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Short Communication

Expression of green fluorescent protein in pollen of oilseed rape (*Brassica napus* L.) and its utility for assessing pollen movement in the field

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Transgene movement via pollen is an important component of gene flow from transgenic plants. Here, we present proof-of-concept studies that demonstrate the monitoring of short distant movement of pollen expressing a genetically encoded fluorescent tag in oilseed rape (*Brassica napus* L. cv. Westar). Transgenic oilseed rape plants were produced using *Agrobacterium*-mediated transformation method with the pBINDC1 construct containing a green fluorescent protein (GFP) variant, mGFP5-ER, under the control of the pollen-specific LAT59 promoter from tomato. Transgenic pollen was differentiated from non-transgenic pollen *in vivo* by a unique spectral signature, and was shown to be an effective tool to monitor pollen movement in the greenhouse and field. GFP-tagged pollen also served as a practical marker to determine the zygosity of plants. In a greenhouse pollen flow study, more pollen was captured at closer distances from the source plant plot with consistent wind generated by a fan. Under field conditions, GFP transgenic pollen grains were detected up to a distance of 15 m, the farthest distance from source plants assayed. GFP-tagged pollen was easily distinguishable from non-transgenic pollen using an epifluorescence microscope.

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Pollen flow is a prominent mode for transgene movement in the environment, and it is desirable to track transgene movement under field conditions to assess potential ecological risks such as the interspecific hybridization with weedy relatives and potential impact on non-target organisms [1]. The pattern of pollen movement from a transgenic crop variety is a direct measure of out-crossing potential to conspecific crops and wild relatives. To date, most gene flow studies have been performed by progeny analysis using conventional molecular techniques. Although these studies reflect actual hybridization events, the capacity of the pollen to move within the environment has been measured indirectly, with little information on pollination vectors (*i.e.*, wind or insects). We have proposed that green fluorescent protein (GFP) expressed in pollen grains may be used as a marker to directly measure pollen movement under environmental conditions of interest [2], and have performed proof-of-concept studies to test this proposal in an agronomic crop with the propensity for intra-and interspecific hybridization: *Brassica napus*.

Oilseed rape (OSR; *Brassica napus* L. AACC 2n=38) transgene flow research under field conditions has often been performed using an herbicide tolerance trait as a detection method [3], which requires seed collection and progeny analysis using a destructive method. GFP or other fluorescent protein markers might be useful replacements for conventional molecular techniques and herbi-

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Abbreviations: **CaMV**, cauliflower mosaic virus; **GFP**, green fluorescent protein; **OSR**, oilseed rape; **RFP**, red fluorescent protein

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cide resistance markers as a real-time *in vivo* marker for the presence and expression of transgenes [4–7]. Using GFP expressed in vegetative tissues under the control of the CaMV 35S promoter, transgene flow has been assessed in transgenic OSR using progeny analysis [8]; however, GFP was not expressed in the pollen. A system including pollen tagged with GFP could be used in monitoring transgenic pollen directly as well as pollen distribution biology, pollen viability, and pollen competition biology. The tomato LAT59 promoter [9], which is preferentially expressed in the anthers and pollen of tomato, has been effectively used to express GFP in tobacco pollen [2]. Using pollen traps to measure pollen movement from transgenic varieties has been considered as a problematic technique because it is sometimes difficult to distinguish species or varieties of pollen from one another [10]. GFP-tagging of pollen could be a useful tool to address these issues in some applications, including the ability to distinguish specific transgenic pollen from others in relatively complex environmental mixtures.

OSR is an appropriate crop for the study of transgene escape or movement, since it has many wild relatives such as wild turnip (*Brassica rapa* L.), which occurs as weed populations in or near OSR cultivation areas and has an overlapping flowering period [11–13]. OSR is a partially self-fertilizing species, at rates ranging from 53% to 88%, depending on cultivar and environmental conditions [14]. Approximately half of OSR pollen grains from a given plant fall onto plant surfaces and the ground within 3 m from the source plant [15]. A small percentage of OSR pollen becomes airborne and can move relatively long distances (up to 3 km) via the wind [15, 16]. In addition, insect pollinators, especially bees, can transmit OSR pollen for long distances [17]. In this study, we describe the production of transgenic OSR lines that express GFP in pollen grains, detection of GFP-tagged pollen in the greenhouse and field, and assessment of zygosity via segregation analysis.

Plant transformation and regeneration methods were based on an existing protocol, in which *Agrobacterium*-mediated transformation was performed using hypocotyl segments with antibiotic selection and subsequent plant recovery via organogenesis [18, 19]. Plant transformation was carried out with OSR (*B. napus* L. cv. Westar). The pBINDC1 construct [2] that contains the mGFP5-ER variant under the control of the LAT59 pollen-specific promoter was used for transformation experiments. All cultures were maintained at 24±2°C under a 16/8 h (light/dark) photoperiod. The recovered number of explants, shoots, rooted shoots, and fertile plants were recorded. OSR transgenic events with the pBINDC1 plasmid were designated as LH *B. napus*.

Twenty-six T_1 transgenic seeds from the transformed OSR LH event 1 (LH1) were planted and grown in the greenhouse. Five flowers were collected separately from each individual T_1 LH1 plant to assay pollen. Collected flowers were tapped by hand on clean microscope slides (Fisher Scientific, Pittsburgh, PA, USA) to collect the pollen. Collected pollen from each individual plant was observed using an epifluorescence (FITC filtered) microscope (Olympus BX51 model) with blue light excitation at 200× magnification to score pollen for GFP and infer zygosity status of the parent. The pollen population was inferred to come from homozygous, hemizygous, or non-transgenic isogenic lines for the GFP transgene based upon Mendelian segregation. To confirm determinations of zygosity based upon pollen fluorescence, seeds were collected from the plants, germinated on MS media containing 200 mg/L kanamycin, and segregation patterns were noted. One hundred seeds from each plant were surface sterilized and plated. Only germinated seeds were scored. Green, normal seedlings were scored as transgenic and bleached seedlings with diminished roots were scored as non-transgenic segregants. GFP-specific PCR was used as a confirmatory analysis [19].

A greenhouse experiment was conducted in the Racheff research greenhouse at the University of Tennessee at Knoxville, USA. Homozygous LH1 and Westar plants were planted in 4-L pots and placed alternately in rows (Fig. 1). A total of 15 transgenic and 15 non-transgenic plants were used as source plants with a fan to generate air currents (Aloha 30" Pedestal fan) (Fig. 1). Pollen dispersion was measured when each individual plant had at least 30 open flowers for 3 consecutive days. Pollen traps were used as indicators of pollen sinks and placed at various distances (2, 4, 6, 8, 10, and 12 m) from the source plants to measure pollen flow (Fig. 1). Pollen traps were constructed by covering microscope slides with vaseline petroleum jelly (Unilever, Englewood Cliffs, NJ, USA) and attaching them to wooden stakes with twist ties. The slides were attached to wooden stakes at 1 m from the greenhouse floor. Wind speed was measured at each distance using the portable wind meter using the 'Avg10' function, which allowed the measurement of average wind speed for 10 s (Skymate, Speedtech Instruments, Great Falls, VA, USA). New pollen traps were placed at 9:00 am in the morning and collected at 5:00 pm on the same day. Pollen collection was conducted for 3 consecutive days. Collected pollen traps were observed under an epifluorescence microscope with blue light excitation and a FITC filter for GFP. The number of LH1 and Westar pollen grains in each pollen trap was recorded.

A field experiment was performed at the UT Plant Science Farm, Knoxville, TN, USA (35°58'N, 83°55'W) in the spring of 2005. The experimental design included pollen source plants in the center of the plot and sink plants in concentric circles (Fig. 1A). Each center quadrant was 3 m^2 and contained 150 plants, half transgenic and half non-transgenic plants: this design was employed to test whether transgenic and non-transgenic pollen could be differentiated from one another (Fig. 1). Methods used in the field experiment for the construction of pollen traps

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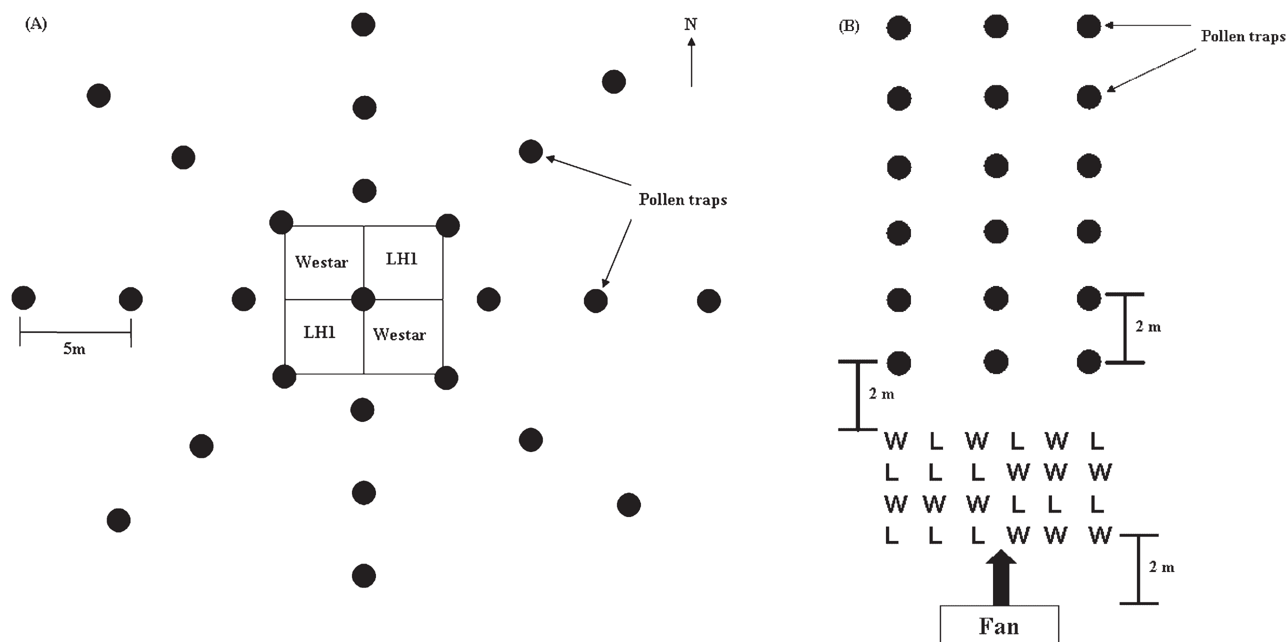

Figure 1. (A) Field design consisted of a central plot split into 4 quadrants. Two quadrants contained LH1 and the remaining two contained Westar. Pollen traps were placed in different directions (N, S, E, W, NW, SW, NE, SE) at distances of 0, 5, 10, 15 m from the center of the source plant plot with two traps located in the center. (B) Greenhouse experiment design with homozygous LH1 and Westar mixed populations. Pollen traps were placed at 2, 4, 6, 8, 10, and 12 m from the source plant plots.

were the same as described in the greenhouse study, but pollen was collected over 4 consecutive days during peak flowering. Pollen traps were placed in different directions (N, S, E, W, NW, SW, NE, SE) at distances of 0, 5, 10, 15 m from the center plant source plot. One slide glass was placed as a pollen trap at each sampling point. Fresh pollen traps were placed at 8:30 am and collected at 5:30 pm on the same day. Wind direction and speed were acquired from the wind information recorder located at the UT Plant Science Farm. Collected pollen traps were screened under an epifluorescence microscope at 200× magnification. The numbers of LH1 and Westar pollen grains were recorded on each pollen trap.

Two LH *B. napus* events were regenerated from separate callus sectors. Out of 1024 explants, 7 shoots were recovered. From these recovered shoots, 3 formed roots. Two rooted T_0 plants produced T_1 seeds and the T_1 generation plants were phenotypically indistinguishable to Westar by visual inspection.

The zygosity of plants was determined based on the GFP pollen to non-GFP pollen ratio under an epifluorescence microscopy using a FITC filter set. T_1 generation seeds from self pollinations were germinated, and these plants were categorized as homozygous, hemizygous, and isogenic lines for the transgene according to the frequency of GFP expression in the pollen (Fig. 2). Both transgenic events apparently harbored transgenes in single loci based upon pollen segregation. Putative homozygous T_1 plants had all transgenic pollen, and hemizygous plants had 50% transgenic pollen, similar to that observed in single-locus transgenic tobacco plants using the same construct [2]. The putative homozygous and hemizygous transgenic plants had yielded progeny with confirmed expected 100% and 3:1, respectively, Mendelian segregation (Chi-squared, $p<0.05$).

In the greenhouse pollen flow experiment, pollen grains detected in the pollen traps were categorized into GFP-tagged transgenic and non-transgenic pollen. Ninety-two GFP-tagged pollen grains were detected at 2 m from the source plants for 3 consecutive days. GFP-tagged pollen grains were easily distinguishable from non-transgenic pollen grains using epifluorescence microscopy (Fig. 2). Pollen traps placed at closer distances to the center source plants trapped more pollen from both LH1 and Westar (Table 1). LH1 pollen traveled up to 10 m from the source plants; however, wind from the fan was not measurable at the 10 m distance. Westar pollen was detected at a distance of 8 m from the source plot on the second day. GFP-tagged and non-transgenic pollen were collected in pollen traps for 8 h each day.

In the field pollen flow experiment, during 4 consecutive days, 417 LH1 pollen grains and 583 non-transgenic Westar pollen grains were detected on pollen traps. As in the greenhouse study, GFP-tagged pollen was easily distinguishable from non-transgenic pollen using epifluorescence microscopy. No significant difference was detected for the number of pollen grains per trap on average between LH1 and Westar (ANOVA, $p=0.34$). The number of

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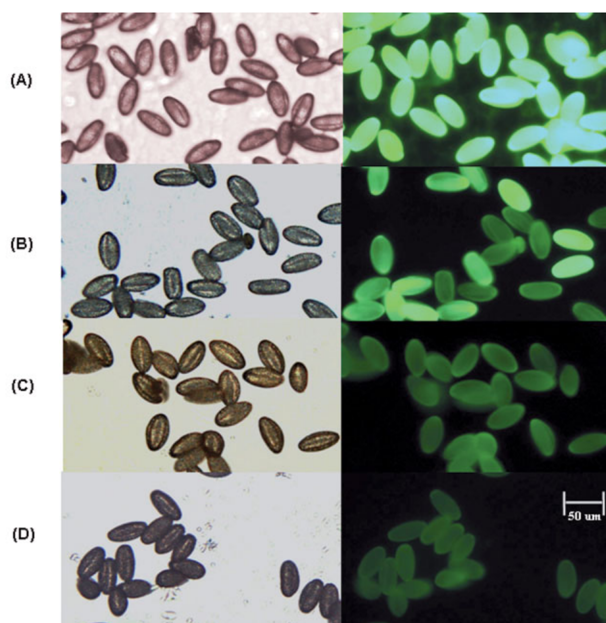

Figure 2. Determination of zygosity based on green fluorescent pollen. Pollen of T_1 LH1 and Westar. (A–C) Pollen of homozygous, hemizygous, and non-transgenic segregant LH1, respectively; (D) Westar pollen. These pictures were taken under white light (left column) and blue light (right column) with exposure times of 16.7 ms and 1.54 s, respectively, with 200× magnification.

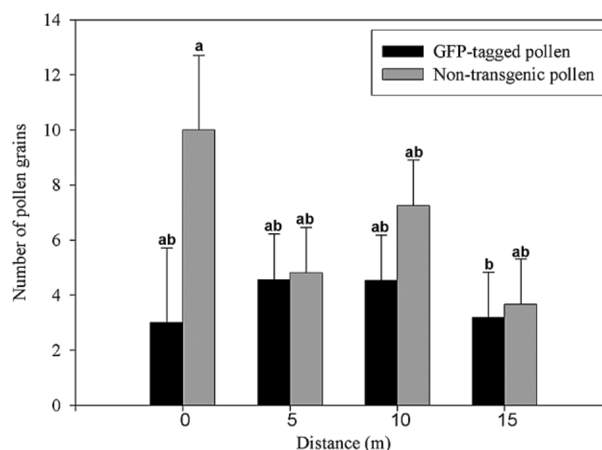

Figure 3. Average number of pollen grains of GFP-tagged and non-transgenic detected per day at each distance under field conditions. Distance indicates the distance from the center of the source plant plot. Standard errors of the mean are shown. No significant difference was detected between different distances (ANOVA, p=0.38).

pollen grains collected on traps from LH1 and Westar in each direction on each day varied; however, no significant difference was observed between the number of pollen grains collected for each distance (ANOVA, p=0.38) (Fig. 3). A significant difference was observed for the number of pollen grains per trap between different directions (ANOVA, p <0.05) (Fig. 4). No consistent effect of wind direction or wind speed for number of pollen was detected (Fig. 5). On day 1, the prevailing wind was toward the west and speed ranged from 0.29 to 5.16 m/s; however, no pollen grains were detected on pollen traps placed west from the center plot. On day 3, wind direction was between northeast and southeast, and pollen traps placed east from the source plot caught more than 10 pollen grains on average per trap.

Transgenic plants have been used effectively to better understand gene flow [20]. Among marker genes used in previous transgenic research, GFP is one of the most suitable *in vivo* markers to track transgene movement via pollen for risk assessment purposes and to study reproductive biology. GFP is a neutral reporter gene and nontoxic to plants, making it especially useful for studying the development of functional pollen [5, 21–23], although GFP might have some costs in certain cases, *e.g.*, decreased tissue growth has been noted under some circumstances in high-expressing events [24–26]. However, in tobacco (*Nicotiana tabacum*), when GFP was expressed under the control of the pollen-specific LAT59 promoter, pollen fitness and tube germination frequencies were not different from pollen of non-transgenic plants [23]. Similarly, LH1 plants were not visibly different than Westar.

Determining the transgene zygosity status of individual plants is important in plant breeding, and GFP-expressing pollen may be another tool to enable this process. Zygosity in transgenic plants can be visualized based on difference in the intensity of GFP fluorescence

Table 1. Number of pollen grains collected from LH1 and Westar plants under greenhouse conditions. Distance refers to meters from the source plants to pollen traps. Experiment was performed for 3 consecutive days. Detected pollen was screened and counted under epifluorescence microscope with blue light excitation and a FITC filter set

Distance (m)	Wind speed (m/s)	1st day		2nd day		3rd day	
		Westar pollen	LH1 pollen	Westar pollen	LH1 pollen	Westar pollen	LH1 pollen
2	0.76	99	58	85	4	0	30
4	0.58	78	0	1	0	0	0
6	0.36	9	0	0	0	0	0
8	0.18	0	6	12	0	0	1
10	0	0	0	0	1	0	0
12	0	0	0	0	0	0	0

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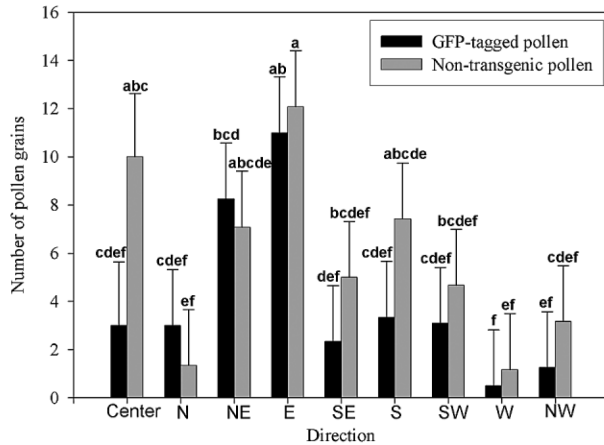

Figure 4. Average number of pollen grains of GFP-tagged and non-transgenic collected in different directions under field conditions. Central indicates the average number of pollen grains collected in two pollen traps at the center of the plot. The remaining directions represent the number of pollen grains collected on average of three pollen traps at 5, 10, and 15 m. Standard errors of the mean are shown. Different letters represent significant differences for the number of pollen grains between directions (ANOVA, Fisher's LSD, $p<0.05$).

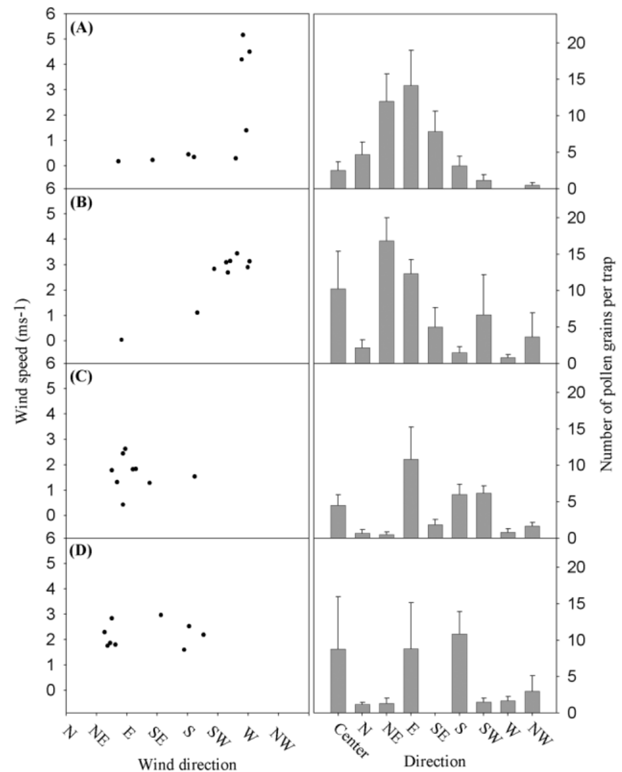

Figure 5. Number of pollen grains collected under field conditions and wind information. Pollen grains were collected in each direction on each day with wind direction and speed measured every hour from 9:00 am to 5:00 pm. (a–d) Wind direction and speed measured at every hour and average number of pollen grains collected from pollen traps for each direction. (a–d) Days 1–4, respectively. SEMs are shown.

[8, 27] when driven by the constitutive CaMV 35S promoter. Also, zygosity in transgenic plants can be analyzed by real-time PCR [28, 29]. The method using the LAT59::GFP construct was shown here to be an efficient method for determination of zygosity in transgenic plants at flowering by analysis of pollen segregation. The GFP-tagged pollen method requires relatively very short time to prepare the samples, and is a rapid, reagent-free alternative to other methods such as real-time PCR.

OSR normally produces an abundant amount of pollen for approximately 4–5 weeks [3]. The majority of the produced pollen is dispersed over a short distance [15]. In commercial OSR fields, cross-pollination was found to occur at higher frequencies in close distances to the source field [16]. Although the greenhouse experiment was performed as a preliminary experiment to see whether GFP-tagged pollen of OSR could be detected and differentiated from non-transgenic pollen, the greenhouse pollen dispersal study is important because several factors such as wind speed and direction, which can have an effect on pollen movement, could be controlled and kept consistent during the experimental process. Scheffler *et al*. [30] estimated the frequency of OSR pollen dispersal to be approximately four-times higher at a distance of 1 m than at 3 m from the transgenic pollen source when surrounded by non-transgenic plants acting as recipients at varying distances. However, in this study, no significant differences in the number of pollen grains were found in the traps at different distances (0, 5, 10, and 15 m) from the source plant plot under field conditions. The estimation of pollen frequency by Scheffler *et al*. [30] may be explained by the close proximity of non-transgenic plants to the pollen source plot creating a buffer zone and then preventing the spread of transgenic pollen over greater distances. In addition, much short distance pollen dispersal is attributable to insects rather than wind in OSR as discussed elsewhere [31], and indeed short distance gene flow may be independent of wind patterns [31]. It is important to develop pollen and gene flow tools for empirical risk assessment research since field experiments have resulted in findings (e.g., [16, 31]) that often differ from modeled expectations (e.g., [32]). Other constitutive promoters, such as the maize *Adh1* promoter, which have shown activity in root, shoot meristems, and pollen [33], could be potential candidates for risk assessment studies in the future in monocots. There are no promoters that regulate strong constitutive expression to that degree in dicots. In this study, GFP under the control of the LAT59 promoter allowed pollen to be distinguished from the same species based on the GFP expression in the pollen in proof-of-concept studies. Experimental improvements are also possible to enhance the ability to detect pollen movement via fluorescence. For example, GFP might not be the optimal fluorescent protein to tag pollen because of autofluorescence in blue light [6, 7]. Other fluorescent proteins such

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as a red fluorescent protein (RFP) might be better markers for expression in plants, including pollen grains, compared with GFP [7, 34] because of decreased spectral background under RFP visualization conditions. Also, new technology such as laser-induced fluorescence spectroscopy [35] that enables GFP observation in ambient light might be useful for detecting GFP or RFP-tagged pollen movement. Marker-tagged pollen technology might not only be useful for transgenic risk assessment research, but possibly also for post-release monitoring [6].

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