Production of HPV-16 L1 virus-like particles in Physcomitrella photobioreactors

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Abstract

Human Papilloma Virus (HPV) infections are the main causal agent of cervical and other anogenital cancers. Modern HPV vaccines are based on virus-like particles (VLPs), which are self-assembling nanoparticles composed of viral structural proteins. VLPs mimic native virions but lack viral DNA, rendering these pseudovirions non-infectious while retaining high immunogenicity. Despite available vaccines, the majority of cervical cancer deaths are due to HPV infections, particularly in low- and middle-income countries where inequalities in access to vaccination, screening, and treatment persist. While current HPV vaccines are produced in yeast or insect cells, alternative production platforms are explored, focusing on cost-effectiveness and scalability. Here, we used the moss plant Physcomitrella (*Physcomitrium patens*) to produce HPV-16 VLPs *via* expression of the major structural protein L1. We characterized Physcomitrella chloroplast transit peptides, which we used for targeting of L1 to chloroplasts, leading to higher recombinant protein accumulation compared to nuclear or cytoplasmic localization. We confirmed subcellular localization with confocal laser scanning microscopy and found L1 to accumulate within the chloroplast stroma. Production in 5 L-photobioreactors yielded more than 0.3 mg L1 per g fresh weight. We established a purification protocol for moss-produced L1 using a combination of ammonium sulphate precipitation and cation exchange chromatography. Purified samples were subjected to a controlled dis- and reassembly, ultimately yielding fully assembled HPV-16 L1 VLPs. This is the first report of production, purification and assembly of pseudovirions in a non-vascular plant.

Keywords: bryophyte, bioreactor, molecular farming, nanoparticle, plant biotechnology, vaccine candidate

Key message: First production of virus-like particles as a vaccine candidate in a non-vascular plant.

Word count: 6809

67 # Introduction

68 Human Papilloma Virus (HPV) infections are a major source of cancer, causing approximately
69 690,000 cases annually (zur Hausen et al., 1981; zur Hausen, 2002; de Martel et al., 2020). The most
70 prevalent type is cervical cancer, accounting for about 80% of HPV-attributable cancers. Other HPV-
71 related cancers include anogenital and oropharyngeal cancers (Wu et al., 2024). The burden of HPV
72 infection is not evenly distributed, with the highest prevalence in low- and middle-income countries, as a
73 result of inequities in access to vaccination, screening, and treatment services (World Health Organisation,
74 2024a).

75 HPV belongs to the *Papillomaviridae*, comprising nearly 200 different types, which are commonly
76 transmitted sexually (Ljubojevic & Skerlev, 2014). The majority of HPV-attributable cancers are linked to
77 two types in particular, namely HPV-16 and HPV-18 (de Martel et al., 2020; Wu et al., 2024). HPV is a non-
78 enveloped double stranded DNA virus of 8000 bp, encoding six early regulatory proteins (E1, E2, E4 – E7)
79 and two late structural proteins (L1, L2) (Roden & Stern, 2018). L1 proteins multimerize into pentamers,
80 the capsomers, and L1 capsomers and L2 monomers are imported into the nucleus *via* nuclear localization
81 signals (NLS), where HPV virions assemble (Cerqueira & Schiller, 2017). While the role of L2 in virion
82 assembly differs from type to type, L1 can self-assemble into virus-like particles (VLPs) in the absence of
83 L2 (Kirnbauer et al., 1992). The final HPV-16 virion is composed of 360 L1 monomers, organized in 72
84 pentamers, and 72 L2 monomers and is reinforced by inter-pentameric disulfide bonds (Baker et al., 1991;
85 Buck et al., 2005). Because the viral life cycle depends on differentiated epithelial cells, it is difficult to
86 obtain large quantities of virions. Therefore, today's HPV vaccines are based on L1 VLPs.

87 VLPs are nanoparticles consisting solely of viral structural proteins (Marsian & Lomonossoff, 2016;
88 Nooraei et al., 2021). Therefore, they cannot replicate and infect cells. These nanoparticles resemble
89 native viruses in size, shape, and surface structure, presenting highly repetitive immunogenic epitopes.
90 Although VLP-based HPV vaccines are currently produced in microbes (World Health Organization,
91 2024b), plant-based expression systems are gaining attention in this respect (Scotti & Rybicki, 2013;
92 Rybicki, 2020). In *Nicotiana benthamiana*, expression of L1 and L2 in combination with a reporter gene
93 generated pseudoviruses (PsVs), which could substitute mammalian cell culture-derived PsVs in
94 neutralisation assays (Lamprecht et al., 2016; Adams et al., 2023). Alternatively, Tobacco Mosaic Virus
95 VLPs produced in *N. benthamiana* were used for encapsidation and targeted delivery of mRNA encoding
96 oncogenic E6, a crucial target in therapeutic HPV vaccine development (Karan et al., 2024).

97 While first attempts to produce L1-based VLPs in plants had low yields (Biemelt et al., 2003;
98 Varsani et al., 2003), considerable improvements have been achieved, e.g. by codon optimization and
99 subcellular targeting strategies in transiently transformed tobacco (Maclean et al., 2007). A human codon-
100 optimized version (62% GC content) resulted in highest yields as compared to *N. benthamiana* optimized
101 (49% GC) and the native sequence (37% GC), presumably not due to the GC content but due to inhibitory
102 5' sequences, which act on transcription and mRNA processing (Maclean et al., 2007; Hitzeroth et al.,
103 2018). Further, these authors found chloroplast-targeted L1 yield highest at 0.5 mg / g fresh weight (FW)
104 followed by cytoplasmic L1 (without the C-terminal 22 aa NLS), whereas L1 targeted to the ER was not
105 detectable. Similarly, Zahin et al. (2016) reported 0.25 mg L1 / g FW *via* chloroplast targeting while leaving
106 the NLS intact. Although tobacco remains a prominent option, other plants are increasingly explored for
107 the efficient production of biopharmaceuticals.

108 The moss Physcomitrella has emerged as an alternative for biopharmaceutical production,
109 offering several advantages over traditional systems (Reski et al., 2015; Reski, 2018; Decker & Reski, 2020).
110 Its haploid dominant phase and high rate of homologous recombination enable precise gene targeting
111 (Strepp et al., 1998; Wiedemann et al., 2018; Rempfer et al., 2022). This unique feature facilitated the
112 modification of the N-glycosylation pathway, eliminating plant-specific modifications, such as β1,2-
113 xylosylation, α1,3-fucosylation, and Lewis A epitopes (Koprivova et al., 2004; Parsons et al., 2012; Decker
114 et al., 2014). Moreover, enzymes necessary for sialic acid synthesis, activation, and linkage to protein N-
115 glycans were introduced (Bohlender et al., 2020). These modifications have resulted in more
116 homogeneous and human-like N-glycan structures on recombinant proteins. The potential of
117 Physcomitrella as a biopharmaceutical production platform is demonstrated by several products in
118 development. For example, moss-produced α-galactosidase A has successfully completed clinical phase
119 Ib (Shen et al., 2016; Hennermann et al., 2019). Other moss-produced proteins include human factor H
120 (FH) and synthetic FH-related multitarget regulators MFHR1 and MFHR13, which are potential treatments
121 for complement-related disorders (Büttner-Mainik et al., 2011; Michelfelder et al., 2017, 2018; Top et al.,
122 2019; Ruiz-Molina et al., 2022a). Importantly, Physcomitrella cultivation in photobioreactors is tightly
123 controlled (Hohe et al., 2002), and thus compliant with Good Manufacturing Practice (GMP) standards,
124 ensuring high-quality production of recombinant proteins (Reski et al., 2018; Decker & Reski, 2020). Plants
125 in general (Rosales-Mendoza et al., 2016) and Physcomitrella in particular (Rosales-Mendoza et al., 2014)
126 are considered promising production hosts for innovative vaccines. The first moss-produced vaccine
127 candidate, an ENV-derived multi-epitope HIV chimeric protein, was immunogenic in mice (Orellana-

128 Escobedo et al., 2015). However, until now, there was no record of VLP-production in Physcomitrella, or
129 any other non-vascular plant.

130 Here, we analysed the expression of HPV-16 L1 in Physcomitrella. We characterized five
131 Physcomitrella chloroplast transit peptides and investigated the effect of subcellular L1 localization on
132 recombinant protein yields. We further established L1 production in photobioreactors and subsequent
133 purification of the product. Finally, the assembly of moss-produced HPV-16 L1 to VLPs was demonstrated
134 with transmission electron microscopy.

135

136 Materials and methods

137 Plant material

138 Physcomitrella (new species name: *Physcomitrium patens* (Hedw.) Mitt.; IMSC acc. no. 41269),
139 was cultivated according to Reski & Abel (1985) and Decker et al. (2015).

140

141 Optimization of HPV-16 L1 coding sequence

142 The CDS of h(human codon-optimized)L1 (GenBank acc. no. DQ067889, Maclean et al., 2007) was
143 used for optimization using physCO (https://www.plant-biotech.uni-freiburg.de/tools/physco/, Top et al.,
144 2021). Putative alternative splicing motifs AGGT were identified *via* search function. MicroRNA-binding
145 sites were predicted *via* psRNATarget with the library of 280 published Physcomitrella microRNAs and the
146 default settings *Schema V2* (2017 release) (http://plantgrn.noble.org/psRNATarget/, Dai & Zhao, 2011;
147 Dai et al., 2018). Alternative splice sites and microRNA-binding sites were removed and substituted by
148 synonymous codons based on http://www.kazusa.or.jp/codon/. Sequence editing was done in Benchling
149 (https://www.benchling.com/).

150

151 Expression vectors

152 Chloroplast transit peptides (CTPs) were selected from a list of proteins with experimentally
153 confirmed cleavage of a predicted CTP (Hoernstein et al., 2024). CTP-eGFP vectors were constructed based
154 on a vector containing an eGFP CDS with an N-terminal linker (sequence: GGGGGA) under control of the
155 PpActin5 promoter (Weise et al., 2006) and nos terminator (Hoernstein et al., 2023). CTP candidates were

156 amplified from Physcomitrella cDNA and inserted at the N-terminus of the linker using Gibson Assembly
157 (Gibson et al., 2009). The Citrine-L1 fusion constructs are based on a vector containing the PpActin5
158 promoter, nos terminator and citrine CDS (Wiedemann et al., 2018). The CDS of pL1, pL1Δ22 and CTP-pL1
159 were amplified using Phusion™ polymerase (Thermo Fisher Scientific, Waltham, USA), cloned into the
160 pJet1.2 backbone (Thermo Fisher Scientific) and ligated into the expression vector using T4 Ligase (Thermo
161 Fisher Scientific), *via Xho*I and *BamH*I sites. The pL1 expression vectors were constructed based on a vector
162 containing the PpActin5 promoter, nos terminator and hpt cassette (Ruiz-Molina et al., 2022a). The pL1
163 sequence was synthesized by Genewiz (Leipzig, Germany). For constructs pL1 and pL1Δ22, the pL1 CDS
164 was amplified using Phusion™ polymerase (Thermo Fisher Scientific) and cloned as described above. For
165 construct CTP-pL1, the pL1 and CTPc5 CDS were amplified and inserted into the backbone using Gibson
166 Assembly. Vectors were sequenced by Eurofins Genomics (Ebersberg, Germany). Vector maps are
167 compiled in **Supplementary Figure S1**. Primers were synthesized by Eurofins Genomics and are compiled
168 in **Supplementary Table S1**.
169

170 Protoplast preparation and transformation

171 Physcomitrella protoplasts were prepared and transformed based on existing protocols (Hohe &
172 Reski, 2002; Schween et al., 2003; Hohe et al., 2004; Decker et al., 2015). Transformed protoplasts were
173 incubated at 22 °C for 24 h in the dark and subsequently for 72 h at a 16/8-h light/dark photoperiod and
174 light intensity of 50–70 µmol m^{-2} s^{-1} before plating on KnopME + 25 mg/L hygromycin agar plates covered
175 with cellophane for two weeks. Surviving colonies were transferred to non-selective conditions for two
176 weeks before a second selection on KnopME + 50 mg/L hygromycin.
177

178 Fluorescence microscopy

179 Protoplasts were analysed 2-3 days after transfection with an AxioCam MRc5 camera on an
180 Axioplan2 stereo microscope (Zeiss, Oberkochen, Germany). Pictures were taken with a 40x objective or
181 a 63x objective with immersion oil. A FITC filter was used for eGFP, a rhodamine filter for chlorophyll
182 autofluorescence, and a FITC_LP filter for the combination of the two. Images were edited with GIMP
183 2.10.22 and ImageJ. For confocal microscopy, an AXIO Observer.Z1 Inverted Fluorescence Motorized
184 Phase Contrast Microscope Pred 7 with the objective Plan-Apochromat 63x/1.4 Oil DIC M27 (Zeiss) was

185 used. EGFP signals were detected at 488 / 509 nm, and chlorophyll autofluorescence at 587 / 610 nm.
186 Images were edited in ZEN 3.5 (Blue edition, ZEN Lite), ImageJ, and GIMP 2.10.22.
187 For analysis of subcellular targeting, an inverted confocal laser scanning microscope LSM 880 with
188 the objective LD LCI Plan-Apochromat 40x/1.2 H_2O autocorr and FastAiryScan mode (Zeiss) was used.
189 Citrine signals were detected at 488 / 516 nm, and chlorophyll autofluorescence at 561 / 595 nm. Images
190 were edited in ZEN 3.9 (Blue edition) and processed *via* deconvolution using default setting and
191 orthogonal projection using maximum intensity projection.
192

193 ## Biomass accumulation

194 Flasks containing 30 mL KnopME medium were inoculated at a density of 60 mg DW/L and
195 cultured for 4 weeks at the conditions described above. Biomass DW was measured by filtering 3x 10 mL
196 of culture through miracloth and subsequent drying at 105 °C for 2 hours. Biomass accumulation was
197 analysed by linear regression analysis. Slopes were compared using ANCOVA and post hoc multiple
198 comparisons test (TukeyHSD). The analysis was conducted in RStudio (version 4.2.2) and GraphPad Prism
199 (version 8.1).
200

201 ## Bioreactor operation

202 Stirred tank bioreactors (Applikon, Schiedam, Netherlands) were inoculated at 60 mg DW/L moss
203 density in 5 L KnopME. The light intensity was set to 160 µmol m^{-2} s^{-1} and increased to 350 µmol m^{-2} s^{-1}
204 after 4 days. At day 4, auxin 1-Naphtalene acetic acid (NAA, Sigma, St. Louis, USA) dissolved in 0.5 M KOH
205 was added to a final concentration of 5 µM. Throughout the bioreactor run, the pH was kept at pH 5.8,
206 temperature at 22 °C, and aeration at 0.3 vvm with 2% CO_2. The culture was continuously agitated with a
207 pitched 3 blade impeller at 500 rpm.
208

209 ## Protein extraction, purification and VLP assembly

210 For small scale extraction, 30 – 60 mg FW of vacuum-filtered moss material was combined with a
211 glass and a steel bead (each 3 mm in diameter) in a 2 mL reaction tube, frozen in liquid nitrogen and the
212 tissue lysed using a tissue lyser for 2 min at 28 Hz. Homogenized material was resuspended in PBS (2.7
213 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.137 M NaCl, pH 7.2), 0.01% Tween20, and protease inhibitor

214 (P9599, Sigma) in a ratio 3 mL/g. Samples were placed in an ice-cold sonication bath for 15 min,
215 centrifuged for 30 min at 14,000 x g and 4 °C, and the supernatant taken off for further analysis.
216 For large scale protein extraction, 5 g FW of vacuum-filtered moss material were frozen in liquid
217 nitrogen and ground to a fine powder. The material was combined with 22 mL PBS and 0.01% Tween20,
218 50 mM DTT and 100 µL protease inhibitor. The mixture was homogenized using an ULTRA-TURRAX (IKA,
219 Staufen, Germany) at 10,000 rpm for 10 min on ice. Next, the extract was treated with a Q500 sonicator
220 using a CL-334 converter (QSonica, Newtown, USA) at amplitude 55%, 10s on & 40s off for 20 min at 8 °C.
221 Debris was removed by two centrifugations at 4500 x g for 10 min and 20,000 x g for 20 min, both at 4 °C.
222 The sample was combined with solid $(NH_4)_2SO_4$ to a final concentration of 45% and proteins precipitated
223 under stirring for 1 h at 8 °C. Precipitated proteins were pelleted by centrifugation at 12,000 x g for 10
224 min at 8 °C. Pellets were dissolved in 4 mL CaEx chromatography binding buffer (2.7 mM KCl, 10 mM
225 Na_2HPO_4, 1.8 mM KH_2PO_4, 0.068 M NaCl, 0.01% Tween 20, 5% Glycerol, 5 mM DTT, pH 6.8) and dialysed
226 (20k MW cut off, 88405, Thermo Fisher Scientific) overnight against the same buffer at 8 °C. The sample
227 was diluted to a final volume of 10 mL using binding buffer, and passed through a 0.22 µm filter (Roth,
228 Karlsruhe, Germany) before loading onto a 1 mL HiTrap SP HP cation exchange column (Cytiva,
229 Marlborough, USA), connected to an ÄKTA™ start (Cytiva). The column was washed with 5 column
230 volumes (CV) of 0.3 M NaCl (based on binding and elution buffer) and L1 eluted *via* 4 CV of elution buffer
231 (2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 1 M NaCl, 0.01% Tween 20, 5% Glycerol, 5 mM DTT, pH
232 6.8)
233 Eluted fractions containing L1 were pooled and dialysed against disassembly buffer (22.7 mM KCl,
234 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.068 M NaCl, 0.05% Tween20, 5 mM DTT, pH 8.5) overnight at 8 °C
235 with 3x buffer exchange. Samples were concentrated 5x using a vacuum concentrator (Concentrator plus,
236 Eppendorf, Hamburg, Germany). For reassembly, samples were dialysed against reassembly buffer (22.7
237 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 1 M NaCl, 0.05% Tween20, pH 6) overnight at 8 °C with 3x
238 buffer exchange.
239

240 SDS-PAGE and Western blot

241 SDS-PAGE and Western blots were performed based on Bohlender et al. (2022). Two systems
242 were used for protein separation, either 7.5% polyacrylamide gels (Mini-PROTEAN® TGX™ Precast Gels,
243 Rio-Rad, Munich, Germany) in TGS buffer at 120 V, or 4 – 12% Bis-Tris gels (mPAGE™ Precast Gels, Merck,
244 Darmstadt, Germany) in MOPS buffer at 150 V. As first antibody served 1:5000 diluted CamVir1 (Thermo

245 Fisher Scientific), as second antibody peroxidase-linked rabbit anti-mouse secondary antibody (Cytiva)
246 diluted 1:12,500. For dot blots, protein samples were blotted on a PVDF membrane (Cytiva) using a
247 filtration manifold kit (SRC-96/1, Schleicher&Schuell, Dassel, Germany). Antibody H16.V5
248 (AntibodySystems, Schiltigheim, France) was diluted 1:5000. Dot blots were developed as described for
249 Western blots. Yeast-produced HPV-16 L1 (Abcam, Cambridge, UK) was used as a positive control.
250

251 ## L1 quantification

252 The ELISA protocol was based on Studentsov et al. (2002). In short, 96-well microtiter plates
253 (Greiner, Kremsmünster, Austria) were coated with 1:500 CamVir1 antibody (Thermo Fisher Scientific) in
254 PBS overnight at 8 °C. Wells were washed twice with washing buffer (1x PBS, 0.05% Tween20) and blocked
255 with blocking buffer (1x PBS, 1% polyvinyl alcohol MW 47,000, 0.05% Tween20) for 3 h at 37 °C. After 3x
256 washing, samples were diluted in blocking buffer and incubated in the wells for 1.5 h at 37 °C. Wells were
257 washed 3x before adding 1:1000 diluted polyclonal anti-HPV-16 L1 antibody from rabbit (Cusabio,
258 Houston, USA) for 1 h at 37 °C. After 5x washing, wells were incubated with 1:1000 diluted anti-rabbit
259 HRP-conjugated antibody NA934 (Cytiva) for 1 h at 37°C. Wells were washed 3x before adding 100 µL TMB
260 substrate (Thermo Fisher Scientific). The reaction was stopped with 50 µL of 2 M H_2SO_4. Absorbance was
261 measured at 450 / 595 nm for reference. The signal of a wildtype sample was subtracted to account for
262 background signal.
263

264 ## Transmission electron microscopy

265 TEM protocols are based on Abel et al. (1989). Negative staining of purified VLPs and moss extracts
266 was performed using 300 Mesh glow discharged formvar/carbon-coated copper grids (Electron
267 Microscopy Sciences, Hatfield, USA). Five µL of sample were applied on a grid and incubated for 5 min at
268 RT. Grids were washed 3x with water and stained with 2% (w/v) uranyl acetate. After 30 sec, uranyl
269 acetate was removed with a filter paper, grids were air dried, and inspected with a Hitachi HT7800 TEM
270 (Tokyo, Japan) coupled to a Xarosa CMOS camera (Emsis, Münster, Germany).
271

Results

Adaptation of L1 CDS for Physcomitrella-optimized expression

The CDS of HPV-16 L1 was optimized for expression in Physcomitrella. The goal was to adapt the CDS regarding codon usage and preventing potential degradation through microRNAs and splicing. A human codon-optimized version (hL1, **Supplementary Figure S2**) was used as a basis for optimization since it had led to higher yields than plant-optimized versions or the native viral sequence in *Nicotiana benthamiana* (Maclean et al., 2007).

For codon optimization, physCO (Top et al., 2021) was used, which changed nine out of 505 codons and slightly increased the GC content from 62.25 % to 62.85 % (**Supplementary Figure S2**). Next, the CDS was scanned for the motif AGGT, which is the most common motif at exon-intron borders for alternative splicing in Physcomitrella (Top et al., 2021). Transformation of a heterologous cDNA containing the AGGT motif led to alternative splicing in Physcomitrella, a process referred to as heterosplicing (Top et al., 2021). Scanning the physCO-optimized hL1 sequence yielded 5 AGGT-motifs which were replaced by synonymous codons (**Supplementary Figure S2**). MicroRNAs bind complementary sequences in the mRNA and thereby cause inhibition of translation or degradation of mRNA (Jones-Rhoades et al., 2006; Khraiwesh et al., 2010). One microRNA binding site was predicted within the CDS using psRNATarget (Dai & Zhao, 2011; Dai et al., 2018). Therefore, we changed three bases within the predicted binding region using synonymous codon substitutions. The final Physcomitrella-optimized CDS was named pL1 (**Supplementary Figure S2**) and differs from hL1 by 17 out of 1515 nucleotides without changing the aa sequence.

Characterization of chloroplast transit peptides

Different constructs were designed to target L1 to specific cellular compartments, including chloroplasts. Fusing chloroplast transit peptides (CTPs) to the L1 CDS led to increased protein yields in tobacco as compared to constructs lacking CTPs (Maclean et al., 2007; Zahin et al., 2016). To assess whether this is true for L1 production in Physcomitrella, a selection of Physcomitrella-derived CTPs was characterized.

Initially, the CTP of FtsZ1-1 (Gremillon et al., 2007) was considered. However, cleavage site analysis *via* TargetP-2.0 (Armenteros et al., 2019) predicted 37 aa of this CTP to remain at the N-terminus of L1 after chloroplast import. This CTP was rejected because the additional aa could change L1 properties and hamper VLP assembly. A list of alternative CTPs was derived from the Physcomitrella N-terminome

303 (Hoernstein et al., 2024) (**Supplementary Table S2**). Here, experimentally detected protein N-termini
304 were compared with TargetP-2.0 predictions for cleavable N-terminal targeting sequences. Five CTPs were
305 selected where an exact match between prediction and experimentally validated N-terminus was
306 observed across different experiments (Hoernstein al., 2024), namely CTPc5, CTPc10.1, CTPc10.2, CTPc21,
307 CTPc22 (**Table 1**). Their sequences were fused *in silico* to the N-terminus of L1 and analysed with TargetP-
308 2.0 to validate the correct cleavage of the fusion.

309 Our *in-silico* analysis indicated CTPc22 to end with a methionine, which could introduce an
310 alternative translation initiation site, resulting in translation of L1 without the CTP (**Table 1**). Therefore,
311 an alternative version lacking the C-terminal methionine (CTPc22noMet) was included. The likelihoods for
312 chloroplast localization were above 70 % for all candidates except CTPc10.1 (**Table 1**), which was rejected.

313 **Table 1: *In-silico* analysis of Physcomitrella-derived chloroplast transit peptides.** CTPs were selected based on mass
314 spectrometry datasets (Hoernstein et al., 2024). *In-silico* predictions of likelihood of chloroplast localization target
315 sequence cleavage were performed with TargetP-2.0.

Name of CTP	Originating from protein model	Predicted cleavage of CTP-L1	Length (aa)	Likelihood of GFP chloroplast localization	Likelihood of L1 chloroplast localization
FtsZ1-1	Pp3c22_4940V3.1	37 aa remain on N-terminus	84	0.95	0.92
CTPc5	Pp3c5_1370V3.1	-	74	0.987	0.943
CTPc10.1	Pp3c10_10490V3.1	-	63	0.456	0.229
CTPc10.2	Pp3c10_2940V3.1	-	70	0.908	0.845
CTPc21	Pp3c21_9980V3.1	-	43	0.956	0.891
CTPc22	Pp3c22_5470V3.1	-	62	0.939	0.739
CTPc22noMet	Pp3c22_5470V3.1	-	61	0.913	0.727

316

317 The remaining five CTPs (CTPc5, CTPc10.2, CTPc21, CTPc22, CTPc22noMet) were fused to the CDS
318 of eGFP (**Supplementary Figure S1**) and expressed in Physcomitrella protoplasts. Analysis *via* fluorescence
319 microscopy confirmed localization of eGFP signals in chloroplasts for CTPc5, CTPc10.2, CTPc22, and
320 CTPc22noMet, but not for CTPc21 (**Supplementary Figure S3**). Of the four remaining CTPs, CTPc5 had the

321 highest likelihood for plastid localization (**Table 1**) and were examined *via* confocal microscopy
322 (**Supplementary Figure S3**). Finally, we selected CTPc5 for the chloroplast-targeting of L1.
323

324 Targeting of L1 to nuclei and chloroplasts

325 To evaluate the impact of L1 localization on the yield, we generated three variants (pL1,
326 pL1Δ22, CTP-pL1). pL1 contains the full-length pL1 CDS, which includes the native HPV-16 NLS (Cerqueira
327 & Schiller, 2017). For pL1Δ22 the NLS was removed. In CTP-pL1 the Physcomitrella CTPc5 was fused to the
328 5' of the pL1 CDS, including the NLS.
329 After generation of these CDS variants, we aimed to evaluate L1 targeting to subcellular
330 compartments. The CDS of pL1, pL1Δ22 and CTP-pL1 were fused to the 5' end of Citrine CDS
331 (**Supplementary Figure S1**). Physcomitrella protoplasts were transfected with the plasmids and Citrine
332 localization was analysed using fluorescence microscopy. A vector encoding Citrine without any L1 variant
333 fusion served as a control.
334 Cells transformed with the control vector exhibited predominantly cytoplasmic Citrine signals,
335 whereas diffuse signals also occurred in nuclei (**Figure 1**). For pL1-Citrine, the signal localized to nuclei,
336 demonstrating that the HPV-16 NLS is functional in Physcomitrella. This is further supported by the fact
337 that removal of the NLS in pL1Δ22-Citrine results in cytoplasmic localization. For CTP-pL1-Citrine, the
338 signal is predominantly in chloroplasts, but is not evenly distributed, as seen for CTP-eGFP fusions lacking
339 L1 (**Supplementary Figure S3**). Instead, the pL1-Citrine fusion concentrates in several spots of high
340 fluorescence intensity and <1 µm in diameter. Overall, we confirmed successful L1 targeting to the
341 intended compartments.
342

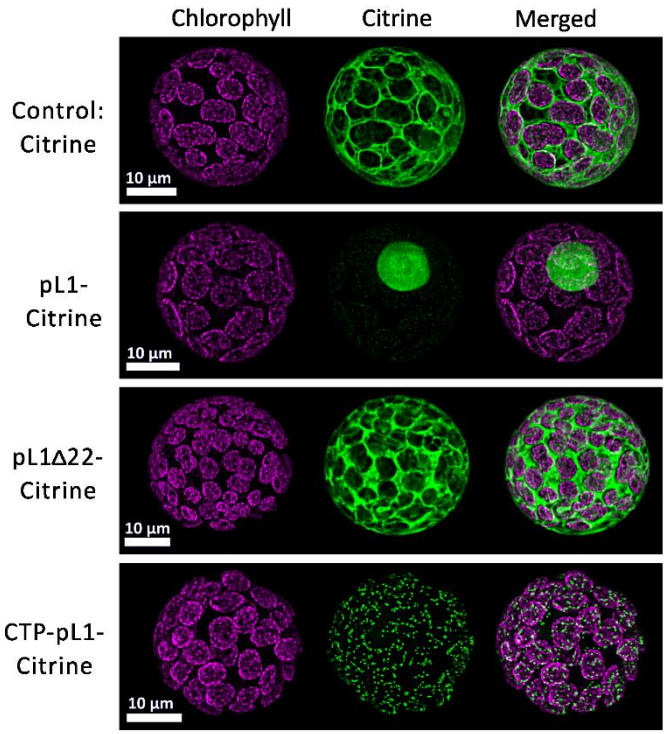


Figure 1: Subcellular localization of L1 variants. Confocal microscopy images of Physcomitrella protoplasts transformed with constructs encoding (CTP-)pL1(Δ22)-Citrine fusion proteins or Citrine alone. For each sample, chlorophyll autofluorescence in magenta is seen on the left, Citrine signals in the middle and the merged signals on the right. In the control and pL1Δ22-Citrine, the Citrine signals do not enter the chloroplasts and are mainly in the cytoplasm. For pL1-Citrine, the Citrine signals are in the nucleus. In the CTP-pL1-Citrine sample, the Citrine signals form small spots of high intensity in the chloroplasts.

Characterization of L1 production

After validating the subcellular localization, pL1, pL1Δ22 and CTP-pL1 expression constructs were cloned (**Supplementary Figure S1**). The linearized constructs were transferred into Physcomitrella protoplasts and obtained plants were screened for productivity by Western blot and ELISA.

Western blot analysis revealed the L1 monomer of 56 kDa (pL1, CTP-pL1) and 54 kDa (pL1Δ22) (**Figure 2**). In addition, pL1 and CTP-pL1 lines exhibited a band at approximately 54 kDa. All lines showed another band at around 45 kDa, even though the signal was relatively weak in CTP lines and more prominent in pL1Δ22 lines. Smaller protein isoforms can originate from degradation or DNA or RNA, resulting in truncated transcripts and, subsequently, truncated L1 isoforms (Murén et al., 2009; Top et al., 2021). The latter possibilities were ruled out by amplifying the L1 CDS *via* PCR from cDNA, which yielded amplicons of the expected sizes, corresponding to the Western blot bands of 56 kDa (pL1, CTP-pL1) and 54 kDa (pL1Δ22), but not additional amplicons (**Supplementary Figure S4**). Also, two bands are visible at

130 – 180 kDa, of which the highest band at 180 kDa is also present in the yeast-produced L1 control. This pattern of two close-by bands repeats another two times at higher molecular weight outside of the marker range (**Figure 2**). ELISA analysis indicates higher yields in CTP-pL1 lines as compared to pL1 or pL1Δ22 lines. In shaking flasks, the best producer is CTP-pL1 30 with 142 µg L1/g FW (**Figure 2**).

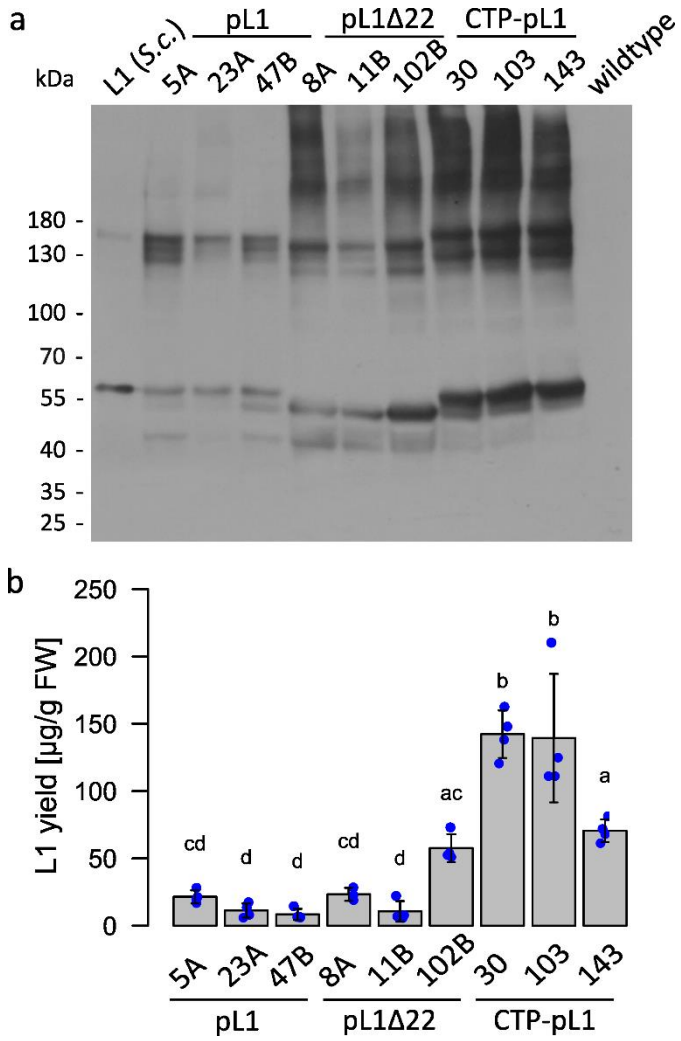
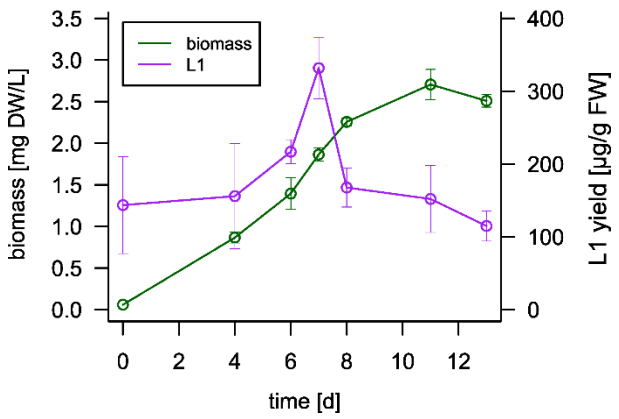


Figure 2: Analysis of HPV-16 L1 production *via* Western blot and ELISA. (**a**) For each of the constructs pL1, pL1Δ22, and CTP-pL1 three lines were tested *via* Western blot for expression of HPV-16 L1. 50 ng *S. cerevisiae*-produced L1 served as a positive control and Physcomitrella wildtype as a negative control. The proteins for the Western blot were separated on a 7.5% TGS polyacrylamide gel under reducing conditions before being transferred to a membrane and developed using CamVir1 antibody (1:5000). (**b**) Production levels of L1 were quantified *via* ELISA. Wildtype background signal was subtracted before analysis *via* one-way ANOVA and post hoc multiple comparisons test (TukeyHSD). Means and standard deviations are calculated based on n ≥ 3 technical replicates from two dilutions. Statistical significance (α = 0.05) is indicated by Compact Letter Display.

376 Besides the amount of recombinant protein per g FW biomass, the accumulation of biomass is
377 important in biomanufacturing as high product yields per biomass can be trivial if biomass accumulation
378 is slow. To study if recombinant protein amount or subcellular localization of L1 hampers plant growth,
379 we studied the time course of biomass accumulation of the nine lines in shaking flasks for 4 weeks.
380 In our conditions, exponential growth could not be measured. This might be due to the culture
381 conditions, to the rather long intervals between sampling, and/or to the intrinsic growth characteristics
382 of Physcomitrella protonema, which grows by apical cell division (Reski, 1998). Therefore, biomass density
383 was fitted using linear regression analysis model (**Supplementary Figure S5**), using the calculated slope to
384 approximate biomass accumulation rates. Comparison of the slopes between the lines pL1, pL1Δ22 and
385 CTP-pL1 showed pL1 lines accumulated biomass faster than pL1Δ22 ($p = <0.0001$) and CTP-pL1 ($p = 0.0001$)
386 lines. If this difference is due to the distinct L1 localization or to lower L1 production in pL1 lines (**Figure**
387 **2**) is unclear. Line pL1 47B showed the highest slope of 255 mg DW/L/week, as compared to line CTP-pL1
388 30 with a slope of 211 mg DW/L/week. However, this difference is negligible considering the yield of pL1
389 47B is 8 µg L1/g FW as to 142 µg L1/g FW for CTP-pL1 30 (**Figure 2**). Consequently, line CTP-pL1 30 was
390 chosen for VLP production.
391

392 ## Upscaled L1 production

393 For production of VLPs, the cultivation of line CTP-pL1 30 was upscaled to 5 L-photobioreactors.
394 The goal was to identify the best time point for harvesting material used for subsequent L1 purification.
395 Biomass increased from initially 60 mg DW/L to around 870 mg DW/L at day 4 (**Figure 3**). L1
396 production remained constant during the first 4 days, in the range of the yield observed for flasks. Upon
397 addition of the phytohormone auxin on day 4, L1 levels increased on day 6 and peaked at day 7 at 332 µg
398 / g FW, 130% higher as compared to day 0. Afterwards, L1 production dropped sharply to levels prior to
399 auxin addition. Biomass continued to accumulate until day 11, after which it dropped slightly. These
400 conditions, including day 7 as the ideal harvesting time point, were used for further L1 production.
401



402

403 **Figure 3: Analysis of biomass accumulation and L1 production of line CTP-pL1 30 in a 5 L-photobioreactor.** The bioreactor was
404 inoculated with 60 mg dry weight (DW)/L and monitored over a course of 13 days. On day 4, 5 µM NAA was added. L1 production
405 peaked at day 7 at 332 µg / g fresh weight (FW). Biomass and L1 measurements are based on n ≥ 3 technical replicates. Production
406 levels of L1 were quantified *via* ELISA.

407

408 Two-step purification removes bulk of cellular proteins

409 Purification of L1 was performed using ammonium-sulphate (AMS) precipitation, followed by
410 cation exchange (CaEx) chromatography. While AMS removes the bulk of cellular proteins, CaEx removes
411 residual proteins and concentrates the sample.

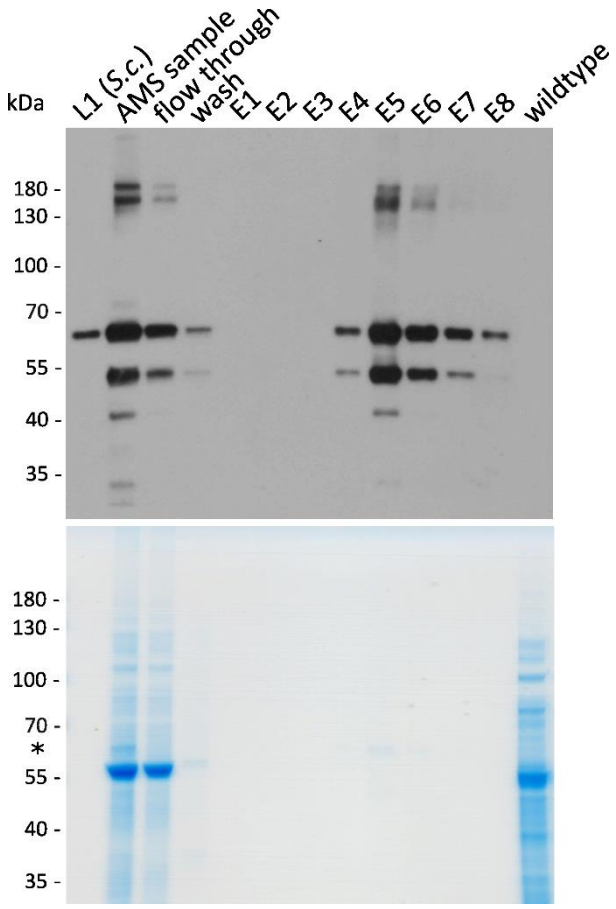
412 We tested the efficacy of 40%, 45% and 50% AMS to precipitate L1 from an extract containing the
413 soluble protein fraction from CTP-pL1 30 plant material produced in the bioreactor. We found 45% AMS
414 to be sufficient to effectively remove L1 from the supernatant, whereas at 40% AMS small amounts of L1
415 remained in the supernatant and at 50% AMS higher amounts of cellular protein were precipitated
416 together with L1 (**Supplementary Figure S6**). In preparation for chromatographic purification, the
417 precipitated proteins were resuspended in CaEx binding buffer and dialysed overnight in binding buffer
418 to remove residual AMS. Cloudiness of samples indicated a reduced solubility of precipitated proteins,
419 and undissolved matter was removed by filtration. The relative amount of L1 and host protein was
420 evaluated *via* Western blots and Coomassie-staining, respectively (**Supplementary Figure S6**). Samples
421 after filtration showed a slight reduction in host protein content, but, except for the sample at 40% AMS,
422 no reduction in L1. Overall, precipitation at 45% AMS was selected, which is in line with purifications of
423 yeast-produced L1 (Park et al., 2008; Kim et al., 2010).

424 Next, CaEx chromatography was used to remove residual cellular proteins. The bulk of cellular
425 proteins did not bind to the column and was detected in the flow through (**Figure 4**, **Supplementary Figure**
426 **S7**). Notably, a large amount of L1 did not bind to the column and was detected in the flow through.

427 Different binding buffer compositions were tested to increase L1 binding to the column, e.g. lowering the
428 pH from 7.2 to 6.8, lowering the concentration of NaCl from 0.137 M to 0.068 M, addition of 5% glycerol,
429 and re-loading of flow-through fraction. Nevertheless, a large part of L1 did not bind to the column and
430 was found in the flow through. During column wash with 5 mL at 0.3 M NaCl, small amounts of
431 contaminating proteins and L1 were washed off the column. For elution, NaCl was increased to 1 M NaCl
432 and 0.5 mL fractions were collected, in total 4 mL. L1 eluted from the column in fractions E4 – E8 (**Figure**
433 **4**), which were pooled for further analysis.

434 In contrast to the confirmation of L1 expression (**Figure 2**) and the establishing of AMS
435 precipitation (**Supplementary Figure S6**), DTT was used throughout the extraction and purification leading
436 to CaEx chromatography. In comparison, the presence of DTT markedly reduces the Western blot signal
437 intensity above 180 kDa (**Figure 4**). We hypothesize that in the absence of DTT, reactive oxygen species in
438 the plant extract cross-link L1 with itself and/or host proteins. Irrespective of the presence or absence of
439 DTT during extraction and purification, two prominent bands in the range of 130 and 180 kDa are present
440 in all Western blots (**Figures 2 and 4, Supplementary Figure S6**).

441

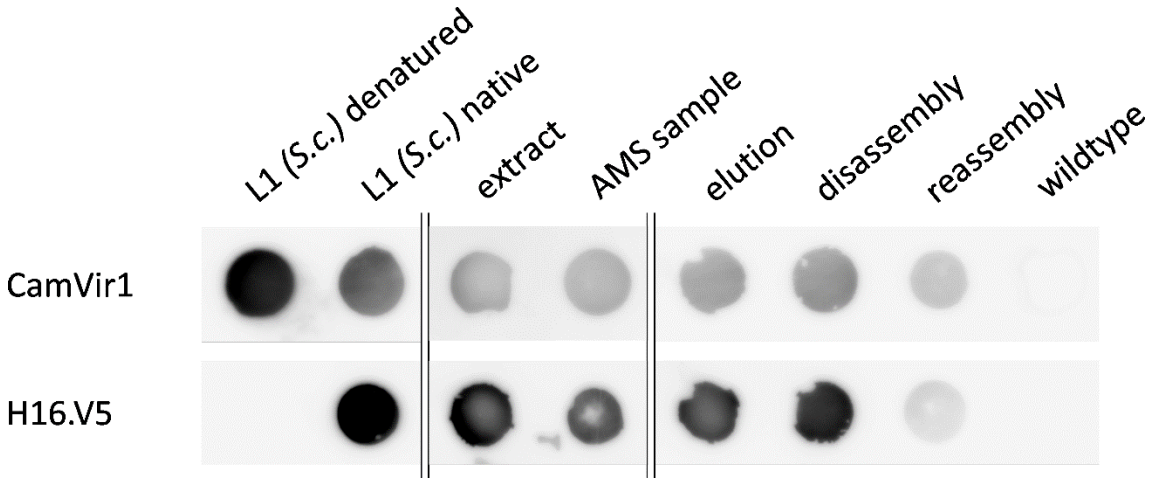


442

443 **Figure 4: Two-step purification of L1 removes bulk of cellular protein.** Analysis of L1 and residual cellular host proteins during
444 purification under reducing conditions *via* Western blot and Coomassie-stained SDS-PAGE (12% Bis-Tris gel). After precipitation
445 with 45% AMS, the filtered sample was subjected to cation exchange chromatography (CaEx) for final purification. Column-bound
446 L1 eluted at 1 M NaCl in fractions E4 – E8. The asterisk in the Coomassie-stained SDS-PAGE marks the band referring to HPV-16
447 L1. 50 ng *S. cerevisiae*-produced L1 served as a positive control and Physcomitrella wildtype as a negative control.
448

449 ## Capsomers and VLPs

450 Next, we performed a dis- and reassembly protocol on the pooled elution fractions, as it yields
451 more uniform and stable VLPs, which are less prone to aggregation (McCarthy et al., 1998; Mach et al.,
452 2006). First, pooled elution fractions were dialysed at low salt concentrations, high pH and DTT, that
453 results in the disassembly of VLPs or other multi-pentameric L1-based structures into capsomers. Second,
454 samples were dialysed at high salt, low pH and in the absence of reducing agent, that leads to the
455 reassembly of capsomers into VLPs.
456 To assess the conformation of L1 throughout the process, dot blots were performed using
457 different monoclonal antibodies. CamVir1 detects a linear epitope in both denatured and native L1 and
458 served as a control. In contrast, H16.V5 detects only conformational epitopes in both, L1 capsomers and
459 VLPs, but not monomeric or denatured L1.
460 The dot blot showed the presence of L1 in all CTP-pL1 30-derived samples throughout the
461 purification process, as well as yeast-derived denatured and native L1 (**Figure 5**). In comparison, H16.V5
462 detects L1 in the native positive control and in all CTP-pL1 30-derived samples, indicating the presence of
463 at least capsomers, if not VLPs, already in the raw extract. Further, capsomers seem to be stable
464 throughout AMS precipitation, CaEx chromatography, disassembly and reassembly.
465



466

467 **Figure 5: Moss-produced HPV-16 L1 assembles into capsomers, which are stable throughout purification.** Dot blots were
468 performed using two different antibodies to analyse the formation of capsomers in yeast-produced L1 and samples from CTP-
469 pL1 30 purification (extract, after AMS precipitation, elution) and dis- and reassembly. The CamVir1 antibody detects a linear
470 epitope in both denatured and native L1. The neutralizing antibody H16.V5 detects specifically conformational epitopes in L1
471 capsomers and VLPs. Positive H16.V5 signals indicate HPV-16 L1 monomers to assemble at least into capsomers, *in vivo* or after
472 extraction, which are stable throughout the purification. The experiment only allows qualitative but not quantitative comparison
473 since sample concentrations were adjusted individually. `L1 denatured´ was incubated at 95 °C for 5 min, all other samples were
474 kept at 4 °C. A wildtype sample was used as a negative control.
475
476 The assembly of L1 capsomers into VLPs was verified with Transmission Electron Microscopy
477 (TEM). As a control, yeast-produced L1 was analysed without prior treatment, after disassembly, and after
478 reassembly. In the untreated sample, particles of ca. 60 nm in size were detected. After disassembly,
479 structures of 5 – 10 nm in size were observed, corresponding to capsomers. VLPs reshape upon
480 reassembly treatment, demonstrating the successful dis- and reassembly of yeast-derived L1 VLPs.
481 Notably, reassembled VLPs are heterogenous in size and shape, despite the controlled dis- and reassembly
482 (**Figure 6**).
483

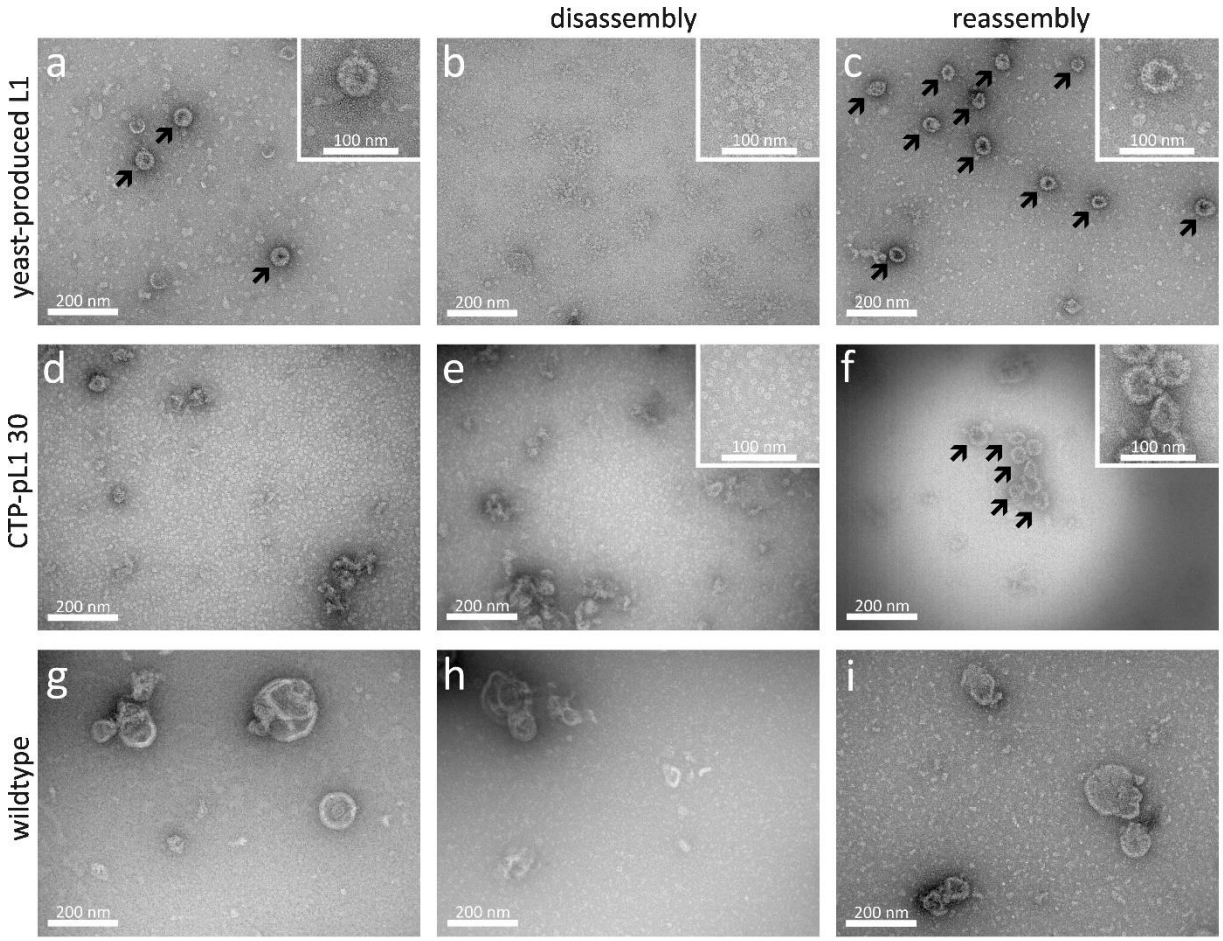


Figure 6: Analysis of dis- and reassembled VLPs from moss and yeast *via* transmission electron microscopy (TEM). Yeast-derived L1 was analysed untreated (**a**), after disassembly (**b**), and after reassembly (**c**) *via* TEM. CTP-pL130 (**d** – **f**) and wildtype (**g** – **i**) extracts were purified *via* AMS precipitation and CaEx chromatography (**d** & **g**), after disassembly (**e** & **h**), and after reassembly (**f** & **i**). Untreated yeast-derived VLPs (**a**) disintegrate into capsomers upon disassembly (**b**) and reshape into VLPs upon reassembly treatment (**c**). TEM shows the presence of moss-derived HPV-16 L1 capsomers in the elution (**d**) and disassembly sample (**e**) and the assembly into VLPs in the reassembly sample (**f**). Capsomer and VLP-like structures are absent from the wildtype control samples. VLPs are indicated by black arrows.

To analyse L1 assembly into VLPs, samples were taken from the pooled CaEx chromatography elution fractions, after disassembly, and after reassembly.

In the elution sample, capsomeric structures comparable to those observed in the disassembled yeast sample were observed, which are not present in the corresponding wildtype control (**Figure 6**). The abundance of capsomers in the elution fractions was surprising given the fact that the elution buffer has a high ionic strength, which favours reassembly. However, before column loading, the sample was dialysed overnight in binding buffer, which favours disassembly. The exposure to high ionic strength

500 during elution might have been too short to allow VLP assembly. In addition, both binding and elution
501 buffer contained DTT, which induces disassembly (McCarthy et al., 1998; Mach et al., 2006). The
502 disassembled CTP-pL1 30-derived sample depicted capsomers as seen in the elution fraction, and for
503 disassembled yeast-derived L1 (**Figure 6**). **Figure 6f** displays fully assembled, moss-derived HPV-16 L1
504 VLPs. Again, VLPs were heterogenous in size and shape, as seen for reassembled yeast-derived VLPs.
505 Moss-produced VLPs tended to aggregate, which was not observed for reassembled yeast-derived VLPs,
506 but is common for HPV-16 L1 VLPs (Shi et al., 2005). No VLPs were detected in wildtype samples (**Figure**
507 **6**).
508

509 ## Discussion

510 Various strategies were explored for increasing production yields of HPV-16 L1 pseudovirions in
511 plants, one of which is optimization of codon usage (Maclean et al., 2007; Hitzeroth et al., 2018). The latter
512 found that codon optimization of L1 improves transcription, rather than translation. This is due to RNA
513 motifs in the L1 CDS, which posttranscriptionally regulate the virus life cycle and lead to pre-mRNA
514 degradation (Mori et al., 2006; Zhao & Schwartz, 2008). It is argued, that codon optimization removes
515 those motifs, thereby increasing mRNA and protein levels, irrespective of whether the CDS was optimised
516 for expression in humans, tobacco (Hitzeroth et al., 2018) or Physcomitrella (this study). Our
517 Physcomitrella-optimized CDS has a GC content of 62.85% as compared to hL1 with 62.25% (Maclean et
518 al., 2007) and the wildtype sequence with 38.27% (Varsani et al., 2003). Combined with the removal of
519 microRNA binding sites and heterosplice sites we obtained Physcomitrella-optimized pL1 CDS. We argue
520 that pL1 addresses the issues of L1 intrinsic degradation motifs as well as Physcomitrella-specific
521 posttranscriptional degradation.
522 Besides codon optimization, research focused on localization signals or transit peptides. The most
523 common strategy is sequestration to chloroplasts, either *via* nuclear transformation and posttranslational
524 import into chloroplasts (Maclean et al., 2007; Pineo et al., 2013; Zahin et al., 2016; Hitzeroth et al., 2018;
525 Naupu et al., 2020; Muthamilselvan et al., 2023) or *via* transplastomic lines (Fernández-San Millán et al.,
526 2008; Lenzi et al., 2008). Here, we experimentally characterized five Physcomitrella CTPs, of which we
527 used CTPc5 for sequestration of L1 to chloroplasts. Employing the HPV-16 native NLS, L1 was targeted to
528 nuclei, whereas the removal of the NLS resulted in cytoplasmic L1. Earlier studies confirmed functionality
529 of the NLS in mammalian cells (Leder et al., 2001; Bissa et al., 2015). However, the NLS failed to localize
530 L1 to nuclei in tobacco (Šmídková et al., 2010). Our observation of the HPV-16 native NLS targeting L1 to

531 nuclei is another example for a conserved cross-kingdom functionality of localization signals between
532 Physcomitrella and mammals or human-infecting viruses (Gitzinger et al., 2009).
533 Western blots of L1-producing lines showed a variety of bands, both smaller and larger than the
534 expected 56 kDa or 54 kDa. A comparable banding pattern was observed when expressing L1 in tobacco
535 chloroplasts (Fernández-San Millán et al., 2008). Likewise, yeast-produced L1 exhibited bands with the
536 size of L1 dimers and trimers (Kim et al., 2007; Kim et al., 2010). L1 dimers and trimers are based on inter-
537 capsomeric disulfide bridges which stabilize virions and VLPs (Sapp et al., 1998; Buck et al., 2005; Buck et
538 al., 2013). McCarthy et al. (1998) resolved L1 multimers with DTT. Likewise, Buck et al. (2005)
539 demonstrated absence and presence of L1 dimers and trimers in a comparison of reducing and non-
540 reducing SDS-PAGE and Western blots, respectively. An investigation of cysteine 175 and cysteine 428 L1
541 mutants resulted in the absence of bands at 130 kDa and 180 kDa, thereby linking disulfide bridge-based
542 dimers and trimers to the observed bands (Buck et al., 2005). Surprisingly, reducing agents such as DTT or
543 β-mercaptoethanol did not result in the disappearance of these bands in our and other studies (Kim et al.,
544 2007; Fernández-San Millán et al., 2008; Kim et al., 2010). This indicates a tolerance of L1 dimers and
545 trimers to reducing agents or a different origin of multimerization observed at 130 kDa and 180 kDa.
546 Castells-Graells & Lomonossoff (2021) observed multimerization of *Nudaurelia capensis* omega virus α-
547 peptide produced in tobacco, irrespective of DTT, but not when produced in insect cells. These authors
548 hypothesize that cross-linkage of α-peptide occurs during extraction and purification and occurs in plant-
549 based expression systems. Regarding the observed bands <56 kDa, proteolytic degradation was proposed
550 in other studies observing the same phenomenon (Buck et al., 2005; Fernández-San Millán et al., 2008).
551 In tobacco, a shift from bands above 100 kDa in young leaves to more prominent bands at 45 kDa and 20
552 kDa in older leaves indicated proteolytic degradation of L1 over time (Fernández-San Millán et al., 2008).
553 In our study, bands at 45 kDa are most prominent in pL1Δ22 lines with cytoplasmic L1 and less prominent
554 for plastidic L1. This might result from organelle-specific proteases (Coates at al., 2022).
555 In Physcomitrella, combining the full-length L1 with a CTP significantly increases L1 yield as
556 compared to full length L1 or L1 without NLS, which is in line with findings in tobacco (Maclean et al.,
557 2007; Zahin et al., 2016). In our study, L1 yields above 0.3 mg/g FW were achieved in the photobioreactor.
558 In tobacco, yields of 0.1 – 1.2 mg/g FW (Maclean et al., 2007; Pineo et al., 2013; Zahin et al., 2016;
559 Hitzeroth et al., 2018) and up to 3 mg/g FW in transplastomic lines have been reported (Fernández-San
560 Millán et al., 2008). The success of targeting L1 to chloroplasts over localization to other organelles may
561 have several reasons. One is that plastid L1 may be protected from proteases absent in chloroplasts (van
562 Wijk, 2024). Besides, CTPs also act on the transcriptional level. Upon expression in tobacco, chimeric L1/L2

563 transcripts containing a CTP were more abundant than transcripts lacking a CTP, and consequently yielded
564 more protein (Hitzeroth et al., 2018). So far, it is unclear whether this difference is due to enhanced
565 transcription, increased mRNA half-life or changes in mRNA secondary structure.

566 For purification of L1 from Physcomitrella extracts, a combination of AMS precipitation and CaEx
567 chromatography was employed. We found 45% AMS to be sufficient to efficiently precipitate L1. Studies
568 from yeast (Park et al., 2008; Kim et al., 2010) and tobacco (Zahin et al., 2016) also used 45% AMS, whereas
569 sometimes 50% AMS was used (Kim et al., 2018). CaEx chromatography showed that a large part of L1
570 does not bind to the column. Likewise, Zahin et al. (2016) reported tobacco-produced L1 to bind poorly
571 to CaEx columns. Kim et al. (2010) purified yeast-produced L1 by either CaEx or heparin chromatography.
572 CaEx chromatography showed unbound L1 in the flow through, as observed in our study, whereas no L1
573 was found in the flow through employing heparin chromatography. Pineo et al. (2013) produced L1/L2
574 chimaeras in tobacco and experienced loss of L1 and insufficient removal of contaminating proteins using
575 heparin chromatography. Heparin is a highly sulphated form of heparan sulphate, a proteoglycan found
576 on host cell surfaces and a requirement for HPV virion attachment and infection (Knappe et al., 2007).
577 However, a prerequisite for heparin binding is the assembly of L1 to capsomers or VLPs (Rommel et al.,
578 2005), meaning monomers and not properly folded L1 are lost. Overall, chromatography yields for VLPs
579 differ and are influenced by different factors, including the production host, the used materials and
580 buffers, and purification prior to chromatography, making it difficult to compare purification strategies
581 across publications. Here, we achieved to remove the bulk of cellular proteins from moss extracts, but
582 experienced a substantial loss of L1 in the flow through. To address this, further optimization of
583 purification might be achieved by combinations with other methods, such as heparin chromatography.
584 Our dot blot analysis confirmed the presence of intact capsomers or VLPs in extracts and after AMS
585 precipitation, suggesting heparin chromatography as an option for the second step in purification.

586 TEM analysis of purified and reassembled CTP-pL1 samples shows VLPs of ca. 60 nm in size. These
587 nanoparticles assembled from capsomers as a result of the reassembly treatment. To the best of our
588 knowledge, this is the first report of VLPs produced in a non-vascular plant and of *in vitro* reassembly of
589 HPV-16 L1 VLPs from capsomers produced in any plant system. The VLPs are heterogenous in shape and
590 size. The same is true for yeast-derived VLPs after reassembly, whereas untreated VLPs were not
591 heterogenous. This is surprising, since controlled dis- and reassembly of VLPs can enhance stability and
592 uniformity (McCarthy et al., 1998; Mach et al., 2006; Shi et al., 2007). Besides high ionic strength and low
593 pH buffer, inter-pentameric disulfide bridges positively influence VLP stability and uniformity (Buck et al.,
594 2005). This could be achieved through prolonged incubation of cleared cell lysates before purification or

595 addition of oxidizing agents, which accelerate disulfide bridge formation. However, prolonged incubation
596 causes aggregation of HPV-16 L1 VLPs, as observed here, but could be addressed by improved formulation
597 (Shi et al., 2005).

598 In conclusion, we generated a CDS optimized for expression of HPV-16 L1 in Physcomitrella. We
599 further characterized five CTPs and confirmed the localization of L1 in chloroplasts. In addition, we
600 demonstrated chloroplast localization to yield higher L1 levels than targeting to nuclei or cytoplasm.
601 Cultivation in photobioreactors yielded more than 0.3 mg/g FW, which is in the range of other plant-based
602 expression platforms. We established a purification protocol and demonstrated the successful assembly
603 of moss-produced HPV-16 L1 VLPs. Future efforts should focus on further enhancing purification and
604 formulation of pseudovirions for immunological studies of this vaccine candidate.

605

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610

611 Author Contributions

612 PAN acquired funding, designed research, performed experiments, analysed data and wrote the
613 manuscript. MCW and SC performed experiments. CET, MRF and SNWH performed experiments and
614 analysed data. JP and ELD analysed data and helped writing the manuscript. HTS acquired funding and
615 conceptualized the idea. RR designed and supervised research, acquired funding and wrote the
616 manuscript. All authors read and approved the final version of the manuscript.

617

618 Conflict of interest disclosure

619 All authors declare no conflict of interest.

Data availability statement

Nucleotide sequence data was submitted to GenBank under accession numbers PV029542 (pL1) and PV068547(CTPc5). Moss lines and vectors were submitted to the International Moss Stock Centre (IMSC) under accession numbers 40963 (pL1 5A), 40964 (pL1 23A), 40965 (pL1 47B), 40966 (pL1Δ22 8A), 40967 (pL1Δ22 11B), 40968 (pL1Δ22 102B), 40969 (CTP-pL1 30), 40970 (CTP-pL1 103), 40971 (CTP-pL1 143), 1840 (pAct5_linker_eGFP_35ST_hpt), 2038 (pAct5_CTP_linker_eGFP_35ST_hpt), 1193 (pAct5_Citrine_nosT_hpt), 2249 (pAct5_pL1-Citrine_nosT_hpt), 2250 (pAct5_pL1Δ22-Citrine_nosT_hpt), 2251 (pAct5_CTP-pL1-Citrine_nosT_hpt), 1997 (pAct5_pL1_35ST_hpt), 1998 (pAct5_pL1Δ22_35ST_hpt) and 2048 (pAct5_CTP-pL1_35ST_hpt).

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642 ## Short legends for Supporting Information

643 Supplementary Figure S1 **Vector maps**

644 Supplementary Figure S2 **Generation of Physcomitrella-optimized (p)L1**

645 Supplementary Figure S3 **Characterization of CTPs by analysis of subcellular eGFP localization**

646 Supplementary Figure S4 **Analysis of CDS integrity in L1-producing lines**

647 Supplementary Figure S5 **Comparison of biomass accumulation rates**

648 Supplementary Figure S6 **Ammonium sulphate precipitation**

649 Supplementary Figure S7 **Chromatogram of Cation Exchange purification**

650 Supplementary Table S1 **List of primers**

651 Supplementary Table S2 **Predicted chloroplast transit peptides for six selected proteins in**

652 **Physcomitrella**

653 ## References

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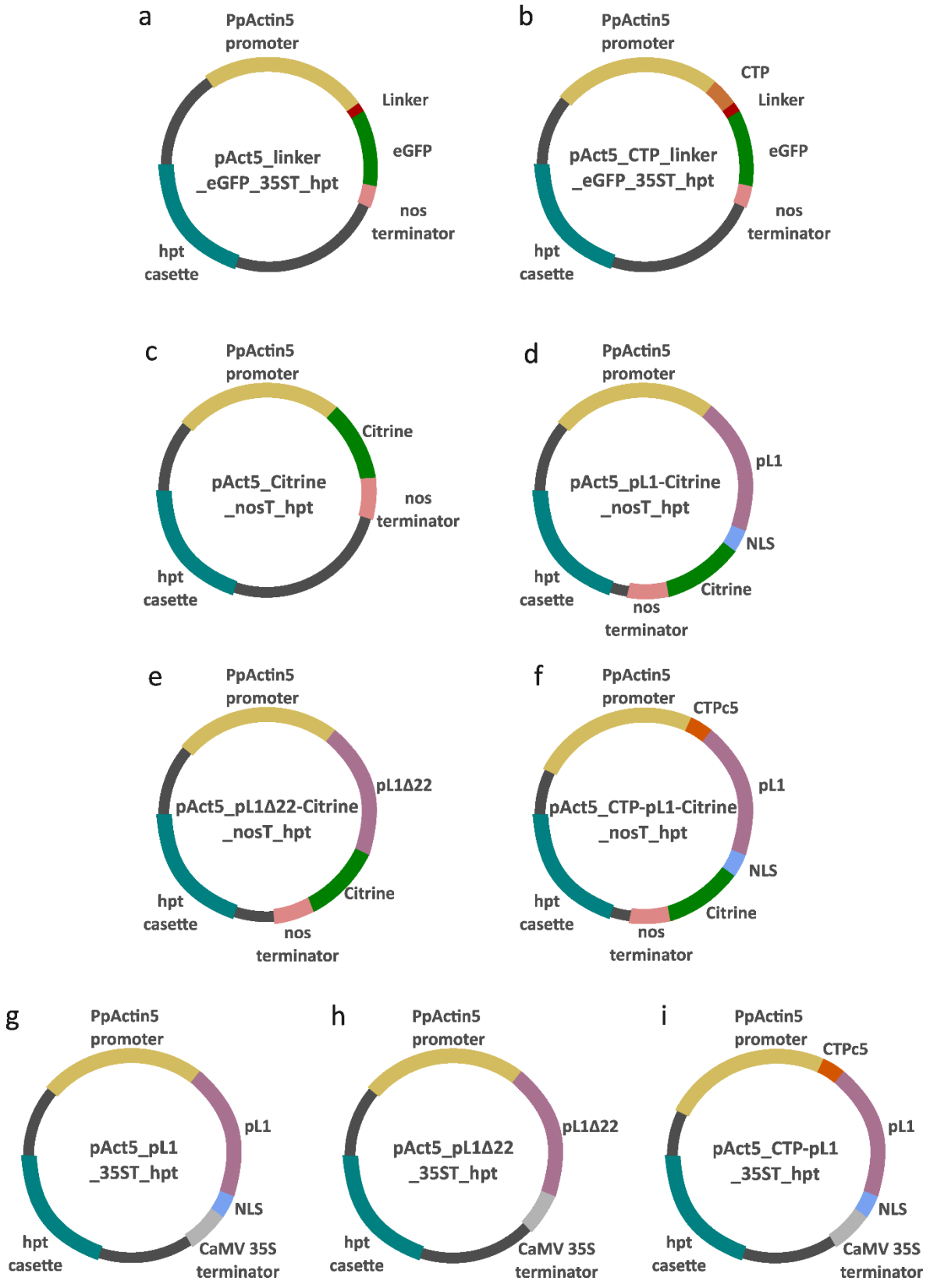
1023 # Supplementary Information

1024 Supplementary Figure S1 – Vector maps



1025

1026 **List of all vectors used in this study.** Vectors **a** & **b** were used for the characterization of chloroplast transit peptides (CTPs).
1027 Vector **a** served as a control and was used as a cloning template for inserting different CTP candidates. Vectors **c**, **d**, **e** & **f** were
1028 used for the analysis of L1 subcellular targeting. Vector **c** served as a control and was used as a cloning template for inserting
1029 either pL1, pL1Δ22 or CTP-pL1 CDS (**d**, **e** & **f**, respectively). Vectors **g**, **h** & **i** were used for generation of stable transgenic
1030 Physcomitrella lines. The vectors contain either the full length pL1 including the native nuclear localization signal (NLS) (**g**), a
1031 truncated version lacking the NLS (**h**), or the full-length version including the native NLS and an N-terminal chloroplast transit
1032 peptide (**i**). All vectors contain the PpActin5 promoter (yellow), the CaMV 35S terminator (grey), and a hygromycin
1033 (hpt)(turquoise) resistance cassette for selection. Other elements are the GGGGGA linker (red), CTP (orange), NLS (blue) and eGFP
1034 or Citrine (green). Genetic elements are not depicted true to scale. Vector sizes: 7260 bp (**a**), 7461 bp (**b**), 7299 bp (**c**), 8802 bp
1035 (**d**), 8736 bp (**e**), 9021 bp (**f**), 7998 bp (**g**), 7932 bp (**h**), 8217 bp (**i**).

Supplementary Figure S2 - Generation of Physcomitrella-optimized (p)L1

HPV-16 hL1

ATGTCCCTGTGGCTGCCCAGCGAGGCCACCGTGTACCTGCCCCCCGTGCCCGTGAGCAAGGTGGTGAGCACCGATGAGTACGTGGCCCGGA
CCAACATCTACTACCACGCCGGCACCTCCAGACTGCTGGCCGTGGGCCACCCCTACTTCCCCATCAAGAAGCCCAACAACAACAAGATCCTGG
TGCCCAAGGTGAGCGGCCTGCAATACCGGGTGTTCAGAATCCACCTGCCCGACCCCAATAAGTTCGGCTTCCCCGACACCAGCTTCTACAACC
CCGACACCCAGAGACTGGTGTGGGCCTGCGTGGGCGTGGAGGTGGGCAGAGGCCAGCCTCTGGGCGTGGGCATCAGCGGCCACCCTCTGC
TGAACAAGCTGGACGACACCGAGAACGCCAGCGCCTACGCCGCCAACGCCGGCGTGGATAACAGAGAATGCATCAGCATGGACTACAAGCA
GACCCAGCTGTGCCTCATCGGCTGCAAGCCCCCCATCGGCGAGCACTGGGGCAAGGGCAGCCCCTGCACCAACGTGGCCGTGAATCCTGGC
GACTGTCCTCCCCTGGAACTCATCAACACCGTGATCCAGGACGGCGACATGGTGGACACCGGCTTCGGCGCCATGGACTTCACCACCCTCCAG
GCCAATAAGAGCGAGGTGCCCCTGGACATCTGCACCAGCATCTGCAAGTACCCCGACTACATCAAGATGGTGAGCGAGCCCTACGGCGATAG
CCTGTTCTTCTACCTGCGGCGGGAGCAGATGTTCGTGCGGCACCTGTTCAACAGAGCCGGCGCCGTGGGCGAGAACGTGCCCGACGACCTGT
ACATCAAGGGCAGCGGCAGCACCGCCAACCTGGCCAGCAGCAACTACTTCCCTACCCCCAGCGGCTCCATGGTGACCAGCGACGCCCAGATC
TTCAACAAGCCCTACTGGCTCCAGAGAGCCCAGGGCCACAACAATGGCATCTGCTGGGGCAACCAGCTGTTCGTGACCGTGGTGGATACCAC
CCGGAGCACCAACATGTCCCTGTGCGCCGCCATCAGCACCAGCGAGACCACCTACAAGAACACCAACTTCAAGGAGTACCTGAGGCACGGCG
AGGAGTACGACCTCCAGTTCATCTTCCAGCTGTGCAAGATCACCCTCACCGCCGACGTGATGACCTACATCCACAGCATGAACAGCACCATCC
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ACCAGTTCCCTCTGGGCAGAAAGTTCCTGCTGCAAGCCGGCCTGAAGGCCAAGCCTAAGTTCACCCTGGGCAAGAGAAAGGCCACCCCCACC
ACAAGCAGCACCAGCACCACCGCCAAGCGGAAGAAGCGCAAGCTGTGA

HPV-16 pL1

ATGTCCCTGTGGCTGCCCAGCGAGGCCACCGTGTACCTGCCCCCCGTGCCCGTGAGCAAAGTGGTGAGCACCGATGAGTACGTGGCCCGGAC
CAACATCTACTACCACGCCGGCACCTCCAGACTGCTGGCCGTGGGCCACCCCTACTTCCCCATCAAGAAGCCCAACAACAACAAGATCCTGGT
GCCCAAAGTGAGCGGCCTGCAGTACCGGGTGTTCAGAATCCACCTGCCCGACCCCAACAAGTTCGGCTTCCCCGACACCAGCTTCTACAACCC
CGACACCCAGAGACTGGTGTGGGCCTGCGTGGGCGTGGAAGTGGGCAGAGGCCAGCCTCTGGGCGTGGGCATCAGCGGCCACCCTCTGCT
GAACAAGCTGGACGACACCGAGAACGCCAGCGCCTACGCCGCCAACGCCGGCGTGGATAACAGAGAGTGCATCAGCATGGACTACAAGCA
GACCCAGCTGTGCCTCATCGGCTGCAAGCCCCCCATCGGCGAGCACTGGGGCAAGGGCAGCCCCTGCACCAACGTGGCCGTGAACCCTGGC
GACTGCCCTCCCCTGGAGCTCATCAACACCGTGATCCAGGACGGCGACATGGTGGACACCGGCTTCGGCGCCATGGACTTCACCACCCTCCA
GGCCAACAAGAGCGAAGTGCCCCTGGACATCTGCACCAGCATCTGCAAGTACCCCGACTACATCAAGATGGTGAGCGAGCCCTACGGCGATA
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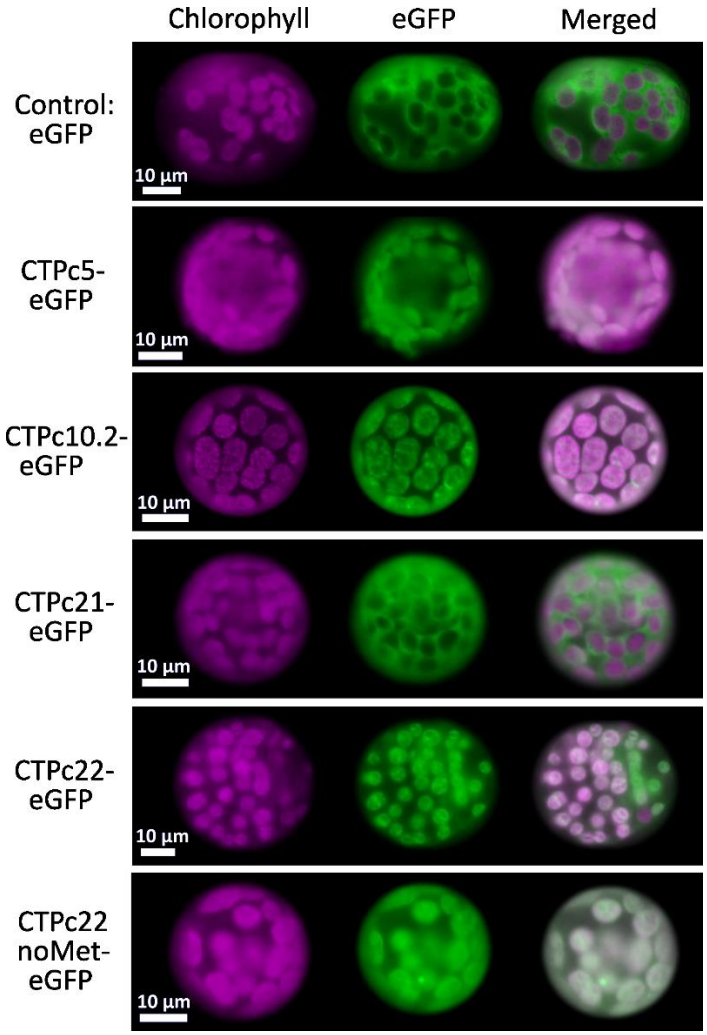
CTTCAACAAGCCCTACTGGCTCCAGAGAGCCCAGGGCCACAACAACGGCATCTGCTGGGGCAACCAGCTGTTCGTGACCGTGGTGGATACCA
CCCGGAGCACCAACATGTCCCTGTGCGCCGCCATCAGCACCAGCGAGACCACCTACAAGAACACCAACTTCAAGGAGTACCTGAGGCACGGC
GAGGAGTACGACCTCCAGTTCATCTTCCAGCTGTGCAAGATCACCCTCACCGCCGACGTGATGACCTACATCCACAGCATGAACAGCACCATC
CTGGAGGACTGGAACTTCGGCCTGCAGCCCCCTCCTGGCGGCACCCTGGAGGACACCTACAGATTCGTGACCAGCCAGGCCATCGCATGCCA
GAAGCACACCCCTCCCGCCCCTAAGGAGGACCCCCTGAAGAAGTACACCTTCTGGGAAGTGAACCTGAAGGAGAAGTTCAGCGCCGACCTG
GACCAGTTCCCTCTGGGCAGAAAGTTCCTGCTGCAGGCCGGCCTGAAGGCCAAGCCTAAGTTCACCCTGGGCAAGAGAAAGGCCACCCCCAC
CACAAGCAGCACCAGCACCACCGCCAAGCGGAAGAAGCGCAAGCTGTGA

1074 **Human codon-optimized HPV-16 (h)L1 CDS and Physcomitrella-optimized HPV-16 (p)L1 CDS.** Grey marked nucleotides were
1075 changed in HPV-16 pL1 through codon usage optimization by the tool physCO (Top et al., 2021). Turquoise marked motifs refer
1076 to the Physcomitrella alternative splicing recognition sites. The green marked segment refers to a predicted miRNA binding site
1077 identified by the tool psRNATarget (Dai & Zhao, 2011; Dai et al., 2018). Light orange coloured segments represent the nuclear
1078 localization signal. Nucleotide changes are underlined in the HPV-16 pL1 sequence. (hL1, GenBank accession no. DQ067889,
1079 (Maclean et al., 2007))

1080 Supplementary Figure S3 - Characterization of CTPs by analysis of subcellular eGFP

1081 localization

1082



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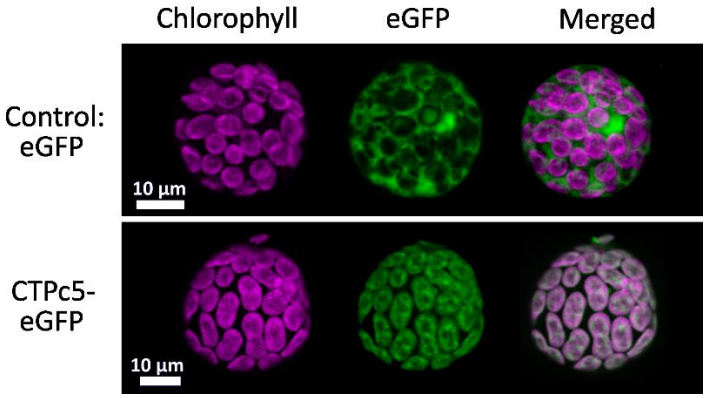

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1086 **Microscopy pictures of Physcomitrella protoplasts transformed with CTP-eGFP constructs.** Samples were analysed using a
1087 rhodamine and a FITC filter to identify chloroplast (magenta) and eGFP localization, respectively, as well as a merge of the two
1088 signals. For sample CTPc21-eGFP no eGFP signal could be observed in chloroplasts. For samples CTPc5-eGFP, CTPc10-eGFP,
1089 CTPc22-eGFP and CTPc22noMet-eGFP green fluorescence signal is observed in chloroplasts. eGFP lacking a CTP localized to the
1090 cytoplasm and served as a negative control. The experiment was repeated two times. CTPc5 was examined a third time using
1091 confocal microscopy. Scale bars represent 10 µm.

1092 ## Supplementary Figure S4 – Analysis of CDS integrity in L1-producing lines

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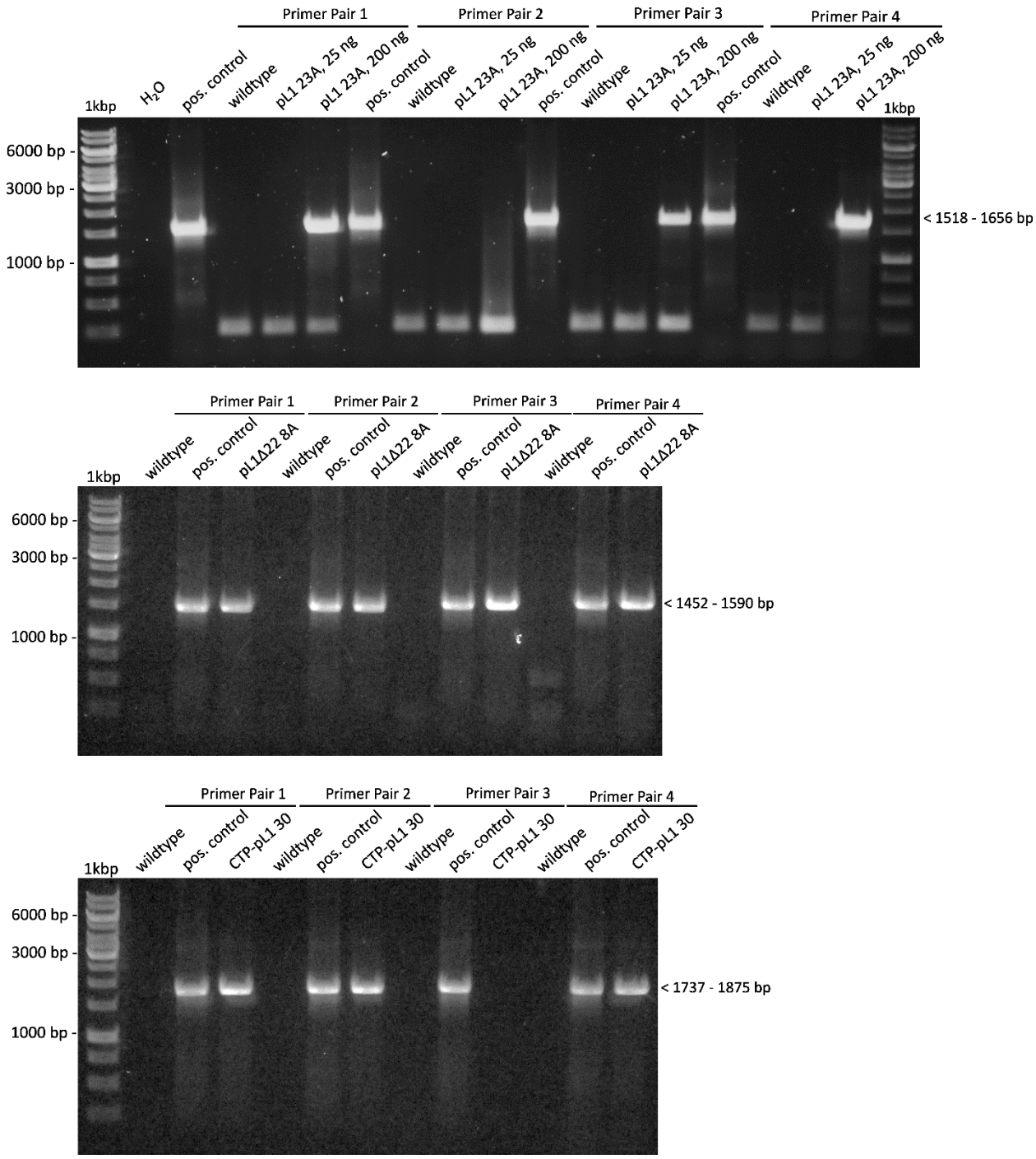

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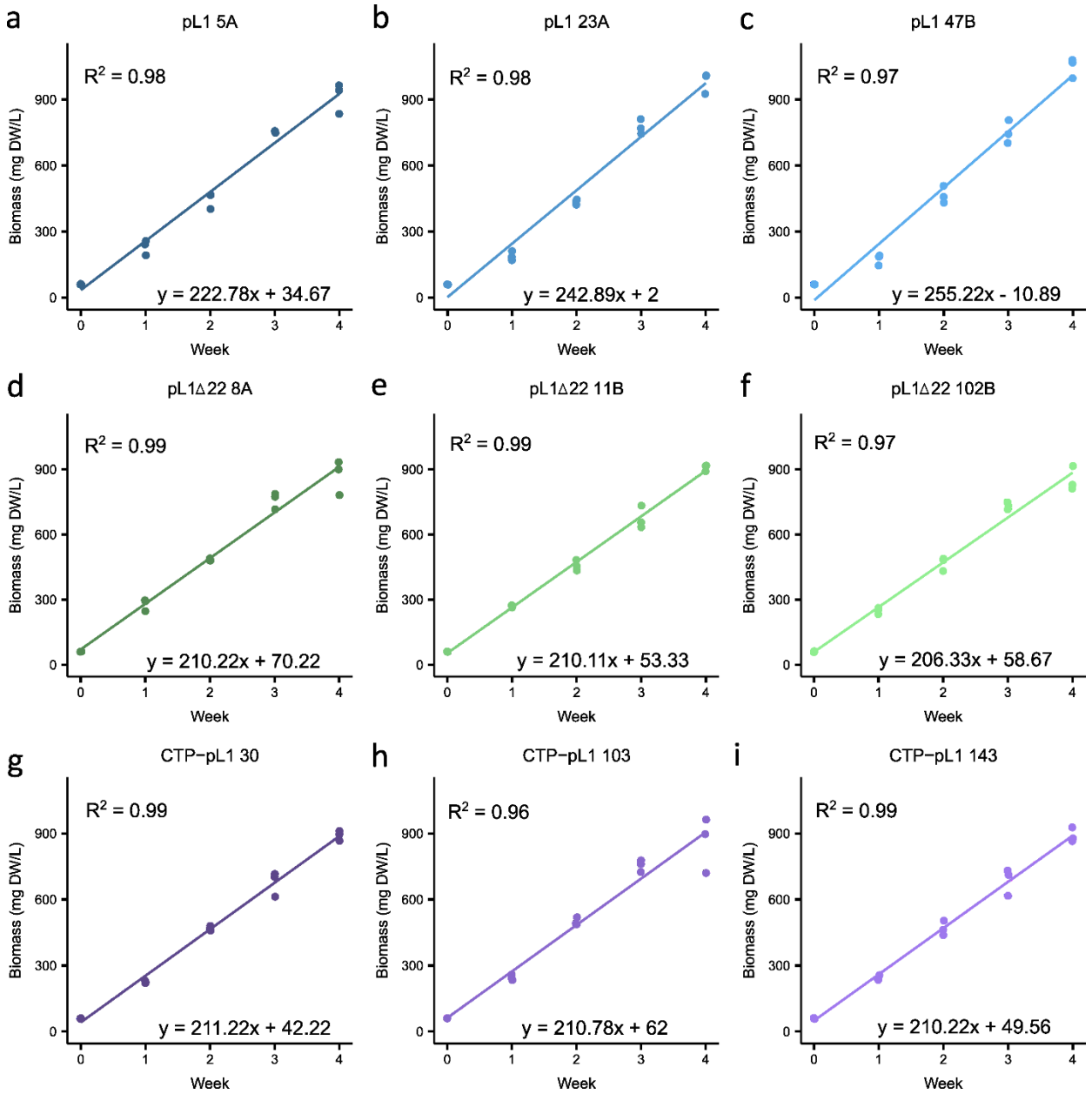
1096 **Analysis of rearrangements in the pL1 CDS using RT PCR.** Lines pL1 23A, pL1Δ22 8A and CTP-pL1 30 were checked for DNA or
1097 RNA rearrangements by performing PCR on cDNA using four different primer pairs. Primer pairs were selected to amplify the
1098 entire cDNA. The forward primer binds at the start codon and is the same in each primer pair (pL1_fwd_1 for lines pL1 23A &

1099 pL1Δ22 8A, CTP_pL1_fwd_1 for line CTP-pL1 30). The reverse primers are different in each pair and bind at the 3' end of the CDS
1100 or within the terminator (**Supplementary Table S1**). For line pL1 23A usage of 25 ng or 200 ng cDNA in PCR reactions was
1101 compared. Following experiments used 200 ng. Absence of distinct bands of a size smaller than the expected size is indicative for
1102 no larger rearrangements on both DNA and RNA level. No amplicon is visible for lines pL1 23A using Primer Pair 2 and CTP-pL1 30
1103 using Primer Pair 3. The constructs used for transformation of Physcomitrella protoplasts (**Supplementary Figure S1 g - i**) served
1104 as a positive control.

1105 ## Supplementary Figure S5 – Comparison of biomass accumulation rates

1106

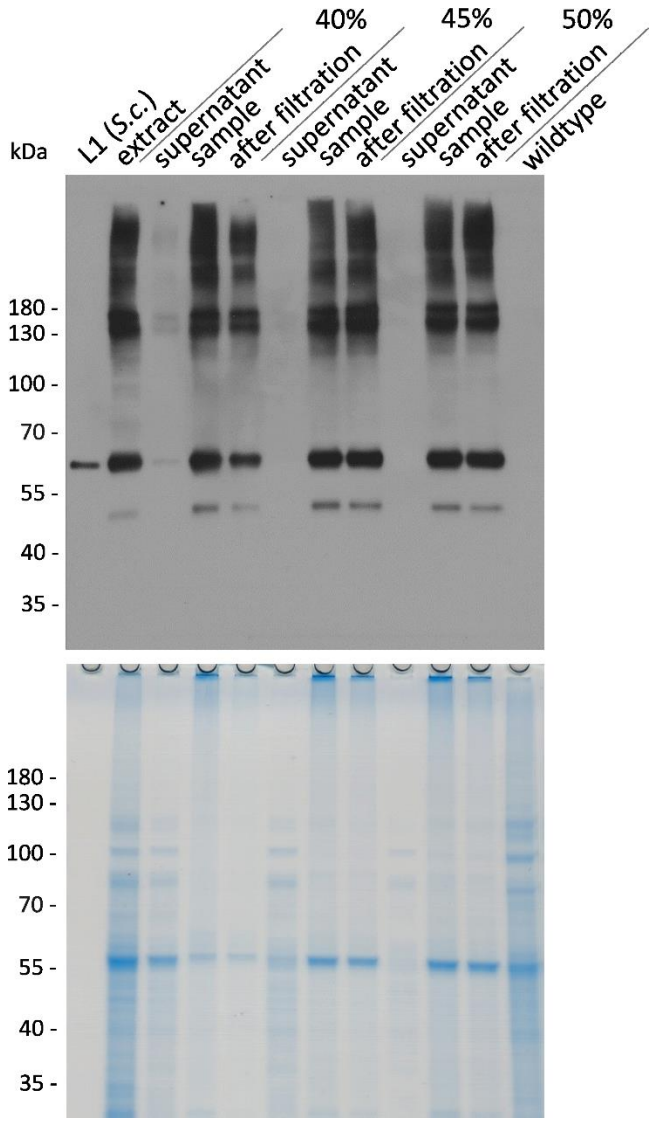


1107

1108 **Analysis of biomass accumulation rates among nine Physcomitrella lines of three different constructs.** Biomass accumulation
1109 rates were observed over a course of 4 weeks for lines pL1 5A, pL1 23A, pL1 47B (blue); pL1Δ22 8A, pL1Δ22 11B, pL1Δ22 102B
1110 (green) and CTP-pL1 30, CTP-pL1 103, CTP-pL1 143 (purple). The data was plotted using linear regression analysis and all slopes
1111 were analysed using ANOVA and post hoc multiple comparisons test (TukeyHSD). Comparison of the slopes between the
1112 constructs of pL1, pL1Δ22 and CTP-pL1 showed pL1 lines accumulated biomass faster than pL1Δ22 ($p = <0.0001$) and CTP-pL1 (p
1113 $= 0.0001$) lines. Per week $n = 3$ biological samples were taken.

1114 ## Supplementary Figure S6 – Ammonium sulphate precipitation

1115



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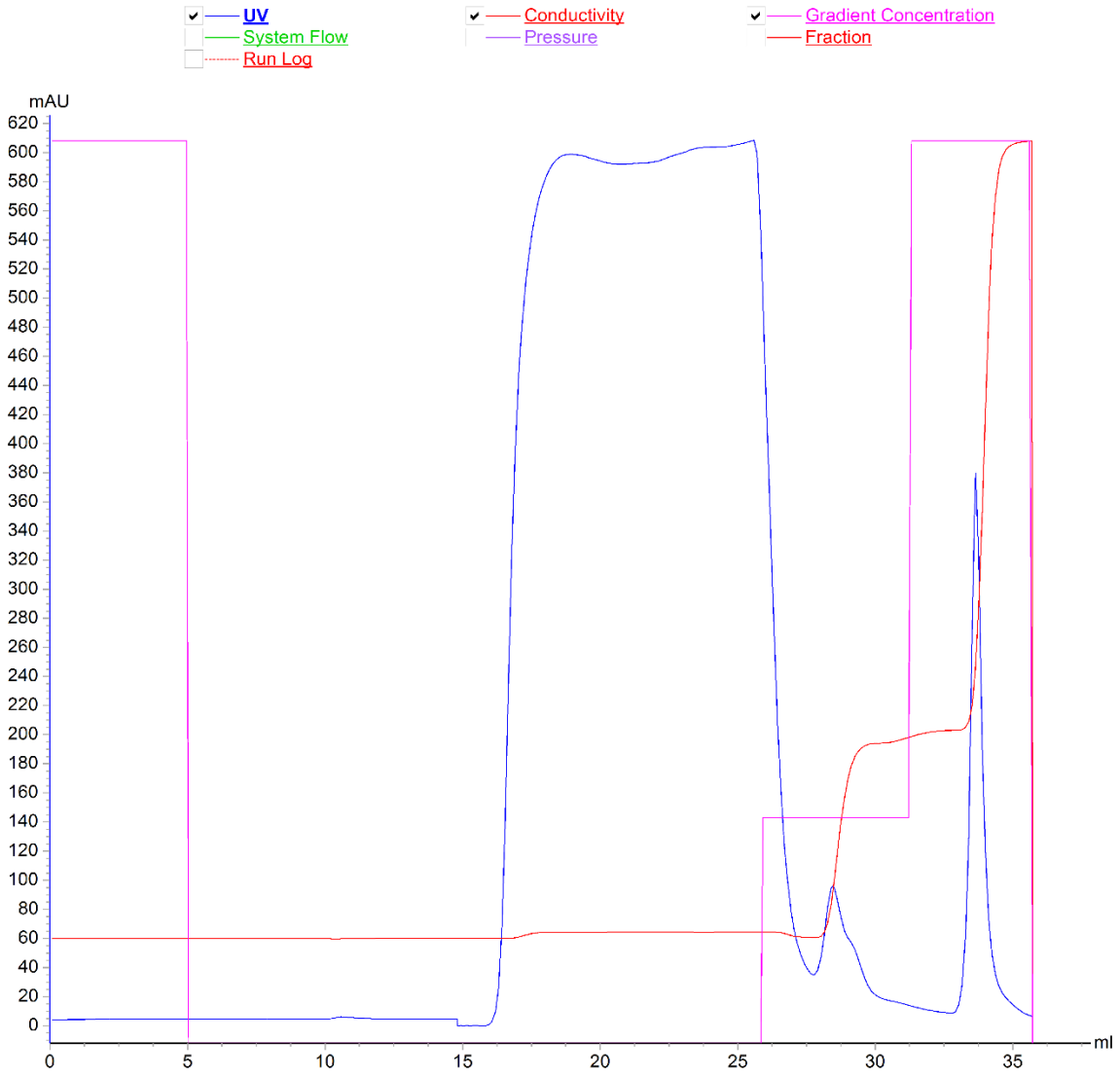
1118 **Ammonium sulphate precipitation of L1 from moss extracts.** Samples from the supernatant and the resuspended and dialysed
1119 pellet before and after filtration were analysed for the presence of L1 content and residual host proteins under reducing
1120 conditions *via* Western blot and Coomassie-stained gel, respectively. CTP-pL1 30 extracts were precipitated with either 40%, 45%,
1121 or 50% ammonium sulphate (AMS). After centrifugation, the supernatant and the precipitate were taken up and dialysed against
1122 cation exchange chromatography binding buffer to remove excess AMS. Non-dissolved protein was cleared from the sample using
1123 0.22 µm filters. 45% AMS was found to be sufficient for full precipitation of L1 from crude extracts. 30 ng *S. cerevisiae*-produced
1124 L1 served as a positive control and Physcomitrella wildtype as a negative control.

Supplementary Figure S7 – Chromatogram of Cation Exchange purification



Chromatogram of HPV-16 L1 purification from moss using cation exchange chromatography. The chromatogram shows the UV absorbance, indicative for protein concentration, in blue, gradient concentration in pink, and conductivity, indicative for salt concentration, in red. The y axis depicts milli-Absorbance Units and the x axis depicts the volume in mL. The large peak between 16 – 26 mL shows the sample application or flow through, the following small peak at 28 mL the wash fraction and the larger peak at 33 – 35 mL the elution fractions.

1133 ## Supplementary Table S1 – List of primers

1134 Capital letters refer to the part of the primer binding to the template whereas lower case letters represent overhangs added to
1135 the template via PCR. Abbreviations: fwd or fw (forward), rev or rv (reverse), CTP (chloroplast transit peptide), MMEJ (micro
1136 homology-mediated end joining), GFP (green fluorescent protein), OH (overhang (lower case letters)).

Primer name	Sequence (5'→3')	Application
AA2_3integFP (hpt3'seq)	ACTCGCCGATAGTGGAAACC	Amp of linker-eGFP vector part 1
PAN_PpActin-Pro_Rv	CTGCACAACAACCACCAATATTTAATTTC	Amp of linker-eGFP vector part 1
linker:EGFP_p1840_Fw	GGAGGTGGAGGTGGAGC	Amp of linker-eGFP vector part 2
HPTLUCfwd2	TTGCCCTCGGACGAGTGC	Amp of linker-eGFP vector part 2
CTP_Pp3c10_Fw	aattaaatattggtggttgttgtgcagATGGCGTTATTGGCGCGC	Amp of CTPc10
CTP_Pp3c10_Rv	gctccacctccacctccATTGGTGACCCTCAAAGGTCCAG	Amp of CTPc10
CTP_Pp3c5_Fw	aattaaatattggtggttgttgtgcagATGGCTACGATGAAGATTGCTGTGG	Amp of CTPc5
CTP_Pp3c5_Rv	gctccacctccacctccAGCTCGCACGGAGCTGTTC	Amp of CTPc5
CTP_Pp3c22_Fw	aattaaatattggtggttgttgtgcagATGCGGGCTCTCTGTGGAG	Amp of CTPc22 & CTPc22_noMet
CTP_Pp3c22_Rv	gctccacctccacctccCATAGCGACAATGCACCGTCTTC	Amp of CTPc22
CTP_Pp3c22_noMet_Rv	gctccacctccacctccAGCGACAATGCACCGTCTTCC	Amp of CTPc22_noMet
CTP_Pp3c21_Fw	aattaaatattggtggttgttgtgcagATGGCCGCCGTCGGAATG	Amp of CTPc21
CTP_Pp3c21_Rv	gctccacctccacctccGGCACGGACAGCGAACGC	Amp of CTPc21
Act5_seq	TCCACATGGCTACAGCTG	Colony PCR CTP-eGFP constructs
EGFP_NoLinker_Rev	CTTGTACAGCTCGTCCATG	Colony PCR CTP-eGFP constructs
XhoI-pL1-BamHI_fw	gtgcagctcgagATGTCCCTGTGGCTGCC	Amp of pL1 and pL1Δ22
XhoI-pL1-BamHI _Rv	tctagaggatccTCACAGCTTGCGCTTCTTCC	Amp of pL1
XhoI-pL1delta22-BamHI _Rv	tctagaggatcctcaGCCCAGGGTGAACTTAGGC	Amp of pL1Δ22
pL1_w/oATG_Fw	TCCCTGTGGCTGCCCAG	Amp of CTP-pL1 (no Start codon)
pL1_Rv_35St-OH	GACTGGTGATTTTTGCGGACTCTAG	Amp of pL1, Heterosplicing and MMEJ check of pL1 lines

CTPc5_Fw_Act5p-OH	tattggtggttgttgtgcagctcgagATGGCTACGATGAAGATTGCTGTG	Amp of CTPc5
CTPc5_Rv_pL1-OH	ctcgctgggcagccacagggaAGCTCGCACGGAGCTGTTC	Amp of CTPc5
Seq_hL1+HSP_Fw	CGTGATGACCTACATCCACAG	Colony PCR pL1 constructs
pL1_Rv_35St-OH	GACTGGTGATTTTTGCGGACTCTAG	Colony PCR pL1 constructs
pL1_fwd_1	ATGTCCCTGTGGCTGCCC	Heterosplicing and MMEJ check of pL1, pL1Δ22 lines (primer pair 1-4, forward)
pL1_rev_1	TCACAGCTTGCGCTTCTTCC	Heterosplicing and MMEJ check of pL1, CTP-pL1 lines (primer pair 1, reverse)
pL1d22_rev_1	TCAGCCCAGGGTGAACTTAGG	Heterosplicing and MMEJ check of pL1Δ22 lines (primer pair 1, reverse)
CTP-pL1_fwd_1	ATGGCTACGATGAAGATTGCTGTG	Heterosplicing and MMEJ check of CTP-pL1 lines (primer pair 1-4, forward)
pL1_Rv_35St-OH	GACTGGTGATTTTTGCGGACTCTAG	Heterosplicing and MMEJ check of pL1, pL1Δ22, CTP-pL1 lines (primer pair 2, reverse)
101_35S-T_rev	CCCTTATCTGGGAACTACTCACACATTATTCTGGA	Heterosplicing and MMEJ check of pL1, pL1Δ22, CTP-pL1 lines (primer pair 3, reverse)
3342_LdC-HATag-35ST-rev	GGGTTTCGCTCATGTGTTGAGCA	Heterosplicing and MMEJ check of pL1, pL1Δ22, CTP-pL1 lines (primer pair 4, reverse)
pL1-fwd	GCTCGAGATGTCCCTGTG	pL1(Δ22)-GFP fusion construct
pL1-rev	AGGATCCCAGCTTGCGCTTCTTC	(CTP-)pL1-GFP fusion construct
pL1d22-rev	AGGATCCGCCCAGGGTGAACTTAG	pL1Δ22-GFP fusion construct
CTPc5-pL1 fwd	GCTCGAGATGGCTACGATGA	CTP-pL1-GFP fusion construct

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1138

1139 Supplementary Table S2 – Predicted chloroplast transit peptides for six selected proteins
1140 in Physcomitrella (the predictions were performed with TargetP-2.0; Armenteros et al., 2019)
1141

Name: CTPc5

Protein: **Pp3c5_1370V3.1**

Predicted chloroplast transit peptide (74 aa):

MATMKIAVAAVAGTPAAAVGTRSAAATETARLCASSAQGLGLSTFVGLRAGSNASRGVTPRVRPLKAENSSVRA

Name: CTPc10.1

Protein: **Pp3c10_10490V3.1**

Predicted chloroplast transit peptide (63 aa):

MEVLPRAIATSHVVVSSSSAPKESSESGSKNNSQVSLSSVPFLRPASALAASIVSSATPAALA

Name: CTPc10.2

Protein: **Pp3c10_2940V3.1**

Predicted chloroplast transit peptide (70 aa):

MALLARGAVAAVAAVGFSNTTSKQGHQNGGSNGVLAFRGNAFCRAVAARTSSVGCQVRVKRGAGPLRVTN

Name: CTPc21

Protein: **Pp3c21_9980V3.1**

Predicted chloroplast transit peptide (43 aa):

MAAVGMTMSLKSSTALKGDFLGAALKQTVAAPKAANVAFAVRA

Name: CTPc22

Protein: **Pp3c22_5470V3.1**

Predicted chloroplast transit peptide (62 aa):

MRALCGVPSCASLSSHRLASPLLAYRACEFALGSSPGLRLEFVGRAVGLQSRSIGRRCIVAM

1142