumed to be of equal probability.

In this report, we propose a method to measure the net GRR of PTs quantitatively using microchannel devices of simple design,

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namely the crossroad device and the T-junction device. Using this method, we report that the net GRR of PTs was measured to be 56–57% under the experimental condition. In addition, we achieved enrichment of attracted PTs in the ovule-containing microchannel reservoir at the percentage of 87% using the crossroad device.

Two types of polydimethylsiloxane (PDMS) microchannel devices were prepared in this study. Both the T-junction and crossroad devices consist of a buffer and style inlet, buffer outlets, and microchannels of 500 μm in width and 25 μm in height. Thus, the device allows for growth assays of multiple PTs passing through the same pistil (Fig. 1). In the T-junction device, the distance between the center of the style inlet and the T-junction was 3.5 mm. In the crossroad device, the distance between the center of the style inlet and the crossroad was 2 mm. The microchannel devices were developed using the fabrication process described elsewhere (15). Briefly, the negative master for the molding of PDMS was fabricated on a silicon wafer with an ultra-thick photoresist (SU-8 3025; Microchem Corp., Newton, MA, USA). The prepolymer of PDMS (Sylgard 184, Dow Corning, Midland, MI, USA) was cast onto the master, which had a frame to hold the prepolymer in place. The prepolymer was degassed in the vacuum chamber for more than 20 min. After incubation at 65°C for 90 min, the cured PDMS was peeled off from the master, and the through-holes for the style (1.2 mm in diameter) and buffer inlets (3 mm in diameter) were punched out manually.

The experimental protocols were as follows. The PDMS microchannel device was placed in the middle of a glass-bottomed dish. After degassing the device in a vacuum chamber at 10 kPa for at least 40 min, 20–40 μL modified Nitsch's medium (16) was injected from the style inlet to fill all of the channels via the power-free pumping mechanism of the PDMS microchannel device (17,18). A hand-pollinated style of *Torenia fournieri* cv. blue and white, cut to a length of 1 cm, was placed in the style inlet of the device. A placenta possessing approximately 500 ovules from a mature *T. fournieri* flower was placed in the buffer inlet. Concentration of attractant at the junction of the crossroad device can be lower than at the junction of the T-junction device. However, since we confirmed that 10–20 ovules in buffer inlet can induce PTs in the T-junction device (Kuzuya et al., unpublished data), a placenta possessing 500 ovules create sufficient concentration of attractant also at the junction of the crossroad device. After pollination, the pollen tubes grew through the style and entered the channels. The dish was kept moist in an incubation chamber at 25 ± 1°C. The measurement of the GRR of PTs was assessed 16–20 h after pollination. For microscopic observation of PTs in the microdevice, we used a stereomicroscope (Axio Zoom V16; Zeiss, Germany, MVX-10; Olympus, Japan) equipped with a CCD camera (AxioCam MRC; Zeiss, DP72; Olympus). For image processing, image analysis software, ImageJ, was used (http://imagej.nih.gov/ij/).

The GRR of PTs can be calculated from the number of PTs entering anovule-containing microchannel (Fig. 1). In the T-junction device, given that non-attracted PTs growing towards or away from the female tissues are assumed to be of equal probability, the proportion of PTs entering the microchannel directed towards the ovules over the total number of PTs (RTt) is expected to be

$$RTt = GRR + \frac{100 - GRR}{2} \quad (1)$$

where GRR represents the net guidance response ratio. This equation is based on the premise that hemorepulsion response between PTs and between PTs and ovules do not take place during our guidance assays, because the repelling response has not been observed in *in-vitro* assays until PTs get close to ovules within short range at a distance ~100 μm (19). The ratio of PTs entering the microchannel directed away from the ovules over the total number of PTs (PTa) is expected to be

$$PTa = \frac{100 - GRR}{2} \quad (2)$$

The measured frequencies of PTs growing towards (PTt) and away from the ovules (PTa) by repeated experiments were 78.1 ± 6.5% and 21.9 ± 6.5% (mean ± S.D., n = 16), respectively (Fig. 2A). In the absence of ovules the respective values were 50.3 ± 9.8% and 49.7 ± 9.8% ($n = 11$) (Fig. 2B). Consequently, PTs were significantly attracted towards the ovules ($p < 0.01$, Student's *t*-test) and the net GRR was calculated to be 56.2% for the assay using the T-junction device. On the other hand, for the crossroad device the ratio of PTs entering the microchannel towards the ovules over the total number of PTs (Rca) is expected to be

$$Rca = GRR + E \quad (3)$$

where E (error) represents the proportion of PTs entering channels both towards and away from ovules by chance without any guidance effect. The ratio of PTs entering the microchannel away from the ovules over the total number of PTs is expected to be equal E,

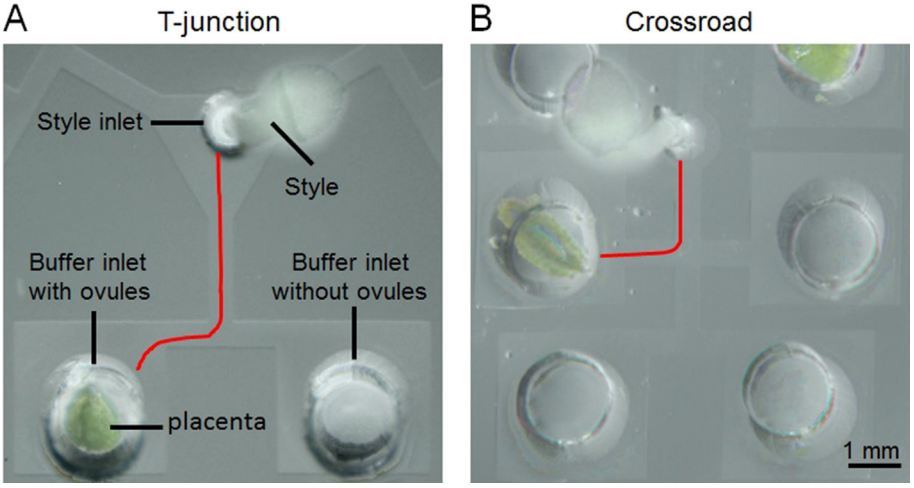

FIG. 1. Photographs of the T-junction device (A) and the crossroad device (B) for measurement pollen tube guidance response ratio. Attracted pollen tubes by guidance signal from ovules are illustrated in red.

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