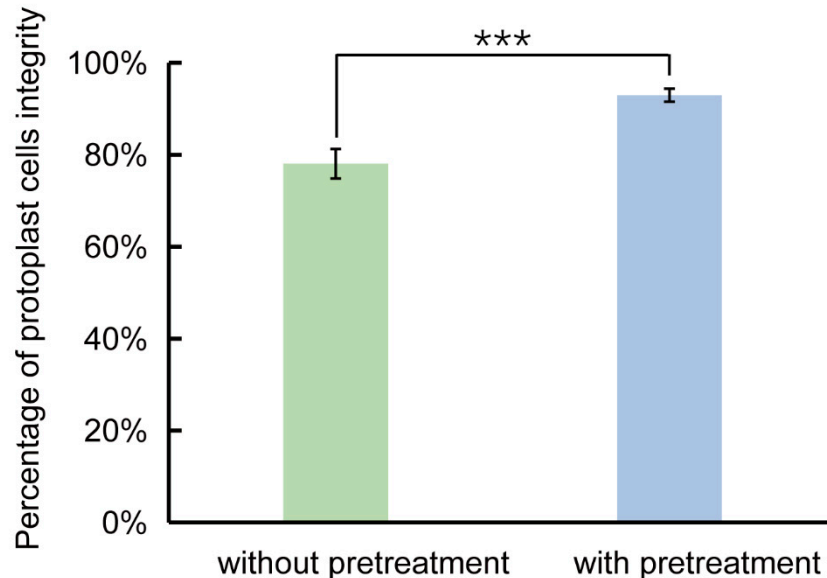


An efficient and universal protoplast isolation protocol suitable for transient gene expression analysis and single-cell RNA sequencing

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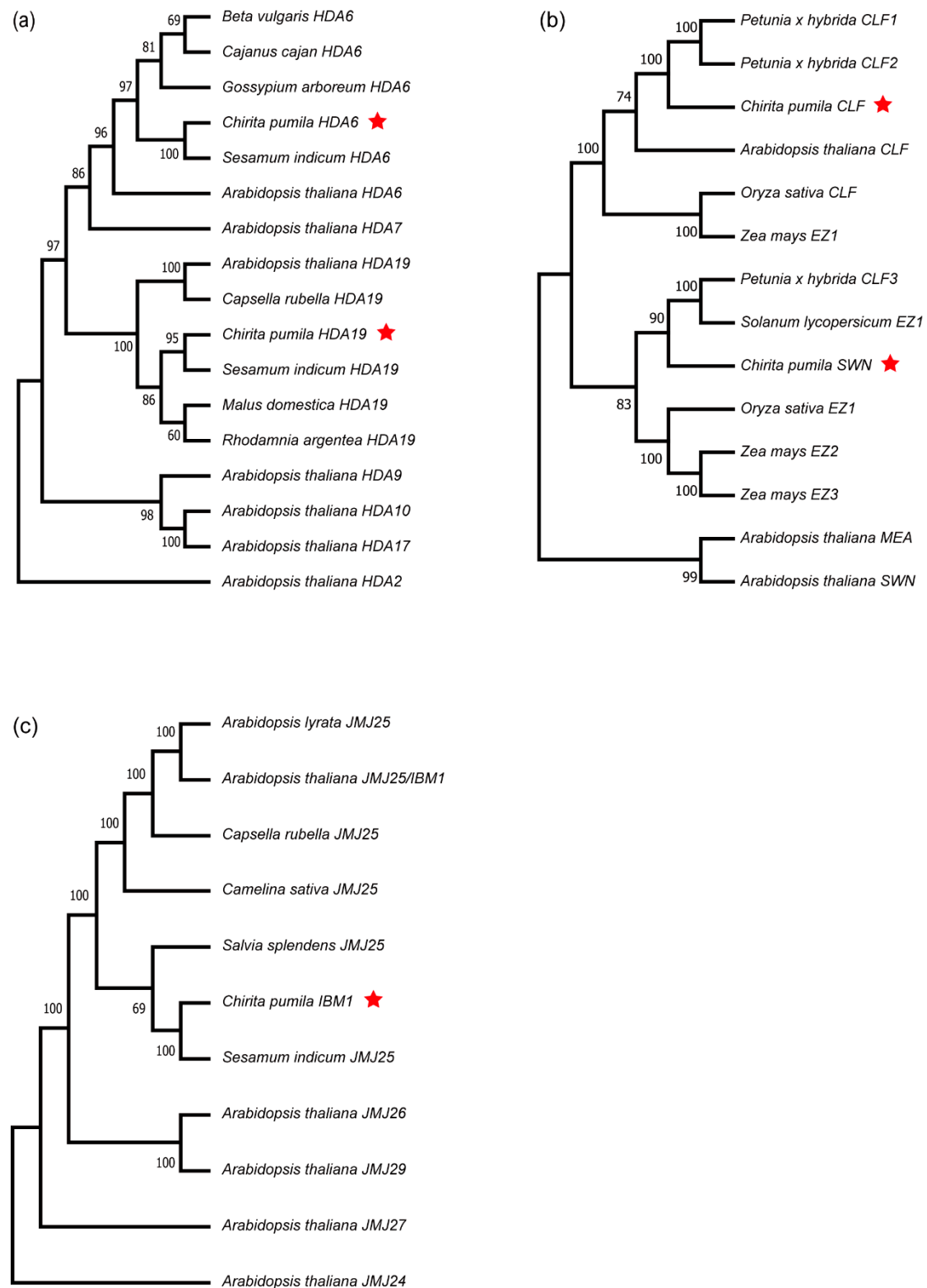
Supplemental Figure S1-S7 and Supplemental Table S1 and S2

Supplemental Figure S1. Statistical analysis of protoplast quality with and without pretreatment procedure



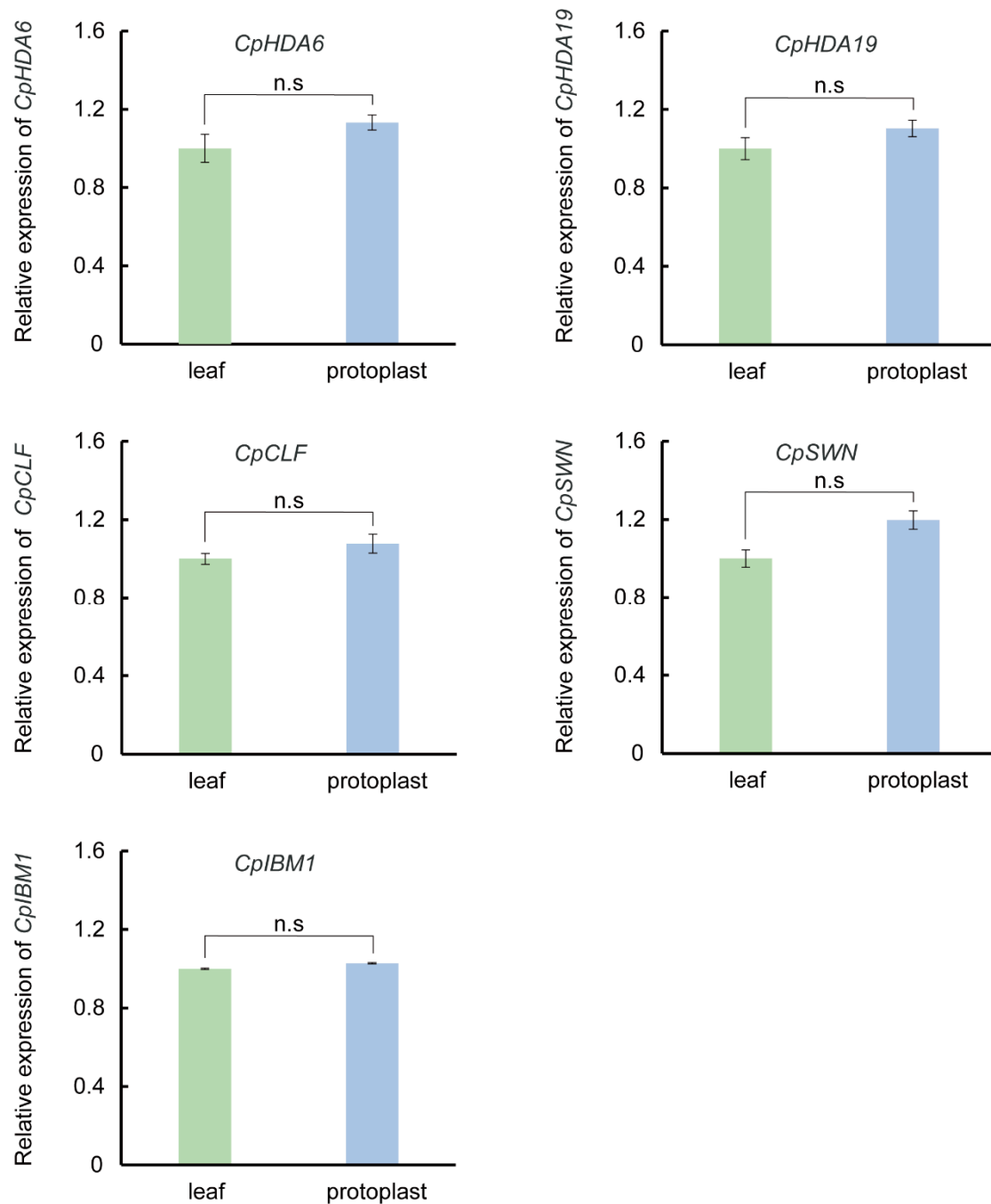
The protoplast quality was evaluated as the percentage of round and green single cells relative to the total protoplasts in the observation field. Please be noted that the protoplasts were immediately subjected to quality check after isolation. The values (mean \pm standard deviation) are the average of three replicates with each replicate containing 10-20 observations. The statistical significance test was performed with Student's *t* test (***, *p* value <0.001).

Supplemental Figure S2. Phylogenetic analysis of genes involved in the epigenetic modification machinery



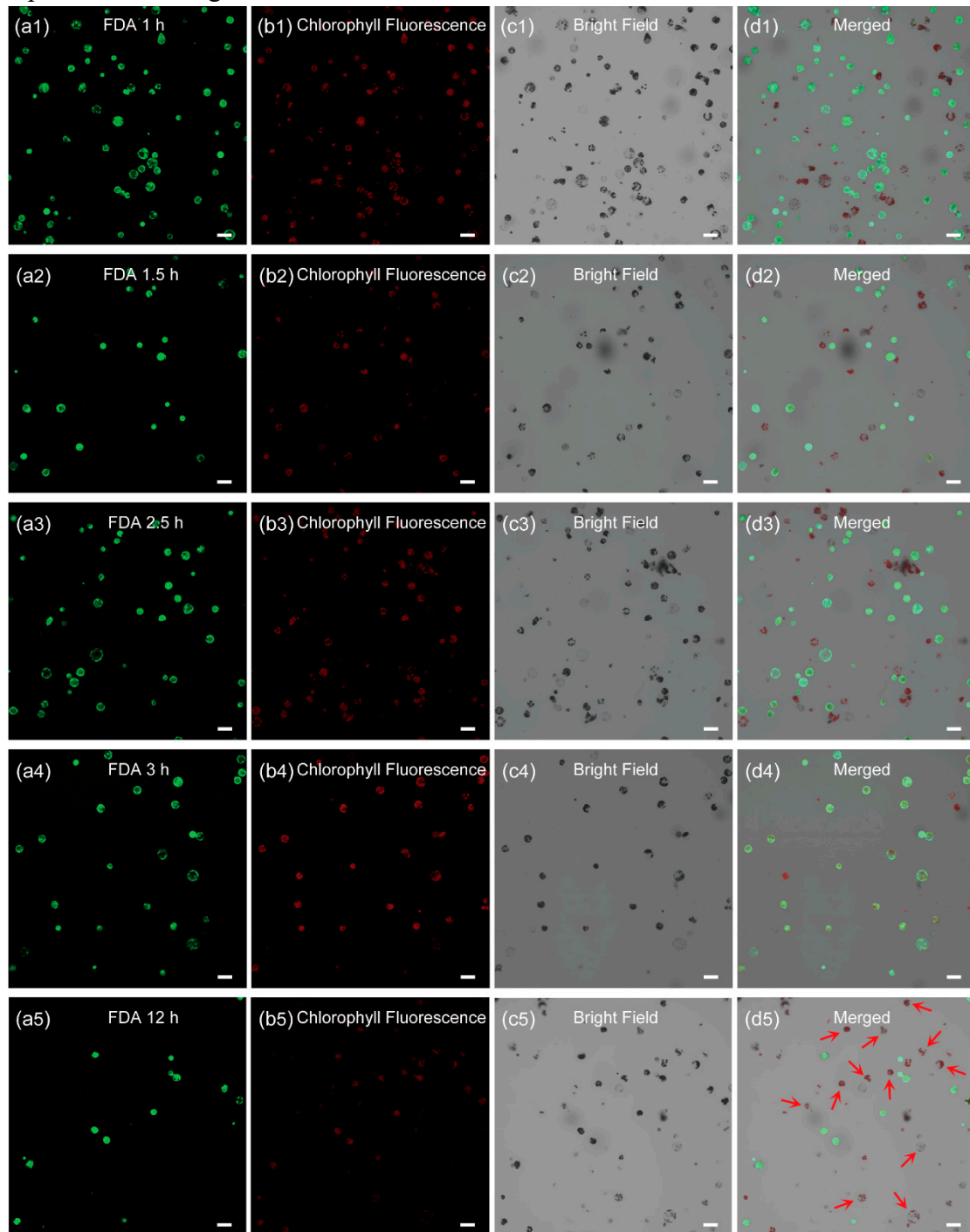
Neighbor-Joining phylogenetic trees of *CpHDA6* and *CpHDA19* (a), *CpCLF* and *CpSWN* (b) and *CpIBM1* (c). The red stars indicate the orthologous sequence of *Arabidopsis* counterpart in *C. pumila*.

Supplemental Figure S3. Expression analysis of genes involved in the epigenetic modification machinery between the digested protoplasts and unprocessed leaf samples in *C. pumila*



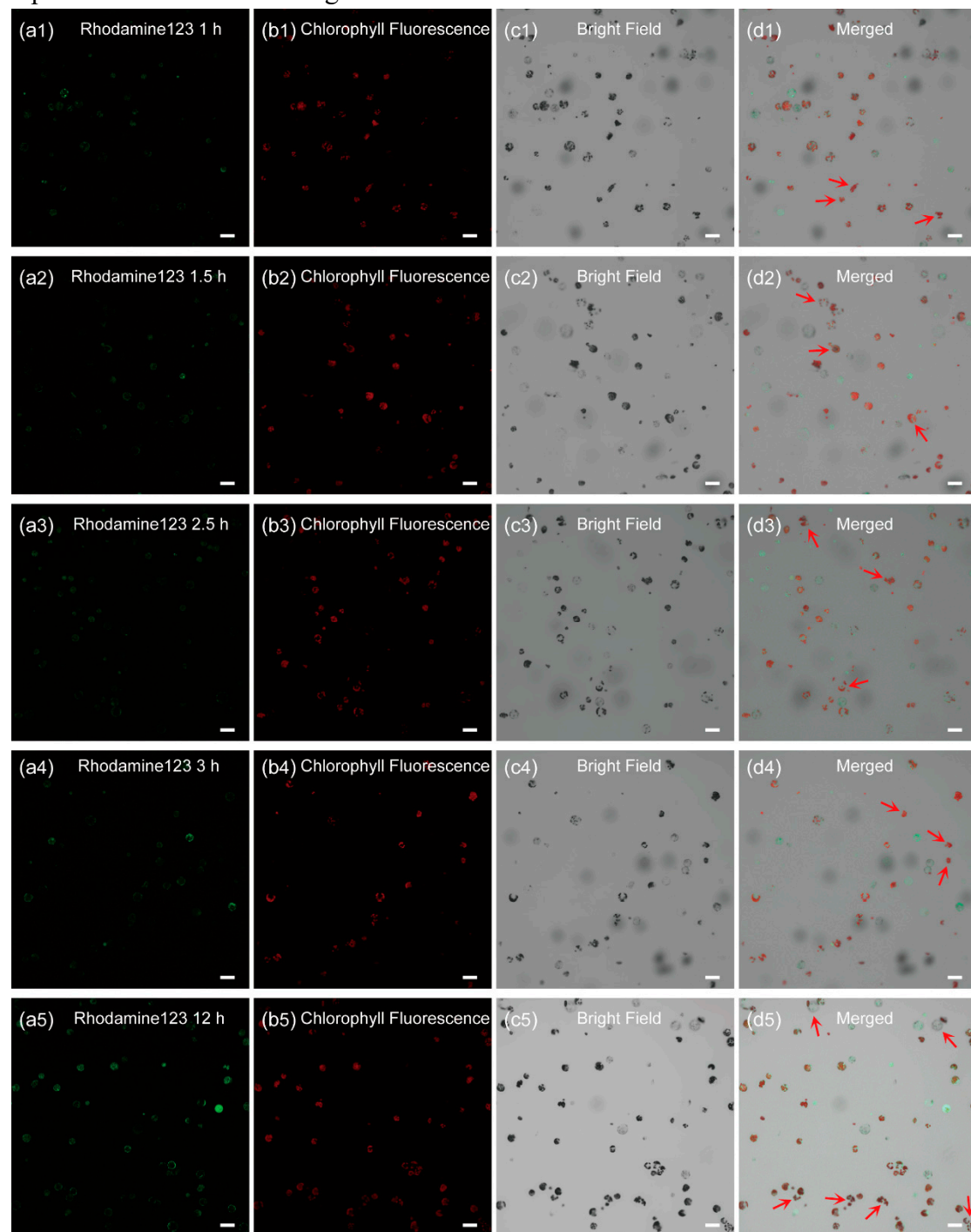
The values shown are the average of three biological replicates. The expression level of each gene in the protoplast were normalized to the relative expression level in the unprocessed leaf samples. The statistical significance test was performed with Student's *t* test (n.s. indicate non-significant, $p > 0.05$).

Supplemental Figure S4. Viability assay of *C. pumila* mesophyll protoplasts by time-lapse FDA staining.



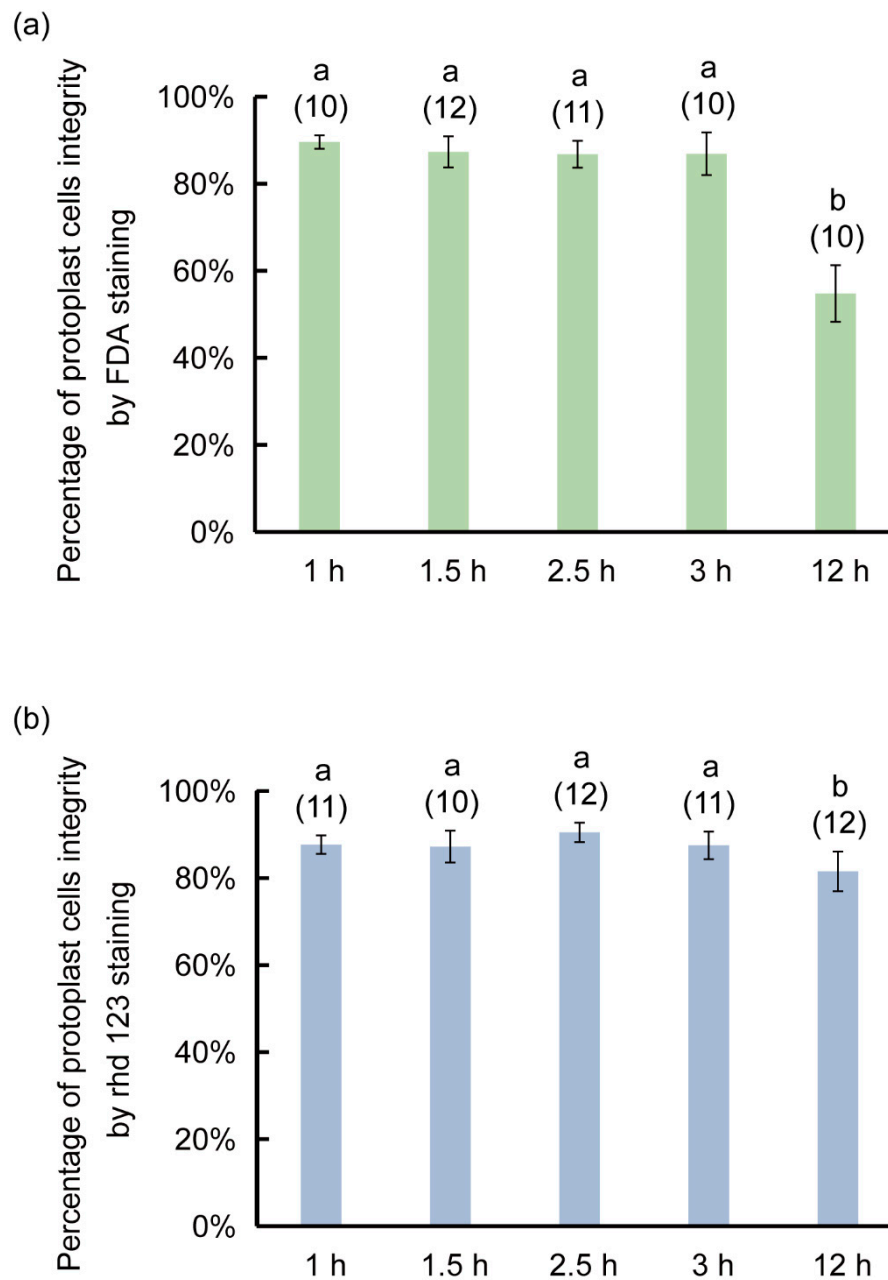
Protoplasts stained with 0.1mg/L FDA were imaged in a time-lapse series (1.0 h, 1.5 h, 2.5 h, 3 h and 12 h). The pictures were taken under GFP channel (a1-a5), chlorophyll auto-fluorescence channel (b1-b5), bright field (c1-c5) and merged channel (d1-d5), respectively. Please be noted that the cell viability was significantly reduced in 12h as indicated the disintegrated cell membrane system (red arrows) possibly due to the existence of acetone in the FDA buffer. Scale bars, 50 μ m.

Supplemental Figure S5. Viability assay of *C. pumila* mesophyll protoplasts by time-lapse rhodamine 123 staining.



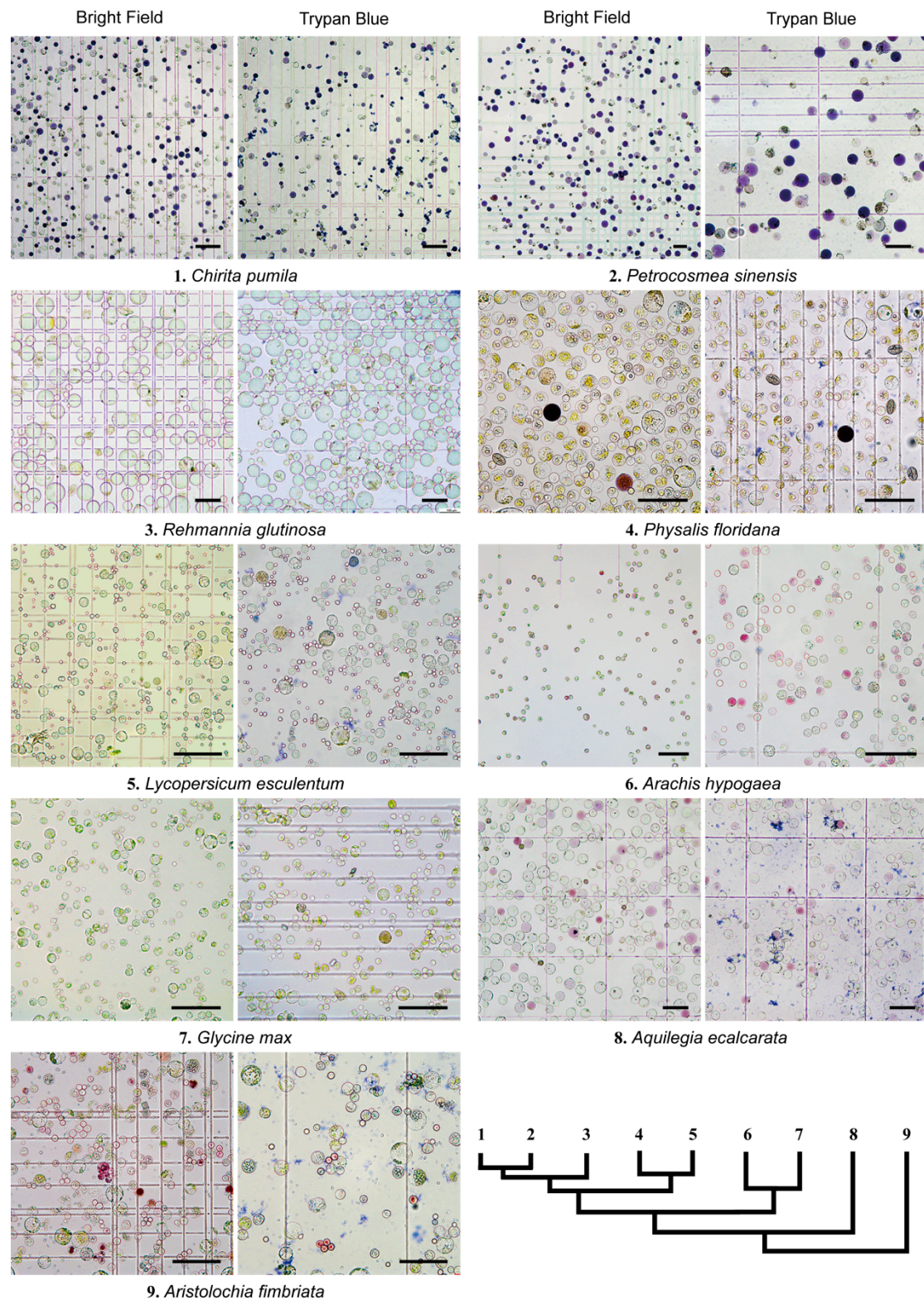
Protoplasts stained with rhodamine 123 (final concentration of 1 μ M) were imaged in a time-lapse series (1.0 h, 1.5 h, 2.5 h, 3 h and 12 h). The pictures were taken under GFP channel (a1-a5), chlorophyll auto-fluorescence channel (b1-b5), bright field (c1-c5) and merged channel (d1-d5), respectively. The red arrows indicate cells without GFP signals, which are associated with disintegrated cell membrane system. Scale bars, 50 μ m.

Supplemental Figure S6. Statistical analysis of protoplast viability with FDA and rhodamine 123 staining, respectively.



The protoplast viability was calculated as the percentage of round cells with GFP signals for FDA staining and clear GFP signals for rhod123 staining, respectively, in a given observation field. The values (mean \pm standard deviation) are the average of three replicates with each replicate containing 10-20 observations. The statistical significance test was performed with Student's *t* test (a, non-significant, $p > 0.05$; b, p value < 0.001 in (a) and p value < 0.05 in (b)).

Supplemental Figure S7. Enlarged images of Figure 6



The phylogenetic relationship of the representative species was based on Angiosperm Phylogeny Group (APG) IV. Please be noted that some of cells are in dark purple or magenta color due to anthocyanin biosynthesis in the samples. Scale bars, 100 μm .

Supplemental Table S1. List of oligos used in this study

Primer name	Sequences (5'-3')	Primer annotation
<i>35S mini</i> (-46bp)	agctctcgag AAGCTT CTCTATATAA -GGAAGTTCATTTTCATTTGGAGAGAACA -CGGGGGACT GGATCC atggtgagca	Plasmid construction
<i>4×ABRE</i>	agctctcgag AAGCTT TGCGTACGTGTT -GTTTGCGTACGTGTTGTTTGCGTACGTGTTG -TTTGCGTACGTGTTGTTTagcttctctatataa	Plasmid construction
<i>4×mABRE</i>	agctctcgag AAGCTT TGCGAATCAATT GTTTGCGAATCAATTGTTTGCGAATCAATTGT -TTGCGAATCAATTGTTTagcttctctatataa	Plasmid construction
<i>AREB1-F</i>	tcccttgctcctg GGATCCTTC -ATGAATTTCCGGGAACG	Plasmid construction
<i>AREB1-R</i>	aatgtttgaacgat CTGCAGCC AGAAT -TGGAGGTACTAAGATAAG	Plasmid construction
<i>p35S-GUS-F</i>	tcccttgctcctg GGATCC GGACTCT -TGACCATGGTAGATCTGAG	Plasmid construction
<i>p35S-GUS-R</i>	aatgtttgaacgat CTGCAGGGTCA CCTG -TAATTCACACG	Plasmid construction
<i>GFP-RT-F</i>	GCTGCTGGGATTACACATGGCATGG	Gene expression analysis
<i>GFP-RT-R</i>	GATAATCATCGCAAGACCGGCAAC	Gene expression analysis
<i>GUS-RT-F</i>	CGCTGGACTGGCATGAACTTCGGTG	Gene expression analysis
<i>GUS-RT-R</i>	GGATCCCCAATTCCCGAGGCTG	Gene expression analysis
<i>CpACT-RT-F</i>	AGTTATCACCATTGCCGCCGAGAGG	Gene expression analysis
<i>CpACT-RT-R</i>	GCAATGCCAGGGAACATAGTCGACC	Gene expression analysis
<i>CpIBM1-RT-F</i>	GGATGACGAGGCTGTGGTAGATG	Gene expression analysis
<i>CpIBM1-RT-R</i>	TCATGTGCACCATTGGTTTTATCACT	Gene expression analysis
<i>CpHDA6-RT-F</i>	GGAACAGGGCACATCAAAGACA	Gene expression analysis
<i>CpHDA6-RT-R</i>	GAATCTGCTCCACATTGAAGAACAAC	Gene expression analysis
<i>CpHDA19-RT-F</i>	GGCAATTCACCTTCATCTGGAAC	Gene expression analysis
<i>CpHDA19-RT-R</i>	CAGTAATCCGTAGTGAGCAAGAAGAGC	Gene expression

<i>CpCLF-RT-F</i>	TTATTGGCTCTGATCGAAGTGTGGA	analysis Gene expression analysis
<i>CpCLF-RT-R</i>	TGGACGAACGGCATTCTTGACT	Gene expression analysis
<i>CpSWN-RT-F</i>	AACCTATGTTCTTGACGCTTACCG	Gene expression analysis
<i>CpSWN-RT-R</i>	TTCTCCAGCTTCAATACGCTCATT	Gene expression analysis

Lowercase letters are adaptor sequences for in-fusion HD cloning and restriction enzyme sites are shown with bold capital letters (*Hind*III: **AAGCTT**; *Bam*HI: **GGATCC**; *Pst*I: **CTGCAG**).

Supplemental Table S2. Accession numbers of genes used for the phylogenetic analysis

Species	Gene	ID
<i>Arabidopsis thaliana</i>	<i>HDA2</i>	832673
<i>Arabidopsis thaliana</i>	<i>HDA6</i>	836431
<i>Arabidopsis thaliana</i>	<i>HDA7</i>	833525
<i>Arabidopsis thaliana</i>	<i>HDA10</i>	823592
<i>Arabidopsis thaliana</i>	<i>HDA17</i>	823574
<i>Arabidopsis thaliana</i>	<i>HDA19</i>	829969
<i>Arabidopsis thaliana</i>	<i>HDA9</i>	823594
<i>Beta vulgaris</i>	<i>HDA6</i>	104894337
<i>Cajanus cajan</i>	<i>HDA6</i>	109798024
<i>Capsella rubella</i>	<i>HDA19</i>	17879361
<i>Gossypium arboreum</i>	<i>HDA6</i>	108483673
<i>Malus domestica</i>	<i>HDA19</i>	103428059
<i>Rhodamnia argentea</i>	<i>HDA19</i>	115756819
<i>Sesamum indicum</i>	<i>HDA19</i>	105165913
<i>Sesamum indicum</i>	<i>HDA6</i>	105179380
<i>Arabidopsis lyrata</i>	<i>JMJ25</i>	9320727
<i>Arabidopsis thaliana</i>	<i>JMJ24</i>	837427
<i>Arabidopsis thaliana</i>	<i>JMJ25/IBM1</i>	819952
<i>Arabidopsis thaliana</i>	<i>JMJ26</i>	837747
<i>Arabidopsis thaliana</i>	<i>JMJ27</i>	827933
<i>Arabidopsis thaliana</i>	<i>JMJ29</i>	842529

<i>Camelina sativa</i>	<i>JMJ25</i>	104793410
<i>Capsella rubella</i>	<i>JMJ25</i>	17890836
<i>Salvia splendens</i>	<i>JMJ25</i>	121744023
<i>Sesamum indicum</i>	<i>JMJ25</i>	105178012
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<i>Arabidopsis thaliana</i>	<i>CLF</i>	816870
<i>Arabidopsis thaliana</i>	<i>MEA</i>	839422
<i>Arabidopsis thaliana</i>	<i>SWN</i>	828165
<i>Oryza sativa</i>	<i>CLF</i>	4340748
<i>Oryza sativa</i>	<i>EZ1</i>	4332612
<i>Petunia x hybrida</i>	<i>CLF1</i>	AB098523.1
<i>Petunia x hybrida</i>	<i>CLF2</i>	AB098524.1
<i>Petunia x hybrida</i>	<i>CLF3</i>	AB098525.1
<i>Solanum lycopersicum</i>	<i>EZ1</i>	100134891
<i>Zea mays</i>	<i>EZ1</i>	541954
<i>Zea mays</i>	<i>EZ2</i>	542659
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