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## **Authors:**

Naoya Sugi<sup>1#</sup>, Andrea R.M. Calhau<sup>2#</sup>, Nathanaël M.A. Jacquier<sup>2</sup>, Marina Millan-Blanquez<sup>2</sup>, Jörg D. Becker<sup>3</sup>, Kevin Begcy<sup>4</sup>, Frédéric Berger<sup>5</sup>, Cécile Bousquet-Antonelli<sup>6</sup>, Daniel Bouyer<sup>2</sup>, Giampiero Cai<sup>7</sup>, Alice Y. Cheung<sup>8</sup>, Sílvia Coimbra<sup>9</sup>, Philipp Denninger<sup>10</sup>, Thomas Dresselhaus<sup>11</sup>, José A. Feijó<sup>12</sup>, John E. Fowler<sup>13</sup>, Danny Geelen<sup>14</sup>, Ueli Grossniklaus<sup>15</sup>, Tetsuya Higashiyama<sup>16</sup>, David Honys<sup>17</sup>, Tomoko Igawa<sup>18</sup>, Gwyneth Ingram<sup>2</sup>, Yvon Jaillais<sup>2</sup>, Mark A. Johnson<sup>19</sup>, Mariko Kato<sup>20</sup>, Miki Kawachi<sup>21</sup>, Tomokazu Kawashima<sup>22</sup>, Yu-Jin Kim<sup>23</sup>, Hong-Ju Li<sup>24</sup>, Sébastien Mongrand<sup>25</sup>, Kazuki Motomura<sup>26</sup>, Shiori Nagahara<sup>27</sup>, Kohdai P. Nakajima<sup>28</sup>, Brad Nelms<sup>29</sup>, Li-Jia Qu<sup>30</sup>, Arp Schnittger<sup>31</sup>, Stefan Scholten<sup>21</sup>, Stefanie Sprunck<sup>11</sup>, Meng-Xiang Sun<sup>32</sup>, David Twell<sup>33</sup>, Dolf Weijers<sup>34</sup>, Wei-Cai Yang<sup>35</sup>, Daisuke Maruyama<sup>1\*</sup>, Thomas Widiez<sup>2\*</sup>.

\* Correspondence to Daisuke Maruyama ([dmaru@yokohama-cu.ac.jp](mailto:dmaru@yokohama-cu.ac.jp)) and Thomas Widiez ([thomas.widiez@ens-lyon.fr](mailto:thomas.widiez@ens-lyon.fr)).

# Contributed equally

## **Affiliations:**

- 1 Kihara Institute for Biological Research, Yokohama City University, Yokohama, Japan.
- 2 Laboratoire Reproduction et Développement des Plantes, Univ Lyon, ENS de Lyon, UCB Lyon 1, CNRS, INRAE, F-69342, Lyon, France.
- 3 Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa (ITQB NOVA), 2780-157 Oeiras, Portugal
- 4 Environmental Horticulture Department, University of Florida, Gainesville, FL 32611, USA.
- 5 Gregor Mendel Institute, Austrian Academy of Sciences, Vienna BioCenter, Vienna 1030, Austria.
- 6 Institute de Biologie Moléculaire des Plantes (IBMP), CNRS, Université de Strasbourg, 67084 Strasbourg, France.
- 7 Department of Life Sciences, University of Siena, via Mattioli 4, 53100 Siena, Italy.
- 8 Department of Biochemistry and Molecular Biology, Molecular Cell Biology and Plant Biology Programs, University of Massachusetts, Amherst, MA, USA.
- 9 LAQV Requite, Faculdade de Ciências da Universidade do Porto, Departamento de Biologia, Universidade do Porto, Rua do Campo Alegre, Porto 4169-007, Portugal.
- 10 Technical University of Munich, School of Life Sciences, Plant Systems Biology, 85354 Freising, Germany.
- 11 Department of Cell Biology and Plant Biochemistry, University of Regensburg, 93053 Regensburg, Germany.
- 12 Department of Cell Biology & Molecular Genetics, University of Maryland, 2136 Bioscience Research Bldg, College Park, MD 20742-5815, USA.
- 13 Oregon State University, Department of Botany and Plant Pathology, Corvallis, OR 97331, USA.
- 14 Horticult, Ghent University, Coupure Links 653, B-9000 Gent, Belgium.
- 15 Department of Plant and Microbial Biology and Zurich-Basel Plant Science Center, University of Zurich, CH-8008 Zurich, Switzerland.
- 16 Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Hongo 7-3-1, Bunkyo-ku, 113-0033 Tokyo, Japan.
- 17 Institute of Experimental Botany of the Czech Academy of Sciences, 165 02 Prague 6, Czech Republic.
- 18 Division of Plant Science, Graduate School of Horticulture, Chiba University, Matsudo, Japan.
- 19 Department of Molecular Biology, Cell Biology, and Biochemistry, Brown University, Providence, Rhode Island 02912, USA.
- 20 Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan.
- 21 Division of Crop Plant Genetics, Department of Crop Science, Georg-August-University Goettingen, 37075 Goettingen, Germany.
- 22 Department of Plant and Soil Sciences, University of Kentucky, Lexington, KY 40546, USA.
- 23 Department of Life Science and Environmental Biochemistry, Life and Industry Convergence Research Institute, Pusan National University, Miryang 50463, Republic of Korea.
- 24 Key Laboratory of Seed Innovation, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China
- 25 Laboratoire de Biogenèse Membranaire, LBM CNRS-Univ. Bordeaux, UMR 5200, Villenave d'Ornon, France.
- 26 Research Organization of Science and Technology, Ritsumeikan University, Kusatsu, Shiga 525-8577, Japan.
- 27 Division of Biological Sciences, Graduate School of Science, Kyoto University, Japan.
- 28 Department of Biology, Technion - Israel Institute of Technology, Haifa 32000, Israel.
- 29 University of Georgia, Department of Plant Biology, Athens, GA 30602, USA.
- 30 State Key Laboratory for Protein and Plant Gene Research, Peking-Tsinghua Center for Life Sciences, New Cornerstone Science Laboratory, College of Life Sciences, Peking University, Beijing 100871, China.
- 31 Department of Developmental Biology, University of Hamburg, Institute for Plant Sciences and Microbiology, Hamburg, Germany.
- 32 State Key Laboratory of Hybrid Rice, College of Life Sciences, Wuhan University, Wuhan 430072, China.
- 33 Department of Genetics and Genome Biology, University of Leicester, Leicester LE1 7RH, UK.
- 34 Laboratory of Biochemistry, Wageningen University, Stippeneng 4, 6708WE Wageningen, the Netherlands.
- 35 State Key Laboratory of Molecular Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China.

Pollen is the male gametophyte of flowering plants, the typically haploid generation of the plant life cycle that produces the male gametes. Upon germination, pollen grains extend a pollen tube to transport the male gametes (sperm cells) toward the ovules for double fertilization of the female gametes harbored by the female gametophyte or the embryo sac. Pollen grains exhibit a unique “cell(s) within a cell” organization. After meiosis, each single-celled microspore divides highly asymmetrically to give rise to a vegetative and a generative cell. The generative cell is subsequently engulfed by the vegetative cell, separates from the external pollen wall and thus becomes enclosed within the large vegetative cell while migrating to the center of the pollen grain. Later in development, the generative cell divides into a pair of sperm cells. As a result of this unique developmental process, a unique membrane surrounds the male germ cells (the generative cell and subsequently the two sperm cells). Here we propose to standardize and unify the name of this membrane as the **peri-germ cell membrane (Fig. 1a)**.

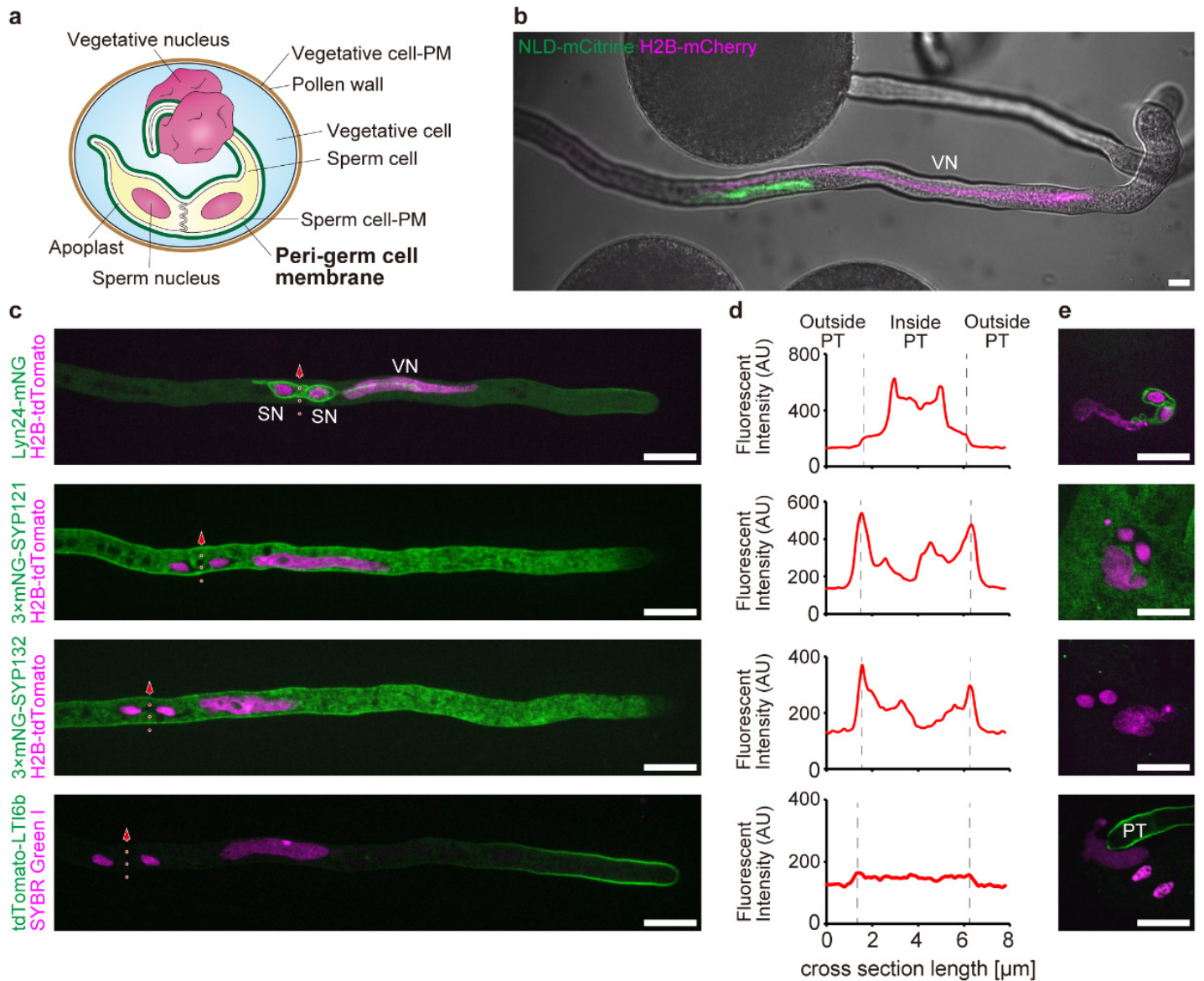
Over the past 50 years, only a few studies have examined the peri-germ cell membrane. Nonetheless, it has been assigned multiple names. For example, the peri-germ cell membrane was identified in 1969<sup>1</sup> as the “*generative cell envelope*”. Subsequent ultrastructural work in the 1980s led to new peri-germ cell membrane names, including “*internal plasma membrane of the vegetative cell*”, “*inner plasma membrane of the pollen grain*” and “*inner vegetative cell plasma membrane*”<sup>2-6</sup>. Another study referred to it as the “*pollen tube inner plasma membrane*” when considering the pollen tube<sup>7</sup>. When the *Arabidopsis* small GTPase RHO OF PLANTS 9 (AtROP9) was identified as the first protein reported to locate to this membrane, it was named by the authors as the “*Invaginated Pollen Tube Plasma Membrane*”<sup>8</sup>. More recently, when the maize NOT-LIKE-DAD/MATRILINEAL/PHOSPHOLIPASE-A1 (NLD/MTL/ZmPLA1) protein was found to localize exclusively to the peri-germ cell membrane, the authors named it the “*pollen endo-plasma membrane*”<sup>9</sup>.

This diverse nomenclature causes confusion and calls for the implementation of a consensus on terminology. For example, the term “*generative cell*” is too restrictive as the peri-germ cell membrane also encloses the two sperm cells. Similarly, the term “*pollen tube*” is not appropriate since this membrane is also present before pollen germination. Furthermore, using “*plasma-membrane*” could lead to confusion with the classical plasma membrane (PM) of the generative cell, sperm cells, or vegetative cell. Additionally, the term “*plasma-membrane*” is misleading, given that the peri-germ cell membrane may differ in protein and lipid composition from the classical PM.

In Figure 1, we show that three PM marker proteins expressed in the vegetative cell do not localize to the peri-germ cell membrane (*3×mNG-SYP121*, *3×mNG-SYP132*, and *tdTomato-LTI6b* in **Fig. 1c, d, e**). Conversely, NLD/MTL/ZmPLA1 (**Fig. 1b**), the engineered Lyn24 probe (chimeric protein associates with detergent-resistant membranes in metazoan cells)<sup>8</sup> (**Fig. 1c**), AtROP9<sup>8</sup>, AtLARP6C (La-related proteins)<sup>10</sup> and the glutamate receptor like channels (AtGLR3.3)<sup>11</sup> all localize to the peri-germ cell membrane and are either absent from the PM of the vegetative cell, or only weakly label the vegetative cell PM at pollen tube tips in the cases of Lyn24 and AtROP9<sup>8</sup> (**Fig. 1c**). Lastly, lipid sensors differentially mark the vegetative cell PM and the peri-germ cell membrane, suggesting a distinct lipid signature of the peri-germ cell membrane<sup>9</sup> that may be responsible for the selective sorting of membrane proteins. Taken together, these data underscore the importance of avoiding the term “*plasma-membrane*” when referring to this

membrane. To reach a consensus on terminology and facilitate integration of future independent studies in the field of sexual plant reproduction, we propose to designate the membrane enveloping the generative cell and subsequent sperm cells that represent the flowering plants' male germ cells as the **peri-germ cell membrane**. We opted for the prefix *peri-*, inspired by examples from cell biology where structures are enveloped by plant membranes. For instance, in arbuscular mycorrhiza symbiosis, the fungal hyphae located within the plant cell are encircled by a plant membrane known as the **periarbuscular membrane**<sup>12</sup>, and in the symbiotic association between *Rhizobium* bacteria and roots, rhizobial symbionts are enclosed by a membrane derived from the plant PM, referred to as the **peribacteroid membrane**<sup>13</sup>. We encourage the research community to adopt the term, **peri-germ cell membrane**, for future use to clarify and unify the nomenclature. To avoid confusion, we advise the community not to use abbreviations as much as possible, but in case an abbreviation is needed, then use **PGCM**.

As we move forward, it is essential that we deepen our insight into the function and significance of the peri-germ cell membrane. Previous work indicated that the peri-germ cell membrane may play a structural role in pollen grains since sperm cells not enclosed within the peri-germ cell membrane do not remain connected as a pair<sup>7</sup>. Moreover, this "cell(s)-within-a-cell" organization implies that if there is any form of communication between the vegetative cell and the germ cells (either generative or sperm cells) it must involve the peri-germ cell membrane. In this context, it is also important to note that the inter-membrane space between the peri-germ cell membrane and the PM of germ cells should be considered as an apoplast with unique features due to its origin, the extremely close proximity of the two membranes (~20-50nm)<sup>1,9,14</sup>, and the mobility of the germ cells within the vegetative cell's cytoplasm. Furthermore, the peri-germ cell membrane attaches the germ cells to the neighboring vegetative nucleus in a structure named the male germ unit (MGU; **Fig. 1a, b, c**)<sup>14,15</sup>. Consequently, all male gametophytic nuclei move as a unit within the pollen tube to reach the embryo sac. Upon delivery of the MGU to the female gametophyte, the peri-germ cell membrane is removed to allow gamete fusion<sup>14</sup>. Recent cell biological studies have visualized peri-germ cell membrane breakdown upon fertilization<sup>9,16</sup>, but any mechanism regulating all these phenomena are unknown. Deciphering the roles of the peri-germ cell membrane, both during pollen development and fertilization, will be challenging, but will also be pivotal for advancing plant reproductive biology.



**Fig. 1 | Peri-germ cell membrane labelling differs from canonical plasma membrane makers.**

- a**, Diagram highlighting the peri-germ cell membrane in a pollen grain.
- b**, Representative confocal images of a maize pollen tube expressing the peri-germ cell membrane-localized protein NLD-mCitrine and a vegetative nucleus-localized H2B-mCherry under control of the maize vegetative cell-specific *pNLD* promoter. Scale bar, 10  $\mu$ m.
- c**, Representative confocal images of pollen tubes expressing the plasma membrane marker proteins 3xmNG-SYP121, 3xmNG-SYP132, and tdTomato-LTI6b under control of the vegetative cell-specific *pLAT52* promoter. The marker Lyn24-mNG, expressed under the vegetative cell-specific *pACA3* promoter, is used as a marker of the peri-germ cell membrane. Sperm and vegetative nuclei were labeled with SYBR Green I in the tdTomato-LTI6b marker line or with the ubiquitous H2B-tdTomato nuclear marker in the other three lines. Scale bars, 10  $\mu$ m.
- d**, Plots of fluorescent intensities measuring transverse pollen tube sections at sperm cell connections indicated by red dotted arrows in (c). X-axis represents the length along these arrows, zero indicates the starting point of the section. Dashed lines indicate limits between outside and inside of the pollen tube.
- e**, Confocal images of male germ units (sperm cells and vegetative nuclei) immediately after the discharge from the pollen tubes after their rupture. Scale bars, 10  $\mu$ m.
- Abbreviations: VN, vegetative nucleus; SN, sperm nucleus; PT, Pollen tube; PM, Plasma membrane; mNG, monomeric NeonGreen.

## **Materials and methods:**

### **Plasmid construction**

LR recombination was performed between pDM249 destination vector carrying the *pLAT52* promoter and the pOR129 entry vector containing the *3×mNG-SYP132* sequence to produce pDM478, a plasmid containing *pLAT52:3×mNG-SYP132*<sup>17,18</sup>. *SYP121* was amplified from *Arabidopsis thaliana* Columbia-0 genomic DNA by PCR using the primers SYP121\_F\_EcoRI\_48 (GGC GGC AGC GAA TTC ATG AAC GAT TTG TTT TCC AG) and SYP121\_R\_SpeI\_49 (GCG CGC CCA CCC TTA CTA GTT CAA CGC AAT AGA CGC CTT GCC T). The *SYP121* fragment was introduced into the *EcoRI/SpeI* site of pOR129 to produce pSG018, an entry vector containing *3×mNG-SYP121*<sup>18</sup>. LR recombination between pDM249 and pSG018 was performed to generate pSG019.

### **Plant material and growth condition**

*Arabidopsis thaliana* Columbia-0 was used as the wild-type (WT). The *pRPS5A:H2B-tdTomato pACA3:Lyn24-mNG* double marker line was previously described<sup>17</sup>. To generate the *pLAT52:3×mNG-SYP121 pRPS5A:H2B-tdTomato* double marker line, *Agrobacterium* GV3101 strains containing the pSG019 or DKv278 plasmids, the latter carrying *pRPS5A:H2B-tdTomato* were independently cultured. Then, both cultures were used for simultaneous floral dipping of the WT<sup>19,20</sup>. Similarly, *pLAT52:3×mNG-SYP121 pRPS5A:H2B-tdTomato* double marker lines were generated by simultaneous floral dipping using pDM478 and DKv278. Seeds of *pLAT52:tdTomato-LTI6b* transgenic plants were a gift from Yoko Mizuta (Nagoya University) and Shiori Nagahara (Kyoto University). Seeds were germinated on Murashige-Skoog medium. Plants were transferred to soil and grown at 22 °C under continuous light conditions. Maize *pNLD:NLD-mCitrine* and *pNLD:H2B-2xmCherry* double marker lines was previously described<sup>9</sup>.

### **Microscopy**

Fresh maize pollen was collected 3-4 h after changing a tassel bag in the morning. 2 ml of pollen germination medium [12% (w/v) sucrose, 5mM CaCl<sub>2</sub> and 0.01% (w/v) of H<sub>3</sub>BO<sub>3</sub>, pH 5.8] was deposited on a microscopy μ-dish (81158, Idbidi®) and covered by a layer of pollen (5-10 μl) followed by 2-3 h of incubation at room temperature (about 22°C). Germinated pollen tubes were directly observed on the glass bottom μ-dish with an inverted oil immersion objective (Plan-Apochr40x/1.4 huile DIC (UV) VIS-IR (#420762-9900-000)) on a LSM800 confocal microscope (Zeiss). Three channels were sequentially acquired in the following order: mCherry, mCitrine, brightfield. mCherry and mCitrine signals were detected using 561 nm and 488 nm laser excitation and detection at 569-700 nm and 495-555 nm respectively. For *Arabidopsis*, pollen tube growth under semi-*in vivo* conditions was performed as previously described with minor modifications<sup>17</sup>. Briefly, emasculated WT pistils were cut beneath the style using a 27-gauge needle. The pistils were placed on pollen tube growth media [0.01% (w/v) boric acid, 5 mM CaCl<sub>2</sub>, 5 mM KCl, 1 mM MgSO<sub>4</sub>, 10% (w/v) sucrose, 1.5% (w/v) NuSieve GTG agarose, pH 7.5] cast in a glass bottom dish (D11130H; Matsunami Glass, Osaka, Japan). Subsequently, the stigma was pollinated with pollen from the plant. After 4 h of incubation at 22 °C, pollen tubes reached the glass bottom. Z series images of six confocal planes with 1 μm step size

were captured by the split-view mode of green and red channels using an inverted microscope (IX-73; Evident, Tokyo, Japan), equipped with a spinning disk confocal scanning unit (CSU-W1; Yokogawa, Tokyo, Japan), an sCMOS camera (Zyla 4.2; Andor, Belfast, Northern Ireland), and 100× objective lens (UPlanSApo 100×; Evident, Tokyo, Japan). To induce pollen tube discharge, pollen tubes were irradiated with excitation light from a CFP filter set (U-FCFP; Evident, Tokyo, Japan) for 15 s<sup>21</sup>. After blue light irradiation, Z-series confocal images were captured every 3 s until the observation of released sperm cells.

### **Image Processing**

We used Fiji software (<https://fiji.sc>, version 2.9.0) for image processing<sup>22</sup>. Z-projections of maximum intensity in Figure 1c were generated from best focal planes. Fluorescence intensities in Figure 1d were analyzed in regions-of-interest (ROIs) with 10-pixel width set between pairs of arrowheads in Figure 1c: total signals of the 10-pixel width were calculated in every column along the long axis of the ROIs.

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## **AUTHOR CONTRIBUTIONS**

TW and DM took a lead of the renaming project and wrote the manuscript with the help of MMB. NS performed the analysis of *Arabidopsis* pollen tubes and prepared figure panels. ARMC and NMAJ observed pollen tubes in maize. All authors participated in the discussion regarding the term peri-germ cell membrane, contributed to the critical reading of the manuscript, and approved the manuscript submission.

## **DECLARATION OF INTERESTS**

The authors declare no competing interests.

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