miniTurbo-based interactomics of two plasma membrane-localized SNARE proteins in *Marchantia polymorpha*

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34 Summary

35

- 36 • *Marchantia polymorpha* is a model liverwort and its
- 37 overall low genetic redundancy is advantageous for
- 38 dissecting complex pathways. Proximity-dependent *in*
- 39 *vivo* biotin-labelling methods have emerged as powerful
- 40 interactomics tools in recent years. However,
- 41 interactomics studies applying proximity labelling are
- 42 currently limited to angiosperm species in plants.
- 43 • Here, we established and evaluated a miniTurbo-based
- 44 interactomics method in *M. polymorpha* using
- 45 MpSYP12A and MpSYP13B, two plasma membrane-
- 46 localized SNARE proteins, as baits.
- 47 • We show that our method yields a manifold of potential
- 48 interactors of MpSYP12A and MpSYP13B compared to
- 49 a co-immunoprecipitation approach. Our method could
- 50 capture specific candidates for each SNARE.
- 51 • We conclude that a miniTurbo-based method is a
- 52 feasible tool for interactomics in *M. polymorpha* and
- 53 potentially applicable to other model bryophytes. Our
- 54 interactome dataset on MpSYP12A and MpSYP13B
- 55 will be a useful resource to elucidate the evolution of
- 56 SNARE functions.

57

58 **Keywords:** *interactomics, Marchantia polymorpha,*
 59 *membrane-trafficking, miniTurbo, proximity labelling, SNARE*
 60 *protein*

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62

63 Introduction

64 The liverwort *Marchantia polymorpha* is a well-
 65 established model plant. The *M. polymorpha* genome has been
 66 sequenced (Bowman *et al.*, 2017); (Montgomery *et al.*, 2020)
 67 and genetic tools have been developed (Ishizaki *et al.*, 2008;
 68 Ishizaki *et al.*, 2013; Kubota *et al.*, 2013; Ishizaki *et al.*, 2015).
 69 In *M. polymorpha*, there is no evidence for whole genome
 70 duplication during evolution and the number of paralogs for
 71 many regulatory genes is rather low in comparison to other
 72 model plants (Bowman *et al.*, 2017). Accordingly, low genetic
 73 redundancy is a useful feature of *M. polymorpha* in dissecting
 74 basic mechanisms and gene functions underlying complex
 75 pathways.

76 Elucidating complex pathways and protein-interaction
 77 networks remain major challenges in plant research.
 78 Conventional approaches to study protein-protein interactions,
 79 like co-immunoprecipitation (Co-IP) followed by mass
 80 spectrometry (MS) have limitations. Successful enrichment and
 81 purification under non-physiological conditions require a
 82 certain binding affinity between interactors. Therefore, Co-IP is
 83 often effective to capture stable complexes, while weak and
 84 transient associations can easily be lost. The analysis of
 85 interactions between members of subcellular proteomes may
 86 require an enrichment of cellular compartments before Co-IP to
 87 avoid artificial interactions upon cell lysis. In this context,
 88 proximity-dependent *in vivo* labelling (PL) approaches are
 89 gaining an increasing importance as alternative interactomics
 90 approaches.

91 The miniTurbo-based PL method was initially
 92 established in bacterial and mammalian cells, and is based on
 93 biotin ligase-mediated labelling of interaction partners with
 94 exogenously applied biotin (Branon *et al.*, 2018). TurboID is a
 95 promiscuous biotin ligase that has been engineered from *E. coli*

96 BirA with 15 mutations and has a higher ligase activity at a
 97 wide range of temperatures compared to BirA. The miniTurbo
 98 is a smaller version of TurboID with a deleted N-terminal
 99 domain and has 13 mutations compared to BirA. TurboID and
 100 miniTurbo were reported to have higher activities than other
 101 biotin-ligases, namely BioID, BioID2, and BASU, in HEK
 102 293T cells. Compared to TurboID, miniTurbo was found to be
 103 overall 1.5 times less active and showed a lower background
 104 labelling activity without exogenous application of biotin in
 105 HEK 293T cells (Branon *et al.*, 2018). For PL of interaction
 106 partners, the biotin ligase is genetically fused to a bait.
 107 Molecules in proximity of the bait are biotinylated by the ligase
 108 in the presence of biotin. After labelling, biotinylated proteins
 109 can be extracted and enriched by streptavidin-pulldown before
 110 an identification by MS.

111 For pulldown of biotinylated proteins, it is not required
 112 that proteins and complexes remain in their native state.
 113 Because enrichment of biotinylated proteins does not rely on
 114 affinity to the bait proteins. PL approaches can thus also
 115 capture weak or transient interactions. The binding affinity
 116 between biotin and streptavidin is high. Therefore, protein
 117 extraction, binding, and washing steps can be conducted in the
 118 presence of high concentrations of detergents. The ligase
 119 activity can be inactivated during protein extraction, and thus
 120 artificial labelling upon cell lysis will not occur. This is
 121 advantageous for investigating interactions of proteins in
 122 subcellular compartments, including plasma membrane-
 123 localized proteins.

124 Since biotin ligases do not distinguish between real
 125 interaction partners and other molecules residing in proximity
 126 of the bait by chance, a certain level of unspecific labelling is
 127 expected. Unspecific labelling may occur according to an
 128 expression of the bait in a specific cellular compartment, cell-
 129 type, tissue, organ, at a specific developmental stage, or

130 physiological status and will be enhanced under saturating
131 labelling conditions. Therefore, non-saturating labelling
132 conditions are desirable and appropriate controls should be
133 designed to narrow down candidates of high confidence by
134 minimizing false positive identifications (Mair *et al.*, 2019).

135 Plants, unlike animals, can synthesise biotin *de novo*
136 (Baldet *et al.*, 1993; Baldet *et al.*, 1997). High levels of
137 endogenous biotin may lead to background labelling,
138 potentially interfering with PL approaches. The endogenous
139 biotin level in *M. polymorpha* has not been investigated. In *A.*
140 *thaliana*, the sucrose-H⁺ symporter AtSUC5 was identified to
141 mediate uptake of exogenous biotin (Ludwig *et al.*, 2000;
142 Pommerrenig *et al.*, 2013). Until to date, AtSUC5 remains the
143 only sucrose transporter that was shown to function in biotin
144 uptake *in planta*. It is not yet known whether comparable
145 mechanisms for biotin uptake exist in *M. polymorpha*.

146 The TurboID or miniTurbo method has been
147 successfully applied in *A. thaliana*, *Nicotiana benthamiana*,
148 and *Solanum lycopersicum* using stable transgenic lines or
149 transient expression systems, to efficiently label and identify
150 subcellular proteomes in specific cell types (Mair *et al.*, 2019)
151 as well as interacting partners of cytosolic (Zhang *et al.*, 2019;
152 Arora *et al.*, 2020) and nuclear (Mair *et al.*, 2019) bait proteins
153 by MS. Arora *et al.* (2020) demonstrated that TurboID can be
154 applied to detect known interactions of plasma membrane-
155 localized proteins with a targeted approach using
156 immunoblotting. A direct comparison of interactomics applying
157 Co-IP and PL using the same plant materials is still missing. It
158 remains unclear whether biotin ligase-mediated PL approaches
159 can be sensitive and specific to reveal differences in
160 interactomes of very similar proteins, like closely related
161 homologs. Lastly, in bryophyte species, an interactome study
162 utilizing PL approaches has not yet been reported.

163 In *M. polymorpha*, the two SNARE (soluble N-
164 ethylmaleimide-sensitive factor attachment protein receptor)
165 proteins, MpSYP12A and MpSYP13B, are plasma membrane-
166 localized and ubiquitously expressed throughout the thallus
167 (Kanazawa *et al.*, 2016; Kanazawa *et al.*, 2020). Plant SNAREs
168 modulate membrane-trafficking, intra- and intercellular
169 signalling, and transport. In *A. thaliana*, 65 SNARE proteins
170 have been identified, 9 of which are SYP1 family proteins that
171 are plasma membrane-localized (Uemura *et al.*, 2004). During
172 land plant evolution, the expansion of SNARE proteins and
173 their functional diversification was hypothesized to be linked to
174 multicellularity and likely facilitated the adaptation to a
175 terrestrial lifestyle (Sanderfoot, 2007). Thus, land plant
176 secretory pathways are highly sophisticated, dynamic, and
177 diversely regulated, being involved in a manifold of cellular
178 processes ranging from polarized growth to defence responses
179 (Batoko & Moore, 2001; Collins *et al.*, 2003; Catalano *et al.*,
180 2007; Enami *et al.*, 2009; Silva *et al.*, 2010; Reichardt *et al.*,
181 2011; Uemura *et al.*, 2012; Ichikawa *et al.*, 2014; Johansson *et al.*,
182 2014; Yun *et al.*, 2016; Xia *et al.*, 2019; Hirano *et al.*, 2020;
183 Rubiato *et al.*, 2021). In *M. polymorpha*, the SYP1 protein
184 family is comprised of 4 members: SYP12A, SYP12B,
185 SYP13A, and SYP13B. MpSYP13A and MpSYP13B belong to
186 the SYP13 group and are closely related to AtSYP131 and
187 AtSYP132. MpSYP12A and MpSYP12B belong to the
188 SYP11/12 group, which is phylogenetically separated from the
189 SYP11 or SYP12 group proteins in angiosperms (Kanazawa *et al.*
190 *et al.*, 2016; Bowman *et al.*, 2017). Carella *et al.* (2018) reported
191 that MpSYP13B accumulated in haustoria-like structures upon
192 infection of *M. polymorpha* with the oomycete pathogen
193 *Phytophthora palmivora*. Recently, MpSYP12A was shown to
194 localize to the phragmoplast during cell plate formation
195 (Kanazawa *et al.*, 2020). Interaction partners of SYP1 family
196 proteins in *M. polymorpha* have not yet been identified.

197 In this study, we established a miniTurbo-based PL
 198 method for interactome profiling in *M. polymorpha*. Using
 199 MpSYP12A and MpSYP13B as baits, we evaluated biotin-
 200 labelling conditions and a procedure to enrich biotinylated
 201 proteins, and then potential interactors were identified by MS.
 202 We directly compared the performances of Co-IP and PL
 203 approaches using the same plant materials. Lastly, by
 204 comparing the identified interactomes of MpSYP12A and
 205 MpSYP13B, we found potential interactors that are specific to
 206 each SNARE.
 207
 208

209 **Materials and Methods**

210 **Construction and cloning.** Gateway entry vectors containing
 211 genomic sequences for an expression of MpSYP12A and
 212 MpSYP13B under their own regulatory elements (5'- and 3'-
 213 flanking sequences and introns) in *M. polymorpha* were
 214 provided by Takashi Ueda (Kanazawa *et al.*, 2016). For N-
 215 terminal tagging of MpSYP12A and MpSYP13B with
 216 miniTurbo and Myc-tag, entry vector backbones were
 217 linearized by restriction with SmaI or BamHI enzymes,
 218 respectively. Codon-optimized miniTurbo (Fig. S1) was
 219 synthesized by Thermo GeneArt and PCR-amplified from a
 220 donor plasmid. Gateway entry vectors pMKMM20 (3.5 kb
 221 upstream *SYP13B*:5'UTR:miniTurbo-Myc-*SYP13B*:3'UTR)
 222 and pMKMM21 (3.5 kb upstream *SYP12A*:5'UTR:miniTurbo-
 223 Myc-*SYP12A*:3'UTR) were generated by in-fusion cloning (HD
 224 enzyme mix; Takara Bio). Gateway binary vectors pMKMM22
 225 and pMKMM23 for the expression of miniTurbo-Myc-
 226 MpSYP13B and miniTurbo-Myc-MpSYP12A were generated
 227 by LR-recombination (LR clonase; Invitrogen) of pMKMM20
 228 or pMKMM21 with pMpGWB301 (Fig. S2).

229 ***M. polymorpha* lines used in this study.** The male *M.*
 230 *polymorpha* Tak-1 ecotype was used as a wildtype. Transgenic
 231 lines in Tak-1 background were generated using the cut thallus
 232 method (Kubota *et al.*, 2013) and *Agrobacterium* strain
 233 GV3101 carrying pMKMM22 or pMKMM23. Transformants
 234 were selected using Chlorsulfuron and Cefotaxime antibiotics
 235 for two generations. Selected transformants were screened for
 236 an expression of miniTurbo-Myc-MpSYP12A and miniTurbo-
 237 Myc-MpSYP13B fusion-proteins by immunoblotting.
 238 Transgenic lines were chosen that displayed similar expression
 239 levels of miniTurbo-Myc-MpSYP12A and miniTurbo-Myc-
 240 MpSYP13B for further analyses (Fig. S3a). *M. polymorpha*

241 plants were grown and maintained on Gamborg's B5 (Duchefa;
242 G0209) half strength solid medium containing 1 % plant agar in
243 a walk-in growth chamber under constant white light (50–60
244 $\mu\text{mol photons LED m}^{-2} \text{ s}^{-1}$) at 22–24 °C.

245 **Sample preparation for PL and Co-IP experiments.** *M.*
246 *polymorpha* Tak-1 and transgenic lines were grown from single
247 gemmae on Gamborg's B5 half strength solid medium
248 containing 0.8 % plant agar for 10 days under constant white
249 light (50–60 $\mu\text{mol photons LED m}^{-2} \text{ s}^{-1}$) at 22–24 °C. Ten
250 individual 10-day old thalli were pooled per sample. For Co-IP,
251 untreated thalli were sampled immediately and frozen in liquid
252 nitrogen until further processing. For biotin treatment, thalli
253 were transferred into transparent 6- or 12-well plates (Greiner
254 Bio-One; 657160) and submerged in 0–700 μM biotin (Sigma;
255 B4501) solution in water. The thalli were vacuum-infiltrated
256 with biotin solutions for 5 minutes using a desiccator and the
257 samples were incubated for 0–24 hours at room temperature
258 (RT; 22–25 °C) while shaking. After incubation, the thalli were
259 washed once with ice-cold ultrapure water for 2 minutes to
260 remove excess biotin. For sampling, thalli were transferred onto
261 filter paper (Whatman; 1001-085) and left for 10 seconds to
262 drain off excess liquid. Next, the thalli were pressed onto the
263 filter paper for 5 seconds, immediately transferred to fresh
264 tubes containing 2 stainless-steel beads and snap-frozen in
265 liquid nitrogen. The plant material was ground in liquid
266 nitrogen using a mixing mill (MM400, Retsch) for 5 minutes at
267 30 Hz. For Co-IP, all following steps were conducted at 4 °C.
268 The finely ground powder was mixed with extraction buffer (50
269 mM Tris pH 7.5, 150 mM NaCl, 10 % glycerol, 2 mM
270 ethylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol
271 (DTT), 1 % Triton X-100, 1 % Plant Protease Inhibitor; Sigma
272 P9599) and incubated for 30 minutes for protein extraction. For
273 PL samples, the powder was mixed with 500 μl pre-heated

274 SDT buffer (100 mM Tris pH 7.5, 4 % sodium dodecyl sulfate
275 (SDS), 0.1 M DTT) at 95 °C for 5 minutes and then sonicated
276 for 10 minutes. Cell debris was removed from the extracts by
277 two consecutive centrifugation steps (10,000 g, 10 minutes).
278 The protein concentration in cell extracts for Co-IP and PL was
279 determined using the Pierce 660 nm Assay (Thermo Fisher;
280 22660). Cell extracts with 500 µg of total protein were used as
281 input for Myc-Trap Co-IP and biotin depletion before affinity-
282 pulldown of biotinylated proteins. For biotin-treated thalli, an
283 aliquot of each sample was taken for immunoblotting and
284 whole proteome analysis. Remaining samples were subjected to
285 biotin depletion before pulldown using streptavidin. Biotin
286 dilution series and time course experiments were each done in
287 two independent experiments (Fig. 1c, d; Fig. S4a, b).

288

289 **Co-IP.** Myc-Trap beads (Chromotek; ytma-20) were
290 equilibrated in ice-cold wash buffer (50 mM Tris pH 7.5, 150
291 mM NaCl, 10 % glycerol, 2 mM EDTA) according to the
292 manufacturer's instructions. Cell extracts with 500 µg of total
293 protein in 1 ml volume were mixed with 25 µl of equilibrated
294 Myc-Trap beads and pulldown was performed for 2 hours at
295 4 °C on a rolling wheel. Myc-Trap beads were magnetically
296 separated from the supernatant and washed 3 times in 500 µl
297 wash buffer. An aliquot corresponding to 10 % of the beads
298 was used for immunoblotting and the remaining beads were
299 subjected to on-bead digestion with trypsin. For
300 immunoblotting of Co-IP samples, the proteins were eluted
301 from the beads in 30 µl 4x SDS sample buffer (250 mM Tris-
302 HCl pH 6.8, 40 % glycerol, 8 % SDS, 0.08 % bromophenol
303 blue, 200 mM DTT) by boiling for 10 minutes at 95 °C.

304

305 **Depletion of free biotin.** Biotin depletion methods were tested
306 using a transgenic line expressing miniTurbo-Myc-MpSYP13B
307 and Tak-1. Cell extracts were prepared as described above from

10-day-old thalli treated with 700 μ M biotin for 24 hours. As input, 500 μ g of total protein in 500 μ l SDT buffer (100 mM Tris pH 7.5, 4 % SDS, 0.1 M DTT) was used per sample. For methanol:chloroform precipitation, 666 μ l methanol and 166 μ l chloroform were added to the cell extracts and the samples were mixed. Next, 300 μ l water was further added and mixed, and then centrifuged for 10 minutes at 4,000 rpm. The separated upper and lower liquid phases were removed. The solid white layer containing precipitated proteins that had formed between the liquid phases was kept. The protein pellet was resuspended in 600 μ l methanol and sonicated for 10 minutes. After centrifugation for 10 minutes at 13,000 rpm, the supernatant was removed completely. The protein pellets were air-dried for 5 minutes and resuspended in 500 μ l SDT buffer. After 10 minutes sonication, the samples were incubated for 30 minutes at RT while shaking at 1,000 rpm, until the protein pellets were redissolved. The samples were then diluted with PBS buffer (0.1 M phosphate, 0.15 M NaCl, pH 7.2) to a final concentration of 0.5 % SDS. For biotin depletion with PD-10 desalting columns (VWR; 17085101), the columns were equilibrated with SDT:water (1:5) and the cell extracts were diluted to 2.5 ml with ultrapure water. PD-10 desalting was performed according to the manufacturer's instructions and the proteins were eluted with 3.5 ml PBS buffer containing 0.5 % SDS. For biotin depletion using Zeba spin columns (Thermo; 89893), the columns were equilibrated with SDT:water (1:5) and the cell extracts were diluted to 2.5 ml with ultrapure water. Desalting was then performed according to the manufacturer's instructions. All biotin-depleted samples were adjusted to 4 ml final volume with binding buffer (0.1 M phosphate, 0.15 M NaCl, 0.5 % SDS, pH 7.2) before pulldown. Aliquots of intermediate steps were taken for immunoblotting. All biotin depletion methods were tested at the same time in duplicates.

341 PD-10 column desalting method was employed for the pulled-
342 down samples measured by MS.

343

344 **Pulldown of biotinylated proteins.** Biotinylated proteins were
345 pulled-down using streptavidin-agarose beads (Thermo; 20353).
346 Per sample, 100 μ l of a 50 % slurry were used. The beads were
347 washed and equilibrated in the binding buffer. Next, the biotin-
348 depleted samples were added to the beads and pulldown was
349 performed overnight at 22–25 °C while mixing. The beads
350 were washed once with 6 ml PBS buffer containing 2 % SDS
351 and then 3 times with 10 ml PBS buffer. Aliquots of all
352 samples were taken during intermediate steps and an aliquot
353 corresponding to 10 % of the washed beads was taken after
354 pulldown for immunoblotting. The proteins were eluted from
355 the beads by boiling for 10 minutes at 95 °C in 50 μ l 4x SDS
356 sample buffer containing 20 mM biotin while shaking at 1,000
357 rpm. For immunoblots, 20 % of the IP-eluate was used,
358 corresponding to 2 % of the input bead-amount. The remaining
359 beads from pulldown with bound biotinylated proteins were
360 subjected to on-bead digestion for MS analysis.

361

362 **On-bead digestion.** For Myc-IP and streptavidin-pulldown
363 samples, Myc-Trap beads or streptavidin-agarose beads were
364 resuspended in 25 μ l digest buffer 1 (50 mM Tris pH 7.5, 2 M
365 urea, 1 mM DTT, 5 μ g μ l⁻¹ trypsin) and incubated at 30 °C for
366 30 minutes while shaking at 400 rpm. The supernatant was
367 separated from the beads magnetically or by sedimentation and
368 transferred to a fresh tube. The beads were then mixed with 50
369 μ l digest buffer 2 (50 mM Tris pH 7.5, 2 M Urea, 5 mM CAA),
370 and the supernatant was separated from the beads and
371 combined with the supernatant from the previous step. The
372 combined supernatant was further incubated overnight at 32 °C
373 while shaking at 400 rpm. Trypsin was inactivated by
374 acidification with trifluoroacetic acid (TFA), and the peptide

sample was subsequently desalted using C₁₈ stage tips (Rappsilber *et al.* (2003).

377

Sample preparation for whole proteome analyses. Aliquots of cell extracts from biotin-labelling experiments were used for whole proteome analysis. The extracts were processed using a filter-aided sample preparation (FASP) protocol adapted from Wisniewski *et al.* (2009). In brief, 50 µg of total protein extract were used as input. The proteins were alkylated using chloroacetamide and digested using LysC and trypsin. The peptide solutions were desalted using C₁₈ stage tips. Whole proteome analyses were conducted for one representative replicate per genotype and condition.

388

LC-MS/MS data acquisition. The dried peptides from filter-aided digestion were re-dissolved in buffer A (2 % ACN, 0.1 % TFA) and adjusted to a final peptide concentration of 0.1 µg µl⁻¹ for analysis. The peptide samples from streptavidin- and Myc-Trap pulldowns were dissolved in 10 µl buffer A and measured without dilution.

PL samples were analysed using an EASY-nLC 1200 (Thermo Fisher) coupled to a Q Exactive Plus mass spectrometer (Thermo Fisher). The peptides were separated on 16 cm frit-less silica emitters (New Objective, 75 µm inner diameter), packed in-house with reversed-phase ReproSil-Pur C18 AQ 1.9 µm resin (Dr. Maisch). The peptides were loaded on the column and eluted for 50 minutes using a segmented linear gradient of 5 % to 95 % solvent B (0 minutes: 5 % B; 0–5 minutes -> 5 % B; 5–25 minutes -> 20 % B; 25–35 minutes -> 35 % B; 35–40 minutes -> 95 % B; 40–50 minutes -> 95 % B) (solvent A: 0 % ACN, 0.1 % FA; solvent B: 80 % ACN, 0.1 % FA) at a flow rate of 300 nl per minute. Mass spectra were acquired in data-dependent acquisition mode with a TOP10 method. MS spectra were acquired in the Orbitrap

409 analyser with a mass range of 300–1500 m/z at a resolution of
 410 70,000 FWHM and a target value of 3×10^6 ions. Precursors
 411 were selected with an isolation window of 1.3 m/z. HCD
 412 fragmentation was performed at a normalized collision energy
 413 of 25. MS/MS spectra were acquired with a target value of
 414 5×10^5 ions at a resolution of 17,500 FWHM, a maximum
 415 injection time of 85 milliseconds and a fixed first mass of m/z
 416 100. Peptides with a charge of 1, greater than 6, or with
 417 unassigned charge state were excluded from fragmentation for
 418 MS², dynamic exclusion for 20 seconds prevented repeated
 419 selection of precursors.

420 Myc-IP and whole proteome samples were analysed
 421 using an EASY-nLC 1000 (Thermo Fisher) coupled to a Q
 422 Exactive mass spectrometer (Thermo Fisher). The peptides
 423 were separated on 16 cm frit-less silica emitters (New
 424 Objective, 75 µm inner diameter), packed in-house with
 425 reversed-phase ReproSil-Pur C18 AQ 1.9 µm resin (Dr.
 426 Maisch). Peptides (0.5 µg) were loaded on the column and
 427 eluted for 115 minutes using a segmented linear gradient of 5
 428 % to 95 % solvent B (0 minutes: 5 % B; 0–5 minutes -> 5 % B;
 429 5–65 minutes -> 20 % B; 65–90 minutes -> 35 % B; 90–100
 430 minutes -> 55 % B; 100–105 minutes -> 95 % B, 105–115
 431 minutes -> 95 % B) (solvent A: 0 % ACN, 0.1 % FA; solvent
 432 B: 80 % ACN, 0.1 % FA) at a flow rate of 300 nl per minute.
 433 Mass spectra were acquired in data-dependent acquisition mode
 434 with a TOP15 method. MS spectra were acquired in the
 435 Orbitrap analyser with a mass range of 300–1750 m/z at a
 436 resolution of 70,000 FWHM and a target value of 3×10^6 ions.
 437 Precursors were selected with an isolation window of 2.0 m/z.
 438 HCD fragmentation was performed at a normalized collision
 439 energy of 25. MS/MS spectra were acquired with a target value
 440 of 10^5 ions at a resolution of 17,500 FWHM, a maximum
 441 injection time of 55 milliseconds and a fixed first mass of m/z
 442 100. Peptides with a charge of 1, greater than 6, or with

443 unassigned charge state were excluded from fragmentation for
 444 MS², dynamic exclusion for 30 seconds prevented repeated
 445 selection of precursors.
 446
 447 **Data analysis.** Raw data were processed using MaxQuant
 448 software (version 1.6.3.4, <http://www.maxquant.org/>) (Cox &
 449 Mann, 2008) with label-free quantification (LFQ) and intensity
 450 based absolute quantification (iBAQ) enabled (Tyanova *et al.*,
 451 2016). For PL data, normalization was skipped for the LFQ
 452 quantification. MS/MS spectra were searched by the
 453 Andromeda search engine against a combined database
 454 containing the sequences from *M. polymorpha*
 455 (MpTak1v5.1_r1_primary_transcripts_proteinV2;
 456 <http://marchantia.info/>, (Montgomery *et al.*, 2020)) and
 457 sequences of 248 common contaminant proteins and decoy
 458 sequences and the sequence of miniTurbo. Trypsin specificity
 459 was required and a maximum of 2 missed cleavages allowed.
 460 Minimal peptide length was set to 7 amino acids.
 461 Carbamidomethylation of cysteine residues was set as fixed,
 462 oxidation of methionine and protein N-terminal acetylation as
 463 variable modifications. Peptide-spectrum-matches and proteins
 464 were retained if below a false discovery rate (FDR) of 1 %. For
 465 PL data, the non-normalized MaxLFQ values of all replicates
 466 (4 per condition) were pre-processed in Perseus (version 1.5.8.5,
 467 <http://www.maxquant.org/>) and submitted for normalization
 468 analysis using the Normalyzer tool
 469 (<http://normalyzer.immunoprot.lth.se/>, (Chawade *et al.*, 2014)).
 470 The output was analysed for outliers and 1 replicate per
 471 condition was removed. The final data analysis was conducted
 472 in MaxQuant as described above on the reduced raw dataset.
 473 Statistical analysis of the MaxLFQ values was conducted using
 474 Perseus (version 1.5.8.5, <http://www.maxquant.org/>).
 475 Quantified proteins were filtered for reverse hits and hits
 476 “identified by site” and MaxLFQ values were log2 transformed.

For PL data, transformed MaxLFQ values were normalized by subtraction of the median per column. After grouping samples by condition only those proteins were retained for subsequent analysis that had 3 or 2 valid values in one of the conditions for PL data or Myc-IP data, respectively. Two-sample *t*-tests were performed using a permutation-based FDR of 5 %. For the generation of volcano plots, missing values were imputed from a normal distribution using the default settings in Perseus (1.8 downshift, separately for each column) for data with 3 valid values in one of the conditions. Volcano plots were generated in Perseus using an FDR of 5 % and an $S0 = 1$. The Perseus output was exported and further processed using Microsoft Excel and RStudio (version 1.4.1103, <https://www.rstudio.com/>), based on R (version x64 4.0.3, <https://cran.r-project.org/>). The data was processed in RStudio using tidyverse (version 1.3.0), rio (version 0.5.16) and zoo (version 1.8-8) packages. Relative iBAQ values were calculated per column from MaxQuant output, scaled by factor 10^6 and log10 transformed. The median value of 3 replicates per condition was used to generate volcano plots including relative iBAQ values. Volcano pots were generated in RStudio using the ggplot2 (version 3.3.3), ggrepel (version 0.9.1) and ggsci (version 2.9) packages.

500

Immunoblotting. Proteins were separated by SDS-polyacrylamide-gel electrophoresis (PAGE) and blotted onto PVDF membranes (BioRad; 1704272) using a Trans-Blot Turbo (BioRad) transfer system. The following antibodies were used: streptavidin-HRP (Cell Signaling; 3999S), anti-Myc-tag mouse monoclonal antibody (Cell Signaling; 9B11), HRP-conjugated anti-mouse IgG antibody (Cell Signaling; 7076S). The membranes were probed for biotinylated proteins with streptavidin-HRP for 45 minutes to 3 hours at RT. For detection of miniTurbo-Myc, the membranes were probed with

511 anti-Myc-tag primary antibody overnight at 4 °C and then with
 512 anti-mouse IgG secondary antibody for 1 hour at RT.
 513 Biotinylated proteins or Myc-tagged miniTurbo were visualized
 514 on the membranes using a luminol-based chemiluminescent
 515 substrate that is oxidized by HRP in the presence of peroxide
 516 (Thermo Fisher; 34577). The membranes were stained with
 517 Coomassie staining solution (60 mg l⁻¹ Coomassie brilliant blue,
 518 10 % acetic acid) afterwards.

519

520 **Annotations and gene ontology analyses.** For *M. polymorpha*
 521 protein annotation, gene annotations from MpTak1_v5.1 and
 522 JGI 3.1 (<https://marchantia.info/>) were integrated. Information
 523 of *A. thaliana* homologs was further used for the annotation. A
 524 Basic Local Alignment Search Tool (BLAST) was used to
 525 determine homologs in *A. thaliana* (TAIR10). The best hit with
 526 an e-value $\leq 10^{-10}$ was defined as a homolog. TAIR10
 527 (<https://www.arabidopsis.org/>, (Lamesch *et al.*, 2012)),
 528 PANTHER version 16.0 (<http://pantherdb.org/>, (Mi *et al.*,
 529 2021)), STRING 11.5 (<https://string-db.org/>, (von Mering *et al.*,
 530 2003)), BioGrid 4.4 (<https://thebiogrid.org/>, (Stark *et al.*,
 531 2006)), and IntAct 1.0.2 (<https://www.ebi.ac.uk/intact/home>,
 532 (Orchard *et al.*, 2014)) were used for annotating *A. thaliana*
 533 homologs (Data S1). RStudio (version 1.4.1103,
 534 <https://www.rstudio.com/>), based on R (version x64 4.0.3,
 535 <https://cran.r-project.org/>), the tidyverse (version 1.3.0), rio
 536 (version 0.5.16), and zoo (version 1.8-8) packages, were used
 537 for integrating annotation files. GO-term enrichment
 538 analysis was performed with Metascape 3.5
 539 (<https://metascape.org/>, (Zhou *et al.*, 2019)) using express
 540 analysis settings. Corresponding *A. thaliana* homologs were
 541 used as input protein lists for the analysis. A list of reported and
 542 predicted interactors of AtSYP1 proteins was generated by
 543 integrating information from BioGrid, IntAct, and STRING
 544 databases (Data S2). BLAST was used to determine homologs

545 in *M. polymorpha* (JGI 3.1). The best hit with an e-value $\leq 10^{-10}$
 546 ¹⁰ were defined as a homolog (Data S3). PANTHER version
 547 16.0 and PPDB (<http://ppdb.tc.cornell.edu/>, (Sun *et al.*, 2009))
 548 were used to predict plasma membrane-localization of *M.*
 549 *polymorpha* homologs.
 550

Results

Experimental design for interactomics using miniTurbo-mediated PL and Co-IP in *M. polymorpha*

To potentially enhance transcription and translation efficiency of the bait-ligase fusion-proteins in *M. polymorpha*, we used a codon-optimized version of the original miniTurbo (Fig. S1). We added a single Myc-tag to the C-terminus of miniTurbo, enabling not only the detection of the miniTurbo fusion-proteins by immunoblotting, but also Co-IP experiments (Fig. 1a, Fig. S1). We designed binary vectors to express miniTurbo-Myc-MpSYP12A and miniTurbo-Myc-MpSYP13B fusion-proteins under the native promoters of *MpSYP12A* and *MpSYP13B* genes, respectively (Fig. 1a, Fig. S2). Using these constructs, we generated stable transgenic lines in wildtype Tak-1 background. Expression of the miniTurbo-Myc fusion-proteins in candidate transformant lines was confirmed by immunoblotting using an anti-Myc antibody. To establish and evaluate the miniTurbo-based interactomics method, we selected transgenic lines showing similar expression levels for miniTurbo-Myc-MpSYP12A and miniTurbo-Myc-MpSYP13B. For this study, we used line No. 1 and line No. 3 for miniTurbo-Myc-MpSYP12A and miniTurbo-Myc-MpSYP13B, respectively (Fig. S3a).

In *A. thaliana*, Mair *et al.* (2019) demonstrated that levels of miniTurbo-mediated biotinylation of cellular proteins saturated when plants were treated with 50 μ M biotin solution. Since uptake of exogenous biotin and levels of endogenously produced biotin are unknown in *M. polymorpha*, we treated the selected transgenic lines and Tak-1 with different concentrations of biotin, 0–700 μ M, to find suitable conditions for *in vivo* biotin-labelling. For biotin treatment, thalli were grown from single gemmae for 10 days, and then whole plants were submerged in biotin solutions and vacuum infiltrated for 5

minutes. We incubated the thalli in biotin solutions at 22–25 °C for 24 hours, a time point at which we expected a saturation of biotin-labelling based on previous studies using miniTurbo in other plant species (Mair *et al.*, 2019; Zhang *et al.*, 2019). We subsequently checked the levels of biotinylated proteins in cell extracts by immunoblotting using streptavidin-HRP (Fig. 1c, Fig. S4a). We found increasing levels of biotinylated proteins with increasing biotin concentration in cell extracts of transgenic lines expressing either miniTurbo-Myc-MpSYP12A or miniTurbo-Myc-MpSYP13B but not in Tak-1. This confirmed biotin uptake and miniTurbo biotin ligase activity in *M. polymorpha* under the tested conditions. Given that levels of biotinylated proteins did not saturate for biotin concentrations up to 700 µM in *M. polymorpha*, we used 700 µM biotin solution in all following experiments. To determine suitable biotin treatment times in *M. polymorpha*, we next performed a time-course experiment and checked the levels of biotinylated proteins by immunoblotting after 0–24 hours of biotin treatment (Fig. 1d). We detected increased levels of biotinylated proteins in samples of transgenic lines expressing either miniTurbo-Myc-MpSYP12A or miniTurbo-Myc-MpSYP13B after 30 minutes of treatment (Fig. S4b), which further increased over time up to 24 hours of treatment (Fig. 1d).

Previously published studies using TurboID and miniTurbo identified the depletion of free biotin after labelling as a critical step for pulldown of biotinylated proteins using streptavidin beads (Mair *et al.*, 2019; Zhang *et al.*, 2019; Arora *et al.*, 2020; Zhang *et al.*, 2021). We therefore tested three different approaches, methanol:chloroform precipitation, PD-10 gravity column desalting, and Zeba spin column desalting, to remove excess free biotin from *M. polymorpha* cell extracts. We used the miniTurbo-Myc-MpSYP13B line, which was treated with 700 µM biotin for 24 hours. We evaluated biotin

depletion based on pulldown efficiency, through immunoblotting of biotinylated proteins that could be eluted from the streptavidin-agarose beads after pulldown, and of non-bound biotinylated proteins that remained in the supernatant of the beads (Fig. S3b, c). We found that all three methods could be used before affinity-pulldown to sufficiently enrich biotinylated proteins from *M. polymorpha* samples (Fig. S3b). With respect to easier handling, we used PD-10 column desalting for subsequent experiments.

The miniTurbo-based approach identifies a manifold of potential interactors of MpSYP12A and MpSYP13B in comparison to the Co-IP approach

A direct comparison between the performances of PL and Co-IP approaches for interactomics using the same plant materials has not yet been reported. We therefore performed IP of miniTurbo-Myc-MpSYP12A or miniTurbo-Myc-MpSYP13B using Myc-Trap beads from the same selected transgenic lines. After IP, we checked successful pulldown of the miniTurbo-Myc fusion-proteins by immunoblotting. We detected miniTurbo-Myc-MpSYP12A and miniTurbo-Myc-MpSYP13B fusion-proteins in cell extract that was used as input for IP, and in IP-eluates of samples from the transgenic lines (Fig. S3d). We then identified and quantified the proteins captured by Myc-IP using MS. We found a significant enrichment of the two bait proteins (Fig. 2a), confirming the immunoblotting result. Using Co-IP, we identified 4 and 1 potential interactors of MpSYP12A and MpSYP13B, respectively (Fig. 2a).

For the PL approach, we treated plants with biotin for 4 and 24 hours to identify and quantify proteins after pulldown by MS. As in the case of the Co-IP approach, we found a significant enrichment of both baits using the miniTurbo-based approach (Fig. 2b, c). Four hours of biotin treatment resulted in an identification of 214 and 189 proteins as potential interactors

652 of MpSYP12A and MpSYP13B, respectively (Fig. 2b). By
 653 increasing the treatment time to 24 hours, the numbers of
 654 identified potential interactors nearly tripled (Fig. 2c). As
 655 expected, most of the candidates identified from 4 hours'
 656 samples were also identified from 24 hours' samples (Fig. 2d).
 657 In other words, approximately one third of the candidates that
 658 were identified after 24 hours of biotin treatment could be
 659 identified after 4 hours of treatment. MpCSR1
 660 (CHLORSULFURON RESISTANT 1), a potential interactor of
 661 MpSYP12A and MpSYP13B that we identified using Co-IP,
 662 was identified in the 24 hours' PL interactome dataset as well
 663 (Fig. 2c). Overall, these results demonstrate that PL approaches
 664 have a higher potential to identify undescribed interacting
 665 proteins compared to Co-IP approaches. However, it should be
 666 noted that the miniTurbo-based approach failed to identify
 667 proteins like secretory peroxidases that are predicted to be
 668 secreted into the extracellular space (Fig. 2a). This is
 669 reasonable as miniTurbo was fused to the intracellular domain
 670 of MpSYPs.

671

672 **PL using MpSYP12A and MpSYP13B as baits enriches** 673 **proteins involved in vesicle-mediated transport and plasma** 674 **membrane-localized proteins**

675 We next asked whether the potential interactors that we
 676 identified by PL overall fit to the expected biological function
 677 of MpSYP12A and MpSYP13B. For this, we annotated *M.*
 678 *polymorpha* proteins based on information of *A. thaliana*
 679 homologs (Data S1). We then performed gene ontology (GO)-
 680 term enrichment analysis of the 24 hours interactome dataset
 681 using Metascape. Strikingly, 'vesicle-mediated transport' was
 682 the most significantly enriched GO-term extracted from the
 683 interactome data for both, MpSYP12A and MpSYP13B,
 684 coinciding with SNARE protein functions (Fig. 3a, b; Data S4).
 685 We also performed GO-term enrichment analysis of proteome

686 data that were obtained by measuring the input samples used
687 for the streptavidin-pulldown. Enriched GO-terms from
688 proteome data were mainly related to primary metabolism (Fig.
689 3c), which is clearly distinct from the enriched GO-terms of the
690 interactome dataset (Fig. 3a, b). These results suggest that the
691 potential interactors comprise actual interactors of MpSYP12A
692 and MpSYP13B. Analysis of the 4 hours interactome dataset
693 gave similar results (Fig. S5a, b; Data S4).

694 A number of interactors of *A. thaliana* SNAREs, which
695 are homologous to MpSYP12A and MpSYP13B, have been
696 reported (Kwon *et al.*, 2008; Fujiwara *et al.*, 2014). Based on
697 BioGrid, STRING, and IntAct databases, a total of 334 proteins
698 were reported or predicted to interact with SYP1 proteins in *A.*
699 *thaliana* (Data S2). Of these 334 *A. thaliana* proteins, we could
700 identify 250 homologous proteins conserved in the *M.*
701 *polymorpha* proteome using a BLAST approach (Data S3).
702 Among these 250 proteins, we found that 47 and 40 proteins
703 were identified as potential interactors of MpSYP12A and
704 MpSYP13B, while 39 were shared between both baits,
705 respectively. This means that around 11–15 % of all potential
706 interactors of MpSYP12A and MpSYP13B revealed by the
707 miniTurbo-based approach are homologous to known
708 interactors of *A. thaliana* SYP1 proteins (Fig. 3d).

709 MpSYP12A and MpSYP13B were demonstrated to
710 localize to the plasma membrane and to be ubiquitously
711 expressed throughout the thallus in *M. polymorpha* (Kanazawa
712 *et al.*, 2016). By using PANTHER GO annotations and *A.*
713 *thaliana* plasma membrane proteome data, we predicted plasma
714 membrane localizations of the potential interactors in *M.*
715 *polymorpha* (Data S1). We found that more than one third of
716 the potential interactors are expected to be plasma membrane-
717 localized (Fig. 3e). This result further supported the specificity
718 and utility of the miniTurbo-based approach.

719

The miniTurbo-mediated approach can reveal subtle differences between very similar baits

By comparing the 24 hours interactome data for MpSYP12A and MpSYP13B, we found that both baits share 90–95 % of their potential interactors (Fig. 1c), which is reasonable considering their predicted functions. Still, we captured 52 and 9 proteins that preferentially interact with MpSYP12A and MpSYP13B, respectively (Fig. 4a, Table 1). To investigate exclusive interaction partners of MpSYP12A and MpSYP13B, we implemented iBAQ (Intensity Based Absolute Quantification) values to the volcano plot (Fig. 4b). The iBAQ is a measure of protein abundance, and relative abundance was reflected in different circle sizes. The respective colours indicate abundances in all pulldown samples. By this, we revealed that MpNEK potentially interacts exclusively with MpSYP13B, and we found 13 proteins that potentially interact exclusively with MpSYP12A (Fig. 4b, Table 1).

737

738 Discussion

739 Published studies applying *in planta* TurboID-based PL have
 740 utilized model angiosperm species, and the amenability of
 741 current PL approaches to other plant species remains to be
 742 determined as outlined by Mair and Bergmann (2021). Here,
 743 we successfully applied miniTurbo-based PL in the liverwort
 744 *M. polymorpha*. This confirmed the transferability of biotin
 745 ligase utilized interactomics to a model bryophyte species.

746 We tested different biotin concentrations and treatment
 747 times to investigate biotin-labelling of proteins in *M.*
 748 *polymorpha* cells. Mair *et al.* (2019) reported a saturation of
 749 biotinylated proteins in *A. thaliana* stable transgenic lines after
 750 treatment with 50 μ M biotin solution for 1 hour. Zhang *et al.*
 751 (2019) transiently expressed TurboID-fusion-proteins in *N.*
 752 *benthamiana* leaves and tested biotin concentrations up to 800
 753 μ M. Zhang *et al.* observed that protein biotinylation was
 754 saturated after 8 hours of treatment with 200 μ M biotin, and
 755 that 15 minutes were sufficient for the saturation at 200 μ M
 756 biotin concentration. Meanwhile, in *M. polymorpha*, we did not
 757 observe a saturation of protein biotinylation after 24 hours of
 758 treatment with 700 μ M biotin. The observed differences in
 759 saturation of biotinylated proteins among the different studies
 760 could be partially explained by differences in mechanisms and
 761 efficiencies for biotin uptake and metabolism. Other factors
 762 potentially impacting biotin-labelling activities are plant growth
 763 conditions like light cycle and temperature used during biotin
 764 treatment.

765 For PL approaches, a careful evaluation of false positive
 766 candidates based on well-designed controls is desirable to
 767 generate a set of candidates with high confidence for further
 768 analyses and validation. It would be beneficial to include other
 769 plasma membrane-localized proteins that are independent of
 770 MpSYP12A and MpSYP13B to aid in predicting false positive

771 candidates, which might have been biotinylated randomly due
 772 to their localizations. That is to say, our transgenic lines
 773 expressing miniTurbo-Myc-MpSYP12A and miniTurbo-Myc-
 774 MpSYP13B could be used as suitable controls for interactome
 775 mapping using other plasma membrane-localized baits in the
 776 future. Meanwhile, the overall high similarity between
 777 MpSYP12A and MpSYP13B can be exploited to investigate
 778 specific interactors to understand functional differences
 779 between the two SNAREs. In general, long biotin treatment
 780 times potentially increase false positive labelling. On the other
 781 hand, longer treatment times may be required to efficiently
 782 capture rare or transient interactors.

783 For biotin-labelling approaches, protein extraction can
 784 be conducted in the presence of strong detergents. Strong
 785 detergents facilitate the extraction and solubilization of
 786 membrane-associated proteins, which can be advantageous for
 787 interactomics of plasma membrane-localized or organellar
 788 proteins. Our results confirmed that we could indeed capture a
 789 manifold of proteins that are predicted to be plasma membrane-
 790 localized, which has not been tested on the proteome level in
 791 published studies using PL in plants. In contrast to the Co-IP
 792 approach, the miniTurbo-based method could not capture
 793 extracellular interactors of MpSYP12A and MpSYP13B, which
 794 can be a drawback for certain applications. In the future, it
 795 remains to be determined whether the miniTurbo-based
 796 approach is also suitable for other cellular compartments,
 797 which may have a different intraorganellar pH or temperature,
 798 as discussed by Mair and Bergmann (2021).

799 Until to date, a manifold of interaction partners of
 800 AtSYP1 family proteins has been identified or predicted (Kwon
 801 *et al.*, 2008; *Arabidopsis* Interactome Mapping Consortium,
 802 2011; Fujiwara *et al.*, 2014). With our miniTurbo-based
 803 approach, we were able to capture homologs to well-known
 804 AtSYP1-interacting proteins, such as KEU, NPSN11, SYP61,

805 and VAMP721 (Fujiwara *et al.*, 2014), demonstrating the
 806 reliability of our method. We identified 47 and 40 proteins that
 807 are homologous to previously described AtSYP1-interacting
 808 proteins, while 104 and 92 proteins were linked to ‘vesicle-
 809 mediated transport’ based on our GO-term enrichment analysis.
 810 This indicates that we were able to capture a number of
 811 undescribed potential interactors of MpSYP12A and
 812 MpSYP13B, respectively. Kanazawa *et al.* (2020) were able to
 813 show that MpSYP12A but not MpSYP13B localized to the
 814 phragmoplast during cell plate formation by using fluorescent
 815 reporter-lines. Based on our GO-term enrichment analysis, we
 816 found the same set of 30 proteins related to ‘cell plate
 817 formation’ in interactomes of both, MpSYP12A and
 818 MpSYP13B. In other words, we failed to identify unique
 819 potential interactors of MpSYP12A previously linked to ‘cell
 820 plate formation’. To address a role of MpSYP12A at the
 821 phragmoplast, experimental conditions would need to be
 822 further adjusted to capture the interactome during cell division,
 823 by using cell cycle specific promoters to express proteins in
 824 dividing cells, for instance.

825 Our miniTurbo-based approach identified a number of
 826 potential interactors that are specific for MpSYP12A and
 827 MpSYP13B, which may help to understand functional
 828 differences between the two SNAREs in the future. For
 829 example, we found that homologs to MILDEW RESISTANCE
 830 LOCUS O (MLO) and PENETRATION 3 (PEN3)
 831 preferentially interact with MpSYP12A. *MLO* genes were
 832 shown to be involved in susceptibility to powdery mildew
 833 pathogens in barley (Büschges *et al.*, 1997) and *A. thaliana*
 834 (Consonni *et al.*, 2006). *AtPEN3* was found to be involved in
 835 resistance against barley powdery mildew (Stein *et al.*, 2006)
 836 and cell-death responses upon infection with *P. infestans*
 837 (Kobae *et al.*, 2006). In *A. thaliana*, AtSYP121 or
 838 PENETRATION1 (PEN1) is homologous to MpSYP12A.

839 *AtPEN1* was shown to be required for SNARE-dependent
 840 penetration resistance against barley powdery mildew and
 841 pathogen-induced vesicle accumulation was enhanced in *MLO*
 842 loss-of-function mutants (Collins *et al.*, 2003). Recently,
 843 Rubiato *et al.* (Rubiato *et al.*, 2021) provided evidence for an
 844 evolutionary conserved role of SYP12 proteins in the formation
 845 of papillae and encasements at pathogen penetration sites,
 846 which are effective defence structures against a broad range of
 847 filamentous pathogens. Taken together, our data suggest a
 848 function of MpSYP12A in penetration resistance and responses
 849 to filamentous pathogens, like SYP12 proteins in other plant
 850 species. Besides, we found that MpSYP12A may exclusively
 851 interact with ABSCISIC ACID INSENSITIVE1 (MpABI1),
 852 NUCLEAR MATRIX CONSTITUENT PROTEIN
 853 (MpNMCP), and homologs of OROSOMUCOID-LIKE 1
 854 (ORM1) and SENSITIVE TO FREEZING 2 (SFR2). MpABI1
 855 is involved in abscisic acid signalling (Tougane *et al.*, 2010)
 856 and MpNMCP was found to function in stress signalling in *M.*
 857 *polymorpha* (Wang *et al.*, 2021). In *A. thaliana*, NMCP
 858 homologs play a role in immunity (Choi *et al.*, 2019; Jarad *et al.*, 2019). AtORMs were reported to play roles in sphingolipid
 859 homeostasis and stress responses (Li *et al.*, 2016), and AtSFR2
 860 is a membrane remodelling enzyme responsive to freezing
 861 conditions in *A. thaliana* (Barnes *et al.*, 2019). Accordingly,
 862 our findings may suggest a potential role of MpSYP12A in
 863 lipid homeostasis and stress responses. We identified NIMA-
 864 related protein kinase 1 (MpNEK) as potential exclusive
 865 interactor for MpSYP13B. MpNEK directs tip growth in
 866 rhizoids of *M. polymorpha* (Otani *et al.*, 2018), and thus our
 867 data may suggest a potential role of MpSYP13B in rhizoid tip
 868 growth. It should be noted that potential interactors revealed by
 869 PL need to be validated using complementary approaches. In
 870 summary, our interactome data should be a useful resource for

872 future investigations of functional conservation and
873 diversification of SNARE proteins in plants.
874
875

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883

884 **Author Contribution**

885 KM and HN have designed the research. KM, SCS, AH, and
886 HN have contributed to experimental design and workflow
887 conceptualization. KM, SCS, and AH have conducted
888 experiments. KM, SCS, and HN have analysed the data by
889 mass spectrometry. KM, SCS, and HN wrote the manuscript.
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895

896 **Data Availability**

897 The mass spectrometry proteomics data have been deposited to
898 the ProteomeXchange Consortium via the PRIDE [1] partner
899 repository with the dataset identifier PXD030429.

900

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Figure legends

Fig. 1 Experimental setup for miniTurbo-mediated biotin-labelling in

M. polymorpha. (a) Schematic representation of the constructs used for generating transgenic plants. (b) Overview of the workflow used for evaluating the miniTurbo-based interactomics method in *M. polymorpha*. The figure was created with elements from BioRender (<https://biorender.com>). (c and d) Biotin ligase activity in *M. polymorpha*. Streptavidin (SA) immunoblots (IB) of cell extracts from *M. polymorpha* transgenic lines expressing miniTurbo-Myc-MpSYP12A (upper panels) and miniTurbo-Myc-MpSYP13B (lower panels) that were (c) treated with 0–700 μ M biotin solutions for 24 hours at RT or (d) treated with 700 μ M biotin solution for 0–24 hours at RT. Cell extracts of wildtype Tak-1 (WT) treated with 700 μ M biotin solution for 24 hours were used as a control. Arrows indicate the positions of the biotinylated miniTurbo-Myc-MpSYP12A and miniTurbo-Myc-MpSYP13B fusion-proteins. Coomassie Brilliant Blue-stained (CBB) membranes are shown as loading controls.

Fig. 2 Identification of MpSYP12A or MpSYP13B interacting proteins

by Co-IP and PL approaches. Wildtype Tak-1 was used as a control, and proteins that are significantly co-purified with or biotinylated by baits are highlighted. Potential interacting proteins for MpSYP12A and MpSYP13B are shown in turquoise and magenta, respectively. Venn diagrams show numbers of the potential interactors and their overlaps. (a) Myc-Trap Co-IP. Black text labels in volcano plots indicate the potential interactors for MpSYP12A or MpSYP13B. (b) 4 hours PL. (c) 24 hours PL. (d) Overlaps between 4 hours and 24 hours PL. Black circles in volcano plots indicate the potential interactors that are also identified with 4 hours PL. Dark grey circles indicate proteins that are identified as the potential interactors with 4 hours PL but not with 24 hours PL.

Fig. 3 Features of the identified MpSYP12A or MpSYP13B interacting

proteins (a - c) GO-term enrichment analysis of (a) 24 hours PL MpSYP12A interactome, (b) 24 hours PL MpSYP13B interactome, and (c) measured whole proteome. The top 20 overrepresented GO-terms are shown. (d) *M. polymorpha* homologs of AtSYP1-interacting proteins are highlighted in black or grey on volcano plots of 24 hours PL. Venn diagram shows numbers of potential interactors that are homologous to the AtSYP1-interactors and their overlaps. (e) Potential interactors that are predicted to localize to the plasma membrane are highlighted in black or grey on volcano

plots of 24 hours PL. Venn diagram shows numbers of the predicted plasma membrane-localized proteins and their overlaps.

Fig. 4 Potential interactors that preferentially interact with MpSYP12A or MpSYP13B. (a) Proteins that preferentially interacted with MpSYP12A or MpSYP13B are highlighted in turquoise and magenta, respectively. (b) Relative protein abundances based on iBAQ values are indicated by sizes of circles. Protein abundances in the samples of MpSYP12A, MpSYP13B, and wildtype Tak-1 are shown in turquoise, magenta, and dark grey, respectively. Proteins that were exclusively identified from the samples of MpSYP12A or MpSYP13B but not from the wildtype Tak-1 sample are annotated except for MpSYP12A and MpSYP13B.

Table 1 Potential interactors that preferentially interact with MpSYP12A or MpSYP13B. Proteins that are highlighted in turquoise or magenta in Fig. 4 are listed.

Fig. S1 Nucleotide and amino acid sequence of the miniTurbo biotin ligase used in this study. The nucleotide sequence of the original miniTurbo (Branon et al., 2018) was modified based on preferential codon-usage of *M. polymorpha*. A linker sequence of 15 amino acids length was added to the C-terminus of miniTurbo, to minimize potential sterical impairments of the enzymatic function. The linker sequence was fused to a single Myc-tag peptide of 10 amino acids length, to enable immunoblot detection of miniTurbo-fusion proteins and affinity purification using Myc-Trap.

Fig. S2 Plasmid maps of the binary vectors used in this study. (a) Vector map of pMKMM23 for the expression of miniTurbo-Myc-MpSYP12A. (b) Vector map of pMKMM22 for the expression of miniTurbo-Myc-MpSYP13B.

Fig. S3 Evaluation of the fusion protein expression, biotin depletion methods, and Myc-Trap Co-IP by immunoblotting. (a) Selection of transgenic lines expressing miniTurbo-Myc-MpSYP12A and miniTurbo-Myc-MpSYP13B fusion-proteins. Immunoblot (IB) of cell extracts from 10-day old thalli. MiniTurbo-Myc fusion proteins were detected by using an anti-Myc antibody. Cell extract of wildtype Tak-1 (WT) was used as a control. (b and c) Comparison of biotin depletion methods. Ten-day old thalli of a transgenic line expressing miniTurbo-Myc-MpSYP13B and

1228 wildtype Tak-1 (WT) were treated with 700 μ M biotin solution for 24 hours.
 1229 Total protein was extracted (input) and free biotin was removed from the
 1230 samples by methanol:chloroform precipitation (IP_1), PD-10 column
 1231 desalting (IP_2), or Zeba spin column desalting (IP_3) before pulldown of
 1232 biotinylated proteins using streptavidin-agarose beads. Streptavidin (SA)
 1233 Immunoblots (IB) of biotinylated proteins. **(b)** Biotinylated proteins in cell
 1234 extracts (input) and IP-eluates (IP_1, IP_2, IP_3) for the different biotin
 1235 depletion methods. **(c)** Biotinylated proteins in cell extract (input), the
 1236 supernatant of the streptavidin-agarose beads after affinity pulldown
 1237 (unbound_1, unbound_2, unbound_3), and IP-eluates (IP_1, IP_2, IP_3) for
 1238 the different biotin depletion methods. All depletion methods were tested in
 1239 duplicates. **(d)** Evaluation of Myc-Trap Co-IP by immunoblotting.
 1240 Immunoblot (IB) of cell extracts from 10-day old thalli. MiniTurbo-Myc
 1241 fusion proteins were detected by using an anti-Myc antibody in cell extracts
 1242 (input) and IP-eluates (IP) after affinity-purification using Myc-Trap beads.
 1243 Coomassie Brilliant Blue-stained (CBB) membranes are shown as loading
 1244 controls.

1245
 1246 **Fig. S4 Biotin ligase activity in *M. polymorpha*.** Streptavidin (SA)
 1247 immunoblots (IB) of cell extracts from *M. polymorpha* transgenic lines
 1248 expressing miniTurbo-Myc-MpSYP12A (upper panels) and miniTurbo-
 1249 Myc-MpSYP13B (lower panels) that were **(a)** treated with 0–700 μ M biotin
 1250 solutions for 24 hours at RT or **(b)** treated with 700 μ M biotin solution for
 1251 0–6 hours at RT. Cell extracts of wildtype Tak-1 (WT) treated with 700 μ M
 1252 biotin solution for **(a)** 24 hours or **(b)** 6 hours were used as controls. Arrows
 1253 indicate the positions of the biotinylated miniTurbo-Myc-MpSYP12A and
 1254 miniTurbo-Myc-MpSYP13B fusion-proteins. Coomassie Brilliant Blue-
 1255 stained (CBB) membranes are shown as loading controls.

1256
 1257 **Fig. S5 GO-term enrichment analysis.** GO-term enrichment analysis of (a)
 1258 4 hours PL MpSYP12A interactome, (b) 4 hours PL MpSYP13B
 1259 interactome, and (c) measured whole proteome. The top 20 overrepresented
 1260 GO-terms are shown.

1261
 1262 **Fig. S6 Uncropped images of immunoblots used in figures.**
 1263

Figure 1

Figure 2

Figure 3

Figure 4

Table 1

No.	MpGene ID	<i>A. thaliana</i> homolog (AGI code)	Annotation	Log2 fold-change SYP13B / SYP12A	-log p-value	Exclusive
1	Mp1g19980	-	LAMA3	-5,05	4,41	no
2	Mp2g10520	AT4G31080	membr. Protein	-4,69	3,34	MpSYP12A
3	Mp3g21160	AT1G67230	MpNMCP	-3,92	3,16	MpSYP12A
4	Mp3g05990	AT3G10650	NUP57	-3,52	3,32	no
5	Mp1g10960	-	Mp1g10960	-3,48	1,52	no
6	Mp1g28560	AT1G47750	PEX11	-3,37	3,21	no
7	Mp6g02820	AT3G47700	RINT1	-3,36	6,14	no
8	Mp3g12450	AT4G30190	MpHA20	-3,33	1,21	MpSYP12A
9	Mp2g17240	AT5G01950	LRR-kinase	-3,18	2,04	MpSYP12A
10	Mp6g00050	AT1G61290	MpSYP12A	-3,14	2,74	no
11	Mp3g18380	AT1G28490	MpSYP6A	-3,08	1,65	no
12	Mp1g08670	AT1G73200	TEX2	-3,01	2,70	no
13	Mp6g00330	AT4G04910	NSF	-2,81	2,25	no
14	Mp2g14450	AT5G45420	MpMYB3	-2,77	2,31	no
15	Mp7g04560	AT3G55420	AT3G55420-like	-2,74	2,68	no
16	Mp2g20600	AT5G43360	PHO84	-2,70	2,86	no
17	Mp1g14480	AT5G45160	RDH3/SEY1	-2,70	2,98	no
18	Mp3g07700	AT5G62670	MpHA11	-2,69	2,84	no
19	Mp7g14000	AT3G52190	PREB	-2,54	1,41	MpSYP12A
20	Mp1g10500	AT1G20970	AT1G20970-like	-2,53	1,34	no
21	Mp1g28850	AT5G64930	MpCPR	-2,48	1,82	MpSYP12A
22	Mp7g07570	AT1G48090	VPS-associated	-2,45	3,35	no
23	Mp2g19670	AT1G21790	TLC lipid-sens.	-2,42	1,94	no
24	Mp3g14570	AT1G73020	ANO10	-2,31	1,65	no
25	Mp1g21390	AT3G18610	NSR1	-2,28	3,06	no
26	Mp5g01540	AT5G53760	MLO	-2,25	2,83	no
27	Mp3g11440	AT1G48090	VPS-associated	-2,20	1,87	MpSYP12A
28	Mp3g23010	AT2G46225	ABIL1	-2,19	1,01	MpSYP12A
29	Mp2g00370	AT3G05060	NOP58	-2,00	1,36	MpSYP12A
30	Mp1g11950	AT3G16310	NUP35/53	-1,99	1,40	no
31	Mp7g06360	AT3G11950	TRAF zinc finger	-1,93	1,94	no
32	Mp2g17840	AT3G06510	SFR2	-1,88	1,66	MpSYP12A
33	Mp3g16790	AT5G19760	SLC25A11	-1,85	3,86	MpSYP12A
34	Mp4g16700	AT1G30690	SEC14-related	-1,85	3,32	no
35	Mp2g02640	AT1G01230	ORM	-1,80	2,29	MpSYP12A
36	Mp2g10340	AT3G12490	CYSB-like	-1,76	1,75	no
37	Mp4g12650	AT3G57150	MpCBF5	-1,69	1,68	MpSYP12A
38	Mp6g17400	AT3G20500	APC7	-1,67	2,21	no
39	Mp6g16270	AT2G45540	BEACH	-1,65	1,82	no
40	Mp8g00690	AT5G21990	FKBP-TRP	-1,65	5,37	no
41	Mp3g00870	AT5G13560	Citron	-1,64	4,26	no
42	Mp4g16720	AT1G30690	SEC14-related	-1,63	2,30	no
43	Mp5g13890	AT2G39280	Ypt-activating	-1,49	2,07	no
44	Mp8g06300	AT5G01180	PTR5	-1,48	2,88	no
45	Mp8g18220	AT3G60860	BIG	-1,47	2,38	no
46	Mp4g23500	AT1G59820	DRS2	-1,46	2,26	no
47	Mp3g01730	AT1G28250	AT1G28250-like	-1,43	2,25	no
48	Mp1g04630	AT5G44240	ALA2	-1,40	4,95	no
49	Mp6g07760	AT1G12360	KEU	-1,37	2,90	no
50	Mp5g23560	AT3G62860	Lysophospholipase	-1,33	2,77	no
51	Mp2g00520	-	Mp2g00520	-1,19	4,22	no
52	Mp4g07450	AT3G28860	MpABCB5	-1,02	2,84	no
53	Mp6g13120	AT5G14240	phosducin-like	1,07	3,14	no
54	Mp3g04380	AT3G09030	EAP3	1,17	2,74	no
55	Mp2g17470	AT1G68410	PP2C	1,40	2,68	no
56	Mp2g15040	AT4G01070	UGT72E	1,70	1,89	no
57	Mp5g04500	AT3G04810	MpNEK	1,73	2,42	MpSYP13B
58	Mp6g00440	AT1G22610	Synaptogamin-like	2,21	1,72	no
59	Mp7g09750	AT2G41740	VLN2	2,25	2,61	no
60	Mp3g14990	AT4G14605	mTERF	3,17	0,94	no
61	Mp2g19600	AT3G03800	MpSYP13B	6,69	4,11	no

Figure S2

Figure S3

Figure S4

(a)

(b)

Figure S5

Figure S6
– uncropped immunoblots

Figure 1c

Figure 1d

Figure S3a

Figure S3b

Figure S3c

Figure S3d

Figure S4a

Figure S4b