Comprehensive genome-wide identification of angiosperm upstream ORFs with peptide sequences conserved in various taxonomic ranges using a novel pipeline, ESUCA

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Abstract

Background: Upstream open reading frames (uORFs) in the 5′-untranslated regions (5′-UTRs) of certain eukaryotic mRNAs encode evolutionarily conserved functional peptides, such as cis-acting regulatory peptides that control translation of downstream main ORFs (mORFs). For genome-wide searches for uORFs with conserved peptide sequences (CPuORFs), comparative genomic studies have been conducted, in which uORF sequences were compared between selected species. To increase chances of identifying CPuORFs, we previously developed an approach in which uORF sequences were compared using BLAST between *Arabidopsis* and any other plant species with available transcript sequence databases. If this approach is applied to multiple plant species belonging to phylogenetically distant clades, it is expected to further comprehensively identify CPuORFs conserved in various plant lineages, including those conserved among relatively small taxonomic groups.

Results: To efficiently compare uORF sequences among many species and efficiently identify CPuORFs conserved in various taxonomic lineages, we developed a novel pipeline, ESUCA. We applied ESUCA to the genomes of five angiosperm species, which belong to phylogenetically distant clades, and selected CPuORFs conserved among at least three different orders. Through these analyses, we identified 88 novel CPuORF families. As expected, ESUCA analysis of each of the five angiosperm genomes identified many CPuORFs that were not identified from ESUCA analyses of the other four species. However, unexpectedly, these CPuORFs include those conserved in wide taxonomic ranges, indicating that the approach used here is useful not only for comprehensive identification of narrowly conserved CPuORFs but also for that of widely conserved CPuORFs. Examination of the effects of 11 selected CPuORFs on mORF translation revealed that CPuORFs conserved only in relatively narrow taxonomic ranges can have sequence-dependent regulatory effects, suggesting that most of the identified CPuORFs are conserved because of functional constraints of their encoded peptides.

Conclusions: This study demonstrates that ESUCA is capable of efficiently identifying CPuORFs likely to be conserved because of the functional importance of their encoded peptides. Furthermore, our data show that the approach in which uORF sequences from multiple species are compared with those of many other species, using ESUCA, is highly effective in comprehensively identifying CPuORFs conserved in various taxonomic ranges.

Background

The 5′-untranslated regions (5′-UTRs) of many eukaryotic mRNAs contain upstream open reading frames (uORFs) [1-4]. Although most uORFs are not thought to encode functional proteins or peptides, certain uORFs encode regulatory peptides that have roles in post-transcriptional regulation of gene expression [5-8]. During translation of some of these regulatory uORFs, nascent peptides act inside the ribosomal exit tunnel to cause ribosome stalling [9]. Ribosome stalling on a uORF results in translational repression of the downstream main ORF (mORF) because stalled ribosomes block the access of subsequently loaded ribosomes to the mORF start codon [10]. Additionally, if ribosome stalling occurs at the stop codon of a uORF, nonsense-mediated mRNA decay may be induced [11, 12]. In some genes, uORF-encoded nascent peptides cause ribosome stalling in response to metabolites to down-regulate mORF translation under specific cellular conditions [10, 12-17]. In contrast to the uORFs encoding cis-acting regulatory nascent peptides, a uORF in the *Medicago truncatula MtHAP2-1* gene encodes a trans-acting regulatory peptide, which binds to the 5′-UTR of *MtHAP2-1* mRNA and causes mRNA degradation [18].

To comprehensively identify uORFs that encode functional peptides, genome-wide searches for uORFs with conserved peptide sequences (CPuORFs) have been conducted using comparative genomic approaches in various organisms [19-23]. In plants, approximately 40 CPuORF families have been identified by comparing the uORF-encoded amino acid sequences of orthologous genes in some of *Arabidopsis*, rice, cotton, orange, soybean, grape and tobacco, or those of paralogous genes in *Arabidopsis* [20, 22, 23]. Recently, 29 additional CPuORF families, which include CPuORFs with non-canonical initiation codons, have been identified by comparing 5′-UTR sequences between *Arabidopsis* and 31 other plant species [24].

In conventional comparative genomic approaches, uORF sequences are compared among selected species. Therefore, homology detection depends on the selection of species for comparison. In searches using this approach, if a uORF amino acid sequence is not conserved among the selected species, this uORF is not identified as a CPuORF, even if it is evolutionarily conserved between one of the selected species and other unselected species. To overcome this problem, we previously developed the BAIUCAS (for BLAST-based algorithm for identification of uORFs with conserved amino acid sequences) pipeline [25]. In BAIUCAS, homology searches of uORF amino acid sequences are performed using BLAST between a certain species and any other

81 species for which expressed sequence tag (EST) databases are available, and uORFs conserved beyond a certain
82 taxonomic range are selected. Using BAIUCAS, we searched for *Arabidopsis* CPuORFs conserved beyond the
83 order Brassicales, which *Arabidopsis* belongs to, and identified 13 novel CPuORF families [25]. We examined
84 the sequence-dependent effects of the CPuORFs identified by BAIUCAS on mORF translation using a transient
85 expression assay, and identified six regulatory CPuORFs that repress mORF translation in an amino acid
86 sequence-dependent manner [26, 27]. These sequence-dependent regulatory CPuORFs include ones conserved
87 only among relatively small taxonomic groups, such as a part of eudicots. Therefore, it is expected that
88 sequence-dependent regulatory CPuORFs conserved in various plant lineages, including narrowly conserved
89 ones, will be more comprehensively identified if BAIUCAS is applied to many plant species.

90 Before applying BAIUCAS to many species, improvement of BAIUCAS was desired to more efficiently
91 identify CPuORFs that were conserved because of the functional importance of their encoded peptides. One
92 major problem with identifying CPuORFs is that there are cases where a uORF found in the 5′-UTR of a
93 transcript is fused to the mORF in an isoform of the transcript, and in some of these cases, such uORF sequences
94 are conserved because they actually encode parts of mORF-encoded protein sequences. In other words, there are
95 cases where the protein-coding mORF is split into multiple ORFs in a splice variant and the ORF coding for the
96 N-terminal region of the protein appears like a uORF. Such an ORF can be extracted as a CPuORF if the amino
97 acid sequence in the N-terminal region of the protein is evolutionarily conserved. It is difficult to distinguish
98 between this type of 'spurious' CPuORFs and 'true' CPuORFs because even 'true' CPuORF-containing genes
99 produce splice variants in which a CPuORF is fused to the mORF, as seen in the At2g31280, At5g01710, and
100 At5g03190 genes [20, 25, 28]. Another major point to be improved is the method of calculating nonsynonymous
101 to synonymous nucleotide substitution (K_a/K_s) ratios for CPuORF sequences. These K_a/K_s ratios are used to
102 evaluate whether uORF sequences are conserved at the amino acid level or at the nucleotide level. However,
103 K_a/K_s ratios largely depend on the selection of uORF sequences used for their calculation. If uORF sequences
104 used for the calculation of a K_a/K_s ratio include many sequences from closely related species, the K_a/K_s ratio tends
105 to be high. For appropriate calculation of K_a/K_s ratios, uORF sequences need to be selected using proper criteria.

106 Here, we present an improved BAIUCAS version ESUCA (for evolutionary search for uORFs with
107 conserved amino acid sequences) and genome-wide identification of CPuORFs from five angiosperm genomes
108 using ESUCA. To distinguish between 'spurious' CPuORFs conserved because they code for parts of
109 mORF-encoded proteins and 'true' CPuORFs conserved because of functional constraints of their encoded small
110 peptides, ESUCA includes an algorithm to assess whether, for each uORF, transcripts bearing a uORF-mORF
111 fusion are minor or major forms among orthologous transcripts. Another new function of ESUCA is systematic
112 calculation of K_a/K_s ratios for CPuORF sequences. ESUCA includes an algorithm to select a uORF sequence
113 from each order for calculation of the K_a/K_s ratio of each CPuORF. Additionally, ESUCA is capable of
114 determining the taxonomic range within which each CPuORF is conserved. Although ESUCA can identify
115 CPuORFs conserved only among a small taxonomic group because ESUCA compares uORF sequences
116 between a certain species and any other species with available transcript databases, CPuORFs conserved among a
117 small taxonomic group may be less likely to encode functional peptides than those conserved across a wide
118 taxonomic range. The automatic determination of the taxonomic range of CPuORF conservation provides useful
119 information for the selection of CPuORFs likely to encode functional peptides. The current study demonstrates
120 that ESUCA efficiently identifies CPuORFs likely to be conserved because of functional constraints of their
121 encoded peptides. Furthermore, the data presented here show that the approach in which uORF sequences from
122 multiple species are compared with those of many other species, using ESUCA, is highly effective in
123 comprehensively identifying CPuORFs conserved in various taxonomic lineages.
124

125 ## Results

126 **The ESUCA pipeline**

127 In this study, to efficiently identify CPuORFs likely to be conserved because of functional importance of their
128 encoded peptides, we developed a novel pipeline, ESUCA, which consists of a six-step procedure (Fig. 1). The
129 first step is extraction of uORF sequences from a transcript sequence dataset. The uORFs are extracted by
130 searching the 5′-UTR sequence of each transcript for an ATG codon and its nearest downstream in-frame stop

131 codon. Although uORFs overlapping their downstream mORFs are also usually considered uORFs, we focus on
132 the type of uORFs that has both the start and stop codons within the 5′-UTR to avoid including uORFs whose
133 sequences are conserved because of functional constraints of mORF-encoded proteins. When there are splice
134 variants of a gene, uORFs in all splice variants are extracted. The second step assesses whether, for each uORF,
135 uORF-mORF fusion type transcripts are minor or major forms among orthologous transcripts. If transcripts with
136 a uORF-mORF fusion are found as a major form in a majority of species with their orthologs, the uORF
137 sequence is likely to code for a part of the mORF-encoded protein. Therefore, such a uORF should be discarded
138 as a 'spurious' uORF. In contrast, if transcripts with a uORF-mORF fusion are found in only a small proportion
139 of species with their orthologs, the uORF-mORF fusion type transcripts are considered minor form transcripts
140 and therefore can be ignored. For this assessment, the NCBI reference sequence (RefSeq) database is used, which
141 provides curated non-redundant transcript sequences [29]. For each uORF, the ratio of RefSeq RNAs with a
142 uORF-mORF fusion to all RefSeq RNAs with both sequences similar to the uORF and its downstream mORF is
143 calculated (Fig. 2). We define this ratio as the uORF-mORF fusion ratio. If the uORF-mORF fusion ratio of a
144 uORF is equal to or greater than 0.3, then the uORF is discarded. The third step is uORF amino acid sequence
145 homology searches. In this step, tBLASTn searches are performed against a transcript sequence database, using
146 the amino acid sequences of the uORFs as queries (uORF-tBLASTn analysis). The uORFs with tBLASTn hits
147 from other species are selected. The fourth step is selection of uORFs conserved among homologous genes. To
148 confirm whether the uORF-tBLASTn hits are derived from homologs of the original uORF-containing gene, the
149 downstream sequences of putative uORFs in the uORF-tBLASTn hits are subjected to another tBLASTn
150 analysis, which uses the mORF amino acid sequence of the original uORF-containing transcript as a query
151 (mORF-tBLASTn analysis) (Fig. 3). If a uORF-tBLASTn hit has a partial or intact ORF that contains a sequence
152 similar to the mORF amino acid sequence downstream of the putative uORF, it is considered to be derived from
153 a homolog of the original uORF-containing gene. If uORF-tBLASTn and mORF-tBLASTn hits are found in at
154 least two orders other than that of the original uORF, then the uORF is selected as a candidate CPuORF. This is
155 because at least three uORF sequences from different orders are necessary to confirm at the later manual
156 validation step that the same region is conserved among homologous uORF sequences. The fifth step is K_a/K_s
157 analysis. In this step, K_a/K_s ratios for the selected candidate CPuORFs is calculated to assess whether the
158 candidate CPuORF sequences are conserved at the nucleotide or amino acid level. A K_a/K_s ratio close to 1

159 indicates neutral evolution, whereas a K_a/K_s ratio close to 0 suggests that purifying selection acted on the amino
160 acid sequences. For each candidate CPuORF, a representative uORF-tBLASTn and mORF-tBLASTn hit is
161 selected from each order, and the putative uORF sequences in the representative uORF-tBLASTn and
162 mORF-tBLASTn hits are used for the calculation of the K_a/K_s ratio. If the K_a/K_s ratio of a candidate CPuORF is
163 less than 0.5 and significantly different from that of the negative control with q less than 0.05, then the candidate
164 CPuORF is selected for further analysis. The final step is to determine the taxonomic range of uORF sequence
165 conservation. In this step, the representative uORF-tBLASTn and mORF-tBLASTn hits selected in the fifth step
166 are classified into taxonomic categories (Fig. 4). On the basis of the presence of the uORF-tBLASTn and
167 mORF-tBLASTn hits in each taxonomic category, the taxonomic range of sequence conservation is determined
168 for each CPuORF.
169
170 **Identification of angiosperm CPuORFs using ESUCA**
171 We applied ESUCA to five angiosperm species, *Arabidopsis*, rice, tomato, poplar and grape, which belong to
172 phylogenetically distant clades of angiosperm, and for which entire genomic DNA and transcript sequence
173 datasets were available. Rice is a monocot, whereas the others are eudicots. *Arabidopsis* and poplar belong to two
174 different groups of rosids (marvids and fabids), whereas tomato belongs to asterids. Grape belongs to neither
175 rosids nor asterids. In the first step of ESUCA, we extracted uORF sequences from the 5′-UTR sequence of each
176 transcript of these species, using the transcript sequence datasets described in the Materials and Methods. In these
177 datasets, different transcript IDs are assigned to each splice variant from the same gene. To extract sequences of
178 uORFs and their downstream mORFs from all splice variants, we extracted uORF and mORF sequences from
179 each of the transcripts with different transcript IDs. In the second step, we calculated the uORF-mORF fusion
180 ratio of each uORF-containing transcript, using the extracted uORF and mORF sequences, and removed uORFs
181 with uORF-mORF fusion ratios equal to or greater than 0.3 (Supplementary Table S1). We also discarded
182 uORFs whose numbers of RefSeq RNAs containing both sequences similar to the uORF and its downstream
183 mORF were less than 10. This was done because appropriate evaluations of uORF-mORF fusion ratios were
184 difficult with a few related RefSeq RNAs and such uORFs are unlikely to be evolutionarily conserved. In the
185 third step, using the amino acid sequences of the remaining uORFs as queries, we performed uORF-tBLASTn
186 searches against a plant transcript sequence database that contained contigs of assembled EST and transcriptome

187 shotgun assembly (TSA), singleton EST/TSA sequences, and RefSeq RNAs (See Materials and Methods for
188 details). In the fourth step, the uORF-tBLASTn hits were subjected to mORF-tBLASTn analysis, and
189 uORF-tBLASTn and mORF-tBLASTn hits were extracted. Plant EST and TSA databases can include
190 contaminant sequences from other organisms, such as parasites, plant-feeding insects and infectious
191 microorganisms. We checked the possibility that the extracted uORF-tBLASTn and mORF-tBLASTn hits
192 included contaminant EST/TSA sequences, using BLASTn searches. The BLASTn searches were performed
193 using each uORF-tBLASTn and mORF-tBLASTn hit EST/TSA sequence as a query against EST/TSA and
194 RefSeq RNA sequences from all organisms, with an *E*-value cutoff of 10^{-100} and an identity threshold of 95%.
195 Contaminant EST/TSA sequences were identified by this analysis, as described in Materials and Methods, and
196 were removed from the uORF-tBLASTn and mORF-tBLASTn hits. We selected uORFs whose remaining
197 uORF-tBLASTn and mORF-tBLASTn hits were found in homologs from at least two orders other than that of
198 the original uORF. Thereafter, we generated amino acid sequence alignments of each selected uORF and its
199 homolog, using a putative homologous uORF sequence from each order in which uORF-tBLASTn and
200 mORF-tBLASTn hits were found. When multiple original uORFs derived from splice variants of the same gene
201 partially or completely shared amino acid sequences, the one with the longest conserved region was manually
202 selected on the basis of the uORF amino acid sequence alignments. In the fifth step, the remaining uORFs were
203 subjected to K_a/K_s analysis. The uORFs with K_a/K_s ratios less than 0.5 showing significant differences from those
204 of negative controls ($q < 0.05$) were selected as candidate CPuORFs (Supplementary Table S1). Through
205 ESUCA analyses of *Arabidopsis*, rice, tomato, poplar, and grape genomes, 104, 59, 42, 148, and 79 candidate
206 CPuORFs were extracted, respectively. Of these, 87 *Arabidopsis*, 53 rice, 30 tomato, 77 poplar, and 44 grape
207 uORFs belong to the previously identified CPuORF families, homology groups (HGs) 1 to 53 [20, 22-25]
208 (Supplementary Table S1). The amino acid sequences of the remaining candidate CPuORFs are not similar to
209 those of the known CPuORFs. Therefore, 17, 6, 12, 71, and 35 novel candidate CPuORFs were extracted from
210 *Arabidopsis*, rice, tomato, poplar, and grape genomes, respectively.
211
212 **Validation of candidate CPuORFs**
213 If the amino acid sequence of a uORF is evolutionarily conserved because of functional constraints of the
214 uORF-encoded peptide, it is expected that the amino acid sequence in the functionally important region of the

215 peptide is conserved among the uORF and its orthologous uORFs. Therefore, we manually checked whether the
216 amino acid sequences in the same region are conserved among uORF sequences in the alignment of each novel
217 candidate CPuORF. We found that the alignments of 13 novel candidate CPuORFs contain sequences that do not
218 share the consensus amino acid sequence in the conserved region, and removed these sequences from the
219 alignments. We also removed sequences derived from genes not related to the corresponding original
220 uORF-containing gene from the alignments of five novel candidate CPuORFs. When these changes resulted in
221 the number of orders with the uORF-tBLASTn and mORF-tBLASTn hits becoming less than two, the candidate
222 CPuORFs were discarded. Eight novel candidate CPuORFs were discarded for this reason. Supplementary
223 Figure S1 shows the uORF amino acid sequence alignments without the removed sequences. The K_a/K_s ratios
224 were recalculated after the manual removal of the sequences (Supplementary Table S1), and six additional novel
225 candidate CPuORFs were discarded because their K_a/K_s ratios were greater than 0.5.

226 Using genomic position information from Ensembl Plants (http://plants.ensembl.org/index.html) [30] and
227 Phytozome v12.1 (https://phytozome.jgi.doe.gov/pz/portal.html) [31], we manually checked whether the
228 positions of the remaining novel candidate CPuORFs overlap with those of the mORFs of other genes or the
229 mORFs of splice variants of the same genes. We found that the genomic position of the candidate CPuORF of the
230 *Arabidopsis ROA1* (AT1G60200) gene overlaps with that of an intron in the mORF region of a splice variant.
231 Protein sequences with an N-terminal region similar to the amino acid sequence encoded by the 5′-extended region
232 of the mORF in this splice variant are found in most orders from which the uORF-tBALASTn and
233 mORF-tBLASTn hits of this candidate CPuORF were extracted, suggesting that the splice variant with the
234 5′-extended mORF is not a minor form among orthologous transcripts. Therefore, this candidate CPuORF was
235 discarded.

236 In the second step of ESUCA, we excluded uORF sequences likely to encode parts of the mORF-encoded
237 proteins, by removing uORFs with high uORF-mORF fusion ratios. To confirm that the novel candidate
238 CPuORFs do not code for parts of the mORF-encoded proteins, each of the putative uORF sequences used for
239 the alignment and K_a/K_s analysis was queried against the UniProt protein database (https://www.uniprot.org/),
240 using BLASTx. When putative uORF sequences matched protein sequences with low *E*-values, we manually
241 checked whether amino acid sequences similar to those encoded by the putative uORFs were contained within
242 mORF-encoded protein sequences. In this analysis, mORF-encoded proteins with N-terminal sequences similar

243 to the amino acid sequences encoded by the candidate CPuORFs of the rice *OsUAM2* gene and its poplar
244 ortholog, POPTR_0019s07850, were identified in many orders. This suggests that the sequences encoded by
245 these candidate CPuORFs are likely to function as parts of the mORF-encoded proteins. Therefore, we discarded
246 these candidate CPuORFs. For some other novel candidate CPuORFs, mORF-encoded proteins with sequences
247 similar to those encoded by the candidate CPuORF and/or its homologous putative uORFs were also found.
248 However, we did not exclude these candidate CPuORFs, because such uORF-mORF fusion type proteins were
249 found in only a few species for each candidate.

250 After manual validation, 12, 4, 11, 70, and 34 uORFs were identified as novel CPuORFs in *Arabidopsis*, rice,
251 tomato, poplar and grape, respectively. Among these novel CPuORFs, those with both similar uORF and mORF
252 amino acid sequences were classified into the same HGs. Also, using OrthoFinder ver. 1.1.4 [32], an algorithm
253 for ortholog group inference, we classified the genes with novel CPuORFs and those with previously identified
254 CPuORFs into ortholog groups. The same HG number with a different sub-number was assigned to CPuORFs of
255 genes in the same ortholog group with dissimilar uORF sequences (e.g. HG56.1 and HG56.2). Of the newly
256 identified CPuORF genes, six were classified into the same ortholog groups as previously identified CPuORF
257 genes, but the amino acid sequences of these six CPuORFs are dissimilar to those of the known CPuORFs.
258 Including this type of CPuORFs, we identified 131 novel CPuORFs that belong to 88 novel HGs (HG2.2, HG9.2,
259 HG16.2, HG43.2, HG50.2, HG52.2 and HG54-HG130) (Supplementary Table S1).

260

261 **Determination of the taxonomic range of CPuORF sequence conservation**

262 As the final step of ESUCA, we determined the taxonomic range of the sequence conservation of each CPuORF
263 identified, including previously identified CPuORFs. For this purpose, the uORF-tBLASTn and
264 mORF-tBLASTn hits selected for the K_a/K_s analysis and retained after manual validation were classified into 13
265 plant taxonomic categories (See Materials and Methods for details.), on the basis of taxonomic lineage
266 information of EST, TSA, and RefSeq RNA sequences (Fig. 4). Fig. 5 and Supplementary Table S2 show the
267 taxonomic range of sequence conservation for each HG and each CPuORF, respectively. In general, CPuORFs
268 belonging to previously identified HGs tend to be conserved in a wider range of taxonomic categories than those
269 belonging to the newly identified HGs. For 19 of the novel HGs, CPuORF sequences are conserved both in
270 eudicots and monocots or in wider taxonomic ranges. In contrast, for 69 of the novel HGs, CPuORF sequences

271 are conserved only among eudicots. For 12 of these, CPuORF sequences are conserved in narrower taxonomic
272 ranges, only among rosids or asterids. These results indicate that the taxonomic range of CPuORF sequence
273 conservation varies, and that ESUCA can identify CPuORFs conserved in a relatively narrow taxonomic range.
274
275 **Sequence-dependent effects of CPuORFs on mORF translation**
276 To address the relationship between the taxonomic range of CPuORF sequence conservation and the
277 sequence-dependent effects of CPuORFs on mORF translation, we selected 11 poplar CPuORFs and examined
278 their sequence-dependent effects on expression of the downstream reporter gene using a transient expression
279 assay. Of the selected CPuORFs, those belonging to HG46, HG55, HG57, HG66 and HG103 are conserved in
280 diverse angiosperms or in wider taxonomic ranges (Fig. 5, Supplementary Table S2). The CPuORFs belonging
281 to HG65, HG80, HG81 and HG87 are conserved in a wide range of eudicots, whereas the CPuORFs belonging
282 to HG88 and HG107 are conserved only among rosids (Fig. 5, Supplementary Table S2). In the 5′-UTR of the
283 poplar gene with the HG107 CPuORF, there is another uORF immediately upstream of the CPuORF
284 (Supplementary Table S2K). To focus on the sequence-dependent effect of the CPuORF on mORF translation,
285 the upstream uORF was eliminated by mutating its initiation codon because the presence of the immediate
286 upstream uORF may reduce the translation efficiency of the CPuORF and therefore potentially make the effect of
287 the CPuORF ambiguous. The 5′-UTR sequences containing the selected CPuORFs were fused to the firefly
288 luciferase (Fluc) coding sequence and were placed under the control of the 35S promoter to generate the
289 wild-type (WT) reporter constructs (Fig. 6a, Supplementary Figure S2). To assess the importance of the amino
290 acid sequences for the effects of these CPuORFs on mORF translation, frameshift mutations were introduced into
291 the CPuORFs so that the amino acid sequences of their conserved regions could be altered. A + 1 or − 1
292 frameshift was introduced upstream or within the conserved region of each CPuORF, and another frameshift was
293 introduced before the stop codon to shift the reading frame back to the original frame (Supplementary Figure S2).
294 These reporter constructs were each transfected into protoplasts from *Arabidopsis thaliana* MM2d
295 suspension-cultured cells. After 24 h of incubation, cells were harvested and disrupted to analyze luciferase
296 activity. In five of the 11 CPuORFs, the introduced frameshift mutations significantly upregulated the expression
297 of the reporter gene, indicating that these CPuORFs repress mORF translation in a sequence-dependent manner
298 (Fig. 6b). These five novel sequence-dependent regulatory CPuORF include the HG107 CPuORF, which is one

299 of the CPuORFs conserved only among rosids. Therefore, this result suggests that CPuORFs conserved only
300 among rosids can have sequence-dependent regulatory effects.
301

302 ## Discussion

303 **Comprehensive identification of angiosperm CPuORFs by ESUCA analyses of multiple species' genomes**
304 In this study, we developed ESUCA, a pipeline for efficient genome-wide identification of CPuORFs. By
305 applying ESUCA to five angiosperm genomes, we identified 131 CPuORFs that belong to 88 novel HGs. Of
306 these HGs, 70 were identified through ESUCA analysis of only one of the *Arabidopsis*, rice, tomato, poplar or
307 grape genomes (Fig. 5). This means that CPuORFs belonging to these HGs cannot be identified by comparing
308 uORF sequences of orthologous genes between these five species. Therefore, this result demonstrates that the
309 approach used in this study, in which uORF sequences from multiple species are compared with those of many
310 other species, is highly effective in comprehensively identifying CPuORFs. We expected this approach to be
311 particularly useful for comprehensive identification of CPuORFs conserved in relatively narrow taxonomic
312 ranges. Therefore, we used the five angiosperm species belonging to relatively distant lineages in order to identify
313 CPuORFs conserved only among the taxonomic categories to which each of the five species belong. However,
314 unexpectedly, in 41 of 70 HGs identified through ESUCA analysis of only one of the five plant genomes,
315 CPuORF sequences are conserved in both rosids and asterids, two major groups of eudicots, indicating that these
316 CPuORFs are conserved across diverse eudicots (Fig. 5). One possible explanation of this observation is that
317 sequence conservation of uORFs is often lost in small taxonomic groups, such as orders or families, during
318 evolution. For example, while the CPuORF of the tomato LOC101264451 gene, which belongs to HG43.1,
319 exerts a sequence-dependent repressive effect on mORF translation, the CPuORF of its *Arabidopsis* ortholog,
320 *ANAC096*, lacks the C-terminal half of the amino acid sequence in the highly conserved region and does not have
321 a sequence-dependent regulatory effect [25-27]. In contrast to the *Arabidopsis ANAC096* CPuORF, all the critical
322 amino acid residues in the highly conserved region are retained in the HG43.1 CPuORF of *Tarenaya hassleriana*,
323 which belongs to the same order as *Arabidopsis*, Brassicales, but a different family. CPuORFs involved in
324 preferable but not essential post-transcriptional regulations may be lost in some taxonomic clades during
325 evolution. Such CPuORFs would be difficult to be identified by comparing uORF sequences between a few

326 selected species if a selected species is included in clades where the CPuORF sequences were lost. Therefore, the
327 ESUCA method is advantageous for comprehensive identification of CPuORFs compared with conventional
328 comparative genomic approaches. Our results reveal that the approach used here (*i.e.* ESUCA analyses of
329 multiple species' genomes) is further advantageous and is highly useful not only for comprehensive identification
330 of narrowly conserved CPuORFs but also for that of widely conserved CPuORFs.

331 The transient expression assays in the current study identified five novel poplar regulatory CPuORFs that
332 exert sequence-dependent repressive effects on mORF translation. One of the identified regulatory CPuORFs is
333 conserved only among rosids (*i.e.* only among fabids and malvids). This result suggests that at least, CPuORFs
334 conserved only among fabids and malvids can have sequence-dependent regulatory effects, although we cannot
335 rule out the possibility that CPuORFs conserved in narrower taxonomic ranges can have sequence-dependent
336 regulatory effects. Of the 92 CPuORF HGs identified through ESUCA analysis of the poplar genome, HG101.1
337 is conserved only among fabids, whereas the others are conserved in at least one other taxonomic category in
338 addition to fabids (Fig. 5). Likewise, all the HGs identified through ESUCA analysis of the other four plant
339 genomes are conserved in multiple taxonomic categories. Altogether, these results suggest that most the
340 CPuORFs identified in this study, in which CPuORFs conserved in more than two orders were extracted, are
341 likely to be conserved because of functional constraints of their encoded peptides. Of the 11 poplar CPuORFs
342 analyzed by the transient expression assays, five are conserved beyond eudicots, and three of them exhibited
343 sequence-dependent repressive effects (Figs. 5 and 6b). In addition, we previously examined the effects of 16
344 CPuORFs, which belong to HG27-HG29, HG33-HG43.1, HG44, and HG45, on mORF translation and
345 identified six sequence-dependent regulatory CPuORFs. In this analysis, five of 11 CPuORFs conserved beyond
346 eudicots showed sequence-dependent repressive effects, whereas one of five CPuORFs conserved only among
347 eudicots showed a sequence-dependent repressive effect [26, 27]. These results may suggest that CPuORFs
348 conserved beyond eudicots are more likely to encode functional peptides than CPuORFs conserved only among
349 eudicots. ESUCA is capable of selecting CPuORFs conserved in certain taxonomic ranges on the basis of two
350 criteria, the numbers of orders in which CPuORFs are conserved and/or taxonomic categories in which
351 CPuORFs are conserved. Our results demonstrate that this function of ESUCA is highly useful for the efficient
352 selection of CPuORFs likely to encode functional peptides.

353 Of the CPuORFs analyzed for their sequence-dependent regulatory effects in the present study, the CPuORFs
354 belonging to HG55 and HG66 showed no significant sequence-dependent effect on mORF translation, despite
355 their widespread sequence conservation beyond eudicots (Figs. 5 and 6b). These CPuORFs might encode
356 peptides that have functions other than the control of mORF translation, or they might exert sequence-dependent
357 regulatory effects only under certain conditions. In fact, many known sequence-dependent regulatory uORFs
358 repress mORF translation in response to metabolites, such as polyamine, arginine, and sucrose [10, 14, 15, 17].
359 Likewise, the other CPuORFs that exhibited no significant sequence-dependent regulatory effect might encode
360 peptides that have other functions or exert regulatory effects only under specific conditions.
361
362 **Filtering using the uORF-mORF fusion ratio**
363 To distinguish between 'spurious' CPuORFs conserved because they code for parts of mORF-encoded proteins
364 and 'true' CPuORFs conserved because of functional constraints of their encoded small peptides, we employed
365 the criterion of the uORF-mORF fusion ratio and discarded uORFs with uORF-mORF fusion ratios equal to or
366 greater than 0.3. We checked how effectively the 'spurious' CPuORFs were removed with this criterion, using a
367 protein sequence database. Although uORF-mORF fusion type protein sequences were found in OsUAM2
368 homologs from widespread angiosperm species, no other candidate CPuORFs were extracted in which
369 uORF-mORF fusion type protein sequences were found in many species that have sequences similar to the
370 candidate CPuORFs. This indicates that the uORF-mORF fusion ratio filtering worked effectively to exclude
371 'spurious' CPuORFs that code for parts of the mORF-encoded proteins.
372 ESUCA extracted all known plant cis-acting regulatory CPuORFs that control mORF translation in a
373 sequence-dependent manner (*i.e.* HG1, HG3, HG6, HG12, HG13, HG14, HG15.3, HG18, HG19, HG27, HG34,
374 HG36, HG41, HG42, and HG43.1 CPuORFs [15, 17, 26, 27, 33-38]) (Supplementary Table S1). Of these, the
375 HG3 CPuORFs, associated with an mORF coding for an *S*-adenosylmethionine decarboxylase (AdoMetDC; EC
376 4.1.1.50) [20, 39], showed relatively high uORF-mORF fusion ratios, although still below 0.3 (Supplementary
377 Table S1). In this study, 11 CPuORFs belonging to this HG were extracted using ESUCA and the uORF-mORF
378 fusion ratios of these CPuORFs were in the range of 0.17 to 0.27, with a median value of 0.26 (Supplementary
379 Table S1). Consistent with these relatively high uORF-mORF fusion ratios, uORF-mORF fusion type protein
380 sequences that contain amino acid sequences resembling HG3 CPuORFs are found in widespread angiosperm

381 species. The uORF-mORF fusion ratios of the candidate CPuORFs of rice *OsUAM2* and its poplar ortholog were
382 0.28 (Supplementary Table S1). The rice *OsUAM2* gene codes for a protein similar to UDP-arabinopyranose
383 mutases (EC 5.4.99.30) [40]. Two protein isoforms, a uORF-mORF fusion type and one lacking the
384 uORF-encoded region, produced from splice variants of an *OsUAM2* orthologous gene, are found in diverse
385 angiosperm species. The functions of both of isoforms are not yet known. Considering the example of HG3
386 CPuORFs, we cannot rule out the possibility that a candidate CPuORF functions as a regulatory uORF even if
387 uORF-mORF fusion type protein sequences are found in widespread species. However, to avoid including
388 potential 'spurious' CPuORFs whose amino acid sequences are likely to be evolutionarily conserved because of
389 their function as N-terminal regions of the mORF-encoded proteins, we excluded the candidate CPuORFs of the
390 rice *OsUAM2* gene and its poplar ortholog. In conclusion, the criterion of the uORF-mORF fusion ratio used in
391 this study appears appropriate because all known cis-acting sequence-dependent regulatory CPuORFs were
392 extracted and most 'spurious' CPuORFs were removed.
393

394 ## Conclusion

395 The present study demonstrates that the approach in which uORF sequences from multiple species are compared
396 with those of many other species, using ESUCA, is highly effective in comprehensive identification of CPuORFs.
397 Using this approach, we identified many novel angiosperm CPuORFs, which include CPuORFs conserved
398 among limited clades and widely conserved CPuORFs. Our results also showed that ESUCA is capable of
399 efficiently selecting CPuORFs likely to be conserved because of functional importance of their encoded peptides.
400 The approach used here can be applied to any eukaryotic organism with available genome and transcript
401 sequence databases and therefore is expected to contribute to the comprehensive identification of CPuORFs
402 encoding functional peptides in various organisms. Furthermore, besides CPuORFs, the algorithms developed for
403 ESUCA and the approach used here can be applied to the identification of other sequences conserved in various
404 taxonomic ranges.
405

406 ## Materials and Methods

407 **Extraction of uORF sequences**

408 We used genome sequence files in FASTA format and genomic coordinate files in GFF3 format obtained from
409 Ensemble Plants Release 33 (https://plants.ensembl.org/index.html) [30] to extract *Arabidopsis* (*Arabidopsis*
410 *thaliana*), tomato (*Solanum lycopersicum*), poplar (*Populus trichocarpa*), and grape (*Vitis vinifera*) uORF
411 sequences. We used a genome sequence file in FASTA format and a genomic coordinate files in GFF3 format
412 obtained from Phytozome v11 (https://phytozome.jgi.doe.gov/pz/portal.html) [31] for rice (*Oryza sativa*). We
413 extracted exon sequences from genome sequences, on the basis of genomic coordinate information, and
414 constructed transcript sequence datasets by combining exon sequences. On the basis of the transcription start site
415 and the translation initiation codon of each transcript in the genomic coordinate files, we extracted 5′-UTR
416 sequences from the transcript sequence datasets. Then, we searched the 5′-UTR sequences for an ATG codon and
417 its nearest downstream in-frame stop codon. Sequences starting with an ATG codon and ending with the nearest
418 in-frame stop codon were extracted as uORF sequences. When multiple uORFs from a gene shared the same
419 stop codon, only the longest uORF sequence was used for further analyses.
420
421 **Assembly of EST and TSA sequences**
422 EST, TSA, and RefSeq RNA sequence datasets were obtained from the International Nucleotide Sequence
423 Database Collaboration databases (NCBI release of 2016-12-03 and DDBJ release 106.0 for EST and TSA
424 sequences, NCBI release 79 for RefSeq RNA sequences). On the basis of taxonomic lineage information
425 provided by NCBI Taxonomy (https://www.ncbi.nlm.nih.gov/taxonomy), EST and TSA sequences derived from
426 Viridiplantae were extracted from the databases. EST and TSA sequences from the same species were assembled
427 using Velvet ver. 1.2.10 [41] with k-mer length of 99. The k-mer length was optimized using *A. thaliana* EST and
428 TSA sequences to minimize the total numbers of assembled contigs and unclustered singleton sequences.
429 Unclustered singleton EST/TSA sequences, derived from species for which RefSeq RNA sequences were
430 available, were mapped to the RefSeq RNA sequences using Bowtie2 ver. 2.2.9 [42] and the default parameters.
431 We discarded singleton EST/TSA sequences that matched any RefSeq RNA sequences from the same species.
432 We created a plant transcript sequence database for BLAST searches in our local computers by using the
433 remaining singleton EST/TSA sequences, assembled contigs, Viridiplantae RefSeq RNA sequences, and
434 makeblastdb, a program contained in the NCBI-BLAST package.
435

436 **Calculation of the uORF-mORF fusion ratio**

437 The uORF-mORF fusion ratio for each of the extracted uORFs was assessed as follows. We performed
438 tBLASTx using each uORF sequence as a query against plant (Viridiplantae) RefSeq RNA sequences with an
439 *E*-value cutoff of 2000 (uORF-tBLASTx). We used standalone NCBI-BLAST + ver. 2.6.0 [43] for all BLAST
440 analyses. We next performed tBLASTx with an *E*-value threshold of 10^{-1} using the mORF sequence associated
441 with each uORF as a query against the uORF-tBLASTx hit sequences (mORF-tBLASTx). Using these two-step
442 tBLASTx searches, we selected RefSeq RNAs that contain both sequences similar to the original uORF and its
443 downstream mORF. Then, we examined whether the largest ORF of each of the selected RefSeq RNAs included
444 the region that matched the original mORF in the same reading frame (Fig. 2). We also examined whether the
445 largest ORF included the region that matched the original uORF, irrespective of the reading frame. The RefSeq
446 RNA was considered to have a uORF-mORF fusion if the largest ORF contained both regions that matched the
447 original uORF and mORF (Fig. 2). The RefSeq RNA was considered to have a uORF separated from the
448 downstream mORF if the largest ORF contained the region that matched the original mORF but not the region
449 that matched the original uORF (Fig. 2). RefSeq RNA numbers of the former and latter types were defined as X
450 and Y, respectively. We calculated a uORF-mORF fusion ratio as $X / (X + Y)$ for each of the original
451 uORF-containing transcript sequences.

452

453 **BLAST-based search for uORFs conserved between homologous genes**

454 To search for uORFs with amino acid sequences conserved between homologous genes, we first performed
455 tBLASTn searches against the assembled plant transcript sequence database, using the amino acid sequences of
456 the uORFs as queries. In these uORF-tBLASTn searches, we extracted transcript sequences that matched a
457 uORF with an *E*-value less than 2000 and derived from species other than that of the original uORF. The
458 downstream in-frame stop codon closest to the 5′-end of the matching region of each uORF-tBLASTn hit was
459 selected (Fig. 3). Then, we looked for an in-frame ATG codon upstream of the selected stop codon, without any
460 other in-frame stop codon between them. uORF-tBLASTn hits without such an ATG codon were discarded. If
461 one or more in-frame ATG codons were identified, the 5′-most ATG codon was selected. The ORF beginning
462 with the selected ATG codon and ending with the selected stop codon was extracted as a putative uORF (Fig. 3).
463 The downstream sequences of putative uORFs were subjected to another tBLASTn analysis to examine whether

464 the transcripts were derived from homologs of the original uORF-containing gene. In this analysis, the amino
465 acid sequence of the mORF associated with the original uORF was used as a query sequence, and transcript
466 sequences matching the mORF with an *E*-value less than 10^{-1} were extracted. For each of the uORF-tBLASTn
467 and mORF-tBLASTn hits, the upstream in-frame stop codon closest to the 5′-end of the region matching the
468 original mORF was selected, and the 5′-most in-frame ATG codon located downstream of the selected stop
469 codon was identified as the putative mORF initiation codon (Fig. 3). uORF-tBLASTn and mORF-tBLASTn hits
470 were discarded as uORF-mORF fusion type sequences if the putative mORF overlapped with the putative uORF.
471 The original uORF was selected as a candidate CPuORF if the remaining uORF-tBLASTn and
472 mORF-tBLASTn hits belonged to at least two orders other than that from which the original uORF was derived.
473

474 **Identification of contaminant ESTs and TSAs**

475 In the uORF-tBLASTn and mORF-tBLASTn analyses described above, we excluded tBLASTn hit ESTs and
476 TSAs derived from contaminated organisms. To examine whether each uORF-tBLASTn and mORF-tBLASTn
477 hit sequence is derived from contaminated organisms, we performed BLASTn searches against EST, TSA, and
478 RefSeq RNA sequences from all organisms except for those of metagenomes, using each uORF-tBLASTn and
479 mORF-tBLASTn hit EST/TSA sequence as a query. If a uORF-tBLASTn hit EST/TSA sequence matched an
480 EST, TSA, or RefSeq RNA sequence of a different order from the species of the uORF-tBLASTn and
481 mORF-tBLASTn hit, with an *E*-value less than 10^{-100} and an identity equal to or greater than 95%, it was
482 considered a candidate contaminant sequence. In this case, either the uORF-tBLASTn and mORF-tBLASTn hit
483 or the BLASTn hit may be a contaminant sequence. To distinguish these possibilities, we compared the ratio of
484 the BLASTn hit number to the total EST/TSA and RefSeq RNA sequence number between the species of each
485 uORF-tBLASTn and mORF-tBLASTn hit and the species of its BLASTn hits. Appropriate comparisons are
486 difficult unless species used for this comparison have enough number of EST/TSA and RefSeq RNA sequences.
487 Therefore, if the total EST/TSA and RefSeq RNA sequence number of a species is less than 5000, BLASTn hits
488 derived from the species were not used for this analysis. If the ratio of the BLASTn hit number to the total
489 EST/TSA and RefSeq RNA sequence number of a uORF-tBLASTn and mORF-tBLASTn hit species is less
490 than that of any other BLASTn hit species, the uORF-tBLASTn and mORF-tBLASTn hit sequence was
491 identified as a contaminant sequence.

492

493 **K_a/K_s analysis**

494 For K_a/K_s analysis of each candidate CPuORF, a putative uORF sequence was selected from each order in which
495 uORF-tBLASTn and mORF-tBLASTn hits were found, using the following criteria. First, we selected transcript
496 sequences that matched the mORF associated with the candidate CPuORF with an *E*-value less than 10^{-20} in
497 mORF-tBLASTn analysis. Of these sequences, we then selected the one with the smallest geometric means of
498 mORF-tBLASTn and uORF-tBLASTn *E*-values. When mORF-tBLASTn *E*-values of all uORF-tBLASTn and
499 mORF-tBLASTn hits in an order were equal to or greater than 10^{-20}, we selected the transcript sequence with the
500 smallest geometric means of mORF-tBLASTn and uORF-tBLASTn *E*-values in the order. Putative uORF
501 sequences in the selected transcript sequences were used for generation of uORF amino acid sequence
502 alignments and for K_a/K_s analysis. Multiple alignments were generated by using standalone Clustal Omega
503 (ClustalO) ver. 1.2.2 [44]. For each candidate CPuORF, a median K_a/K_s ratio for all pairwise combinations of the
504 original uORF and its homologous putative uORFs was calculated using the LWL85 algorithm [45] in the seqinR
505 package [46].

506 For statistical tests of K_a/K_s ratios, we calculated the distribution of mutation rates between the original uORF
507 and its homologous putative uORFs and those between the original uORF and its artificially generated mutants,
508 using the observed mutation rate distribution. Then, observed empirical K_a/K_s ratio distributions were compared
509 with null distributions (negative controls) using the Mann-Whitney *U* test to validate statistical significance. The
510 one-sided *U* test was used to investigate whether the observed distributions were significantly lower than the null
511 distributions. Adjustment for multiple comparisons was achieved by controlling the false discovery rate using the
512 Benjamini and Hochberg procedure [47].

513

514 **Determination of the taxonomic range of uORF sequence conservation**

515 To automatically determine the taxonomic range of the sequence conservation of each CPuORF, we first defined
516 13 plant taxonomic categories. The 13 defined taxonomic categories are lamiids, asterids other than lamiids,
517 mavids, fabids, eudicots other than rosids and asterids, commelinids, monocots other than commelinids,
518 Angiospermae other than eudicots and monocots, Gymnospermae, Polypodiopsida, Embryophyta other than
519 Euphyllophyta, Streptophyta other than Embryophyta, and Viridiplantae other than Streptophyta. On the basis of

520 taxonomic lineage information of EST, TSA, and RefSeq RNA sequences, which were provided by NCBI
521 Taxonomy, the uORF-tBLASTn and mORF-tBLASTn hit sequences selected for K_a/K_s analysis were classified
522 into the 13 taxonomic categories (Fig. 4). It should be noted that, in NCBI taxonomy, eudicots, Angiospermae,
523 and Ggymnospermae are referred to as eudicotyledons, Magnoliophyta, and Acrogymnospermae, respectively.
524 For each CPuORF, the numbers of transcript sequences classified into each category were counted and shown in
525 Supplementary Table S2. These numbers represent the numbers of orders in which the amino acid sequence of
526 each CPuORF is conserved.
527
528 **Statistical and informatic analyses**
529 All programs, except for existing stand-alone programs, such as BLAST [43], ClustalO [44] and Jalview [48],
530 were written in R (www.r-project.org). We also used R libraries, GenomicRanges [49], exactRankTests,
531 Biostrings and seqinr [46].
532
533 **Plasmid construction**
534 Plasmid pNH006 harbors the cauliflower mosaic virus 35S RNA (35S) promoter, the Fluc coding sequence, and
535 the polyadenylation signal of the *A. thaliana HSP18.2* gene in pUC19. To construct this plasmid, pMT61 [26]
536 was digested with *Sal*I and *Sac*I, and the *Sal*I-*Sac*I fragment containing the Fluc coding sequence was ligated into
537 the *Sal*I and *Sac*I sites between the 35S promoter and the *HSP18.2* polyadenylation signal of plasmid pKM56
538 [38]. To generate reporter plasmids pNH92-pNH101 (Supplementary Table S3) for transient expression assays,
539 the 5′-UTR sequences of ten poplar genes were amplified by PCR from poplar (*Populus nigra*) full-length
540 cDNA clones pds25559, pds10965, pds14390, pds12940, pds13862, pds15817, pds28294, pds26157, pds14623
541 and pds23234 (Supplementary Table S4), obtained from RIKEN [50]. Primer sets used are shown in
542 Supplementary Tables S3 and S5. To construct pNH92, pNH94-pNH98 and pNH100-pNH101, amplified
543 fragments containing the 5′-UTR sequences were digested with *Xba*I and *Sal*I and ligated between the *Xba*I and
544 *Sal*I sites of pNH006. To create pNH93 and pNH99, amplified fragments containing the 5′-UTR sequences were
545 inserted between the *Xba*I and *Sal*I sites of pNH006 using the SLiCE method [51]. To make pHN102, the
546 5′-UTR sequence of the *Populus trichocarpa* POPTR_0013s08000 gene with a mutation at the initiation codon
547 of the uORF located immediately upstream of the HG107 CPuORF was synthesized by Fasmac (Atsugi, Japan)

548 on the basis of NCBI RefSeq accession no. XM_002319213.3. The synthesized 5´-UTR sequence was amplified
549 by PCR using primers 35S_XbaI_SLiCE-F and FLUC_SalI_SLiCE-R (Supplementary Table S5) and were
550 inserted between the *Xba*I and *Sal*I sites of pNH006 using the SLiCE method [51]. Frameshift mutations were
551 introduced into each CPuORF using overlap extension PCR [52], with primers listed in Supplementary Tables S4
552 and S5, to yield pNH103-pNH112. Sequence analysis confirmed the integrity of the PCR-amplified regions of all
553 constructs.
554
555 **Transient expression assay**
556 Transient expression assays were performed as described in Hayashi *et al*. 2017 [38]. Protoplasts from *A.*
557 *thaliana* MM2d suspension cells [53] were used, as were the reporter plasmids described above and the pKM5
558 [38] internal control plasmid. pKM5 contains the 35S promoter, the *Renilla* luciferase (Rluc) coding sequence,
559 and the *NOS* polyadenylation signal in pUC19. For each experiment, 5 μg each of a reporter plasmid and pKM5
560 were transfected into protoplasts.
561

562 # Declarations

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571 **Authors' contributions**
572 HT, HO, SK, KS, and SN conceived and designed the experiments. NH and YY performed the experiments. HT,
573 HO, TE, and AT analyzed the data. HT, NH, YY, AT, and KF contributed to reagents/materials/analysis tools.
574 HO and HT wrote the paper. All authors read and approved the final manuscript.

575 **Competing interests**

576 The authors declare that they have no competing interests.

577

578 **Ethics approval and consent to participate**

579 Not applicable.

580

581 **Consent for publication**

582 Not applicable.

583

584 **Availability of data and materials**

585 Not applicable.

586

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589

590 **List of abbreviations**

591 35S: cauliflower mosaic virus 35S RNA; 5′-UTR: 5′-untranslated region; CPuORF: conserved peptide upstream
592 open reading frame; EST: expressed sequence tag; Fluc: firefly luciferase; HG: homology group; mORF: main
593 open reading frame; RefSeq: NCBI reference sequence; Rluc: Renilla luciferase; TSA: transcriptome shotgun
594 assembly; uORF: upstream open reading frame

595

596

597

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751 # Figure legends

752

753 **Figure 1.** Outline of the ESUCA pipeline.

754

755

756 **Figure 2.** Schematic representation of the algorithm to calculate uORF-mORF fusion ratios. For each original
757 uORF-containing transcript sequence, RefSeq RNAs are selected that match an original uORF sequence,
758 irrespective of the reading frame, and the original mORF sequence in the same reading frame as the largest ORF
759 of the RefSeq RNA, using tBLASTx. The shaded regions in the open boxes represent the tBLASTx-matching
760 regions. If the uORF-tBLASTx-matching region is within the largest ORF, the RefSeq RNA is considered a
761 uORF-mORF fusion type. The number of this type of RefSeq RNA is defined as '*X*'. If the
762 uORF-tBLASTx-matching region is not within the largest ORF, the RefSeq RNA is considered a uORF-mORF
763 separate type. The number of this type of RefSeq RNA is defined as '*Y*'. For each of the original
764 uORF-containing transcripts, the uORF-mORF fusion ratio is calculated as $X / (X + Y)$.

765

766 **Figure 3.** Schematic representation of BLAST-based search for uORFs conserved between homologous genes.
767 In the third step of ESUCA, tBLASTn searches are conducted against a transcript sequence database that consists
768 of assembled EST/TSA contigs, unclustered singleton EST/TSA sequences and RefSeq RNAs, using original
769 uORF sequences as queries (uORF-tBLASTn). The shaded regions in the open boxes show the
770 tBLASTn-matching regions. Asterisks represent stop codons. (i) The downstream in-frame stop codon closest to
771 the 5′-end of the matching region of each uORF-tBLASTn hit is selected. (ii) The 5′-most in-frame ATG codon
772 located upstream of the stop codon is selected. The ORF beginning with the selected ATG codon and ending with
773 the selected stop codon is extracted as a putative uORF. In the fourth step of ESUCA, the downstream sequences
774 of putative uORFs in the transcript sequences are subjected to mORF-tBLASTn analysis. Transcript sequences
775 matching the original mORF with an *E*-value less than 10^{-1} are extracted. (iii) For each of the uORF-tBLASTn
776 and mORF-tBLASTn hits, the upstream in-frame stop codon closest to the 5′-end of the matching region is
777 selected. (iv) The 5′-most in-frame ATG codon located downstream of the selected stop codon is identified as the

778 initiation codon of the putative partial or intact mORF. If the putative mORF overlaps with the putative uORF, the
779 uORF-tBLASTn and mORF-tBLASTn hit is discarded as a uORF-mORF fusion type.
780
781 **Figure 4.** Schematic representation of the algorithm to determine the taxonomic range of uORF sequence
782 conservation. In the fifth step of ESUCA, for K_a/K_s analysis of each candidate CPuORF, a uORF-tBLASTn and
783 mORF-tBLASTn hit sequence is selected from each order that has uORF-tBLASTn and mORF-tBLASTn hits
784 (See Materials and Methods for the criteria for the selection). In the sixth step of ESUCA, the selected transcript
785 sequences are classified into the 13 plant taxonomic categories to determine the taxonomic range of uORF
786 sequence conservation, using taxonomic lineage information of EST, TSA, and RefSeq RNA sequences.
787
788 **Figure 5.** Taxonomic range of the sequence conservation of the CPuORF families. The blue, yellow, red, green,
789 and purple lines show the conservation range of CPuORF HGs determined by applying ESUCA to *Arabidopsis*
790 (*Arabidopsis thaliana*), rice (*Oryza sativa*), tomato (*Solanum lycopersicum*), poplar (*Populus trichocarpa*), and
791 grape (*Vitis vinifera*) genomes, respectively. The presence of a line within a cell in each taxonomic category
792 indicates the presence of uORF-tBLASTn and mORF-tBLASTn hits for any of the CPuORFs that belong to
793 each HG. In taxonomic categories with a category name with an asterisk, uORF-tBLASTn and
794 mORF-tBLASTn hits found in lower taxonomic categories were excluded. In the case where no
795 uORF-tBLASTn and mORF-tBLASTn hit was found in the taxonomic category that contain a species from
796 which the original uORF was derived, the line showing the species was still drawn in the cell of the taxonomic
797 category because this category contained the species with the original uORF. *Arabidopsis*, rice, tomato, poplar
798 and grape belong to malvids, commelinids, lamiids, fabids and eudicots*, respectively. HG1-HG53 are
799 previously identified HGs, except for HG2.2, HG9.2, HG16.2, HG43.2, HG50.2 and HG52.2, whereas HG2.2,
800 HG9.2, HG16.2, HG43.2, HG50.2, HG52.2 and HG54-HG130 are newly identified HGs.
801
802 **Figure 6.** Sequence-dependent effects of novel CPuORFs on main ORF translation. (A) Schematic
803 representation of the WT (*35S::UTR(WT):Fluc*) and frameshift mutant (*35S::UTR(fs):Fluc*) reporter constructs.
804 The 5′-UTR sequence containing each of the 11 poplar CPuORFs was inserted between the 35S promoter
805 (*35Spro*) and the *Fluc* coding sequence. The hatched box in the frameshift (fs) mutant CPuORF indicates the

806 frame-shifted region. The dotted boxes represent the first five nucleotides of the mORF associated with each of
807 the 11 poplar CPuORFs. See Supplementary Figure S2 for the exact position and length of each CPuORF and the
808 exact frame-shifted region. The polyadenylation signal of the *A. thaliana HSP18.2* gene is designated as 'ter'. (B)
809 Transient expression assay. Each reporter plasmid containing a WT or fs mutant CPuORF, belonging to an
810 indicated HG, was co-transfected into MM2d protoplasts with the *35S::Rluc* internal control plasmid by PEG
811 treatment. After incubation for 24 h, cells were collected and disrupted for dual luciferase assay. Fluc activity was
812 normalized to Rluc activity, and the activity relative to that of the corresponding WT reporter construct was
813 calculated. Means ± SD of three biological replicates are shown. Single and double asterisks indicate significant
814 differences between the WT and fs constructs at $p < 0.05$ and $p < 0.01$, respectively, as determined by Student's
815 *t*-test. Representative results of two experiments using independently prepared protoplasts are shown.

Figure 1

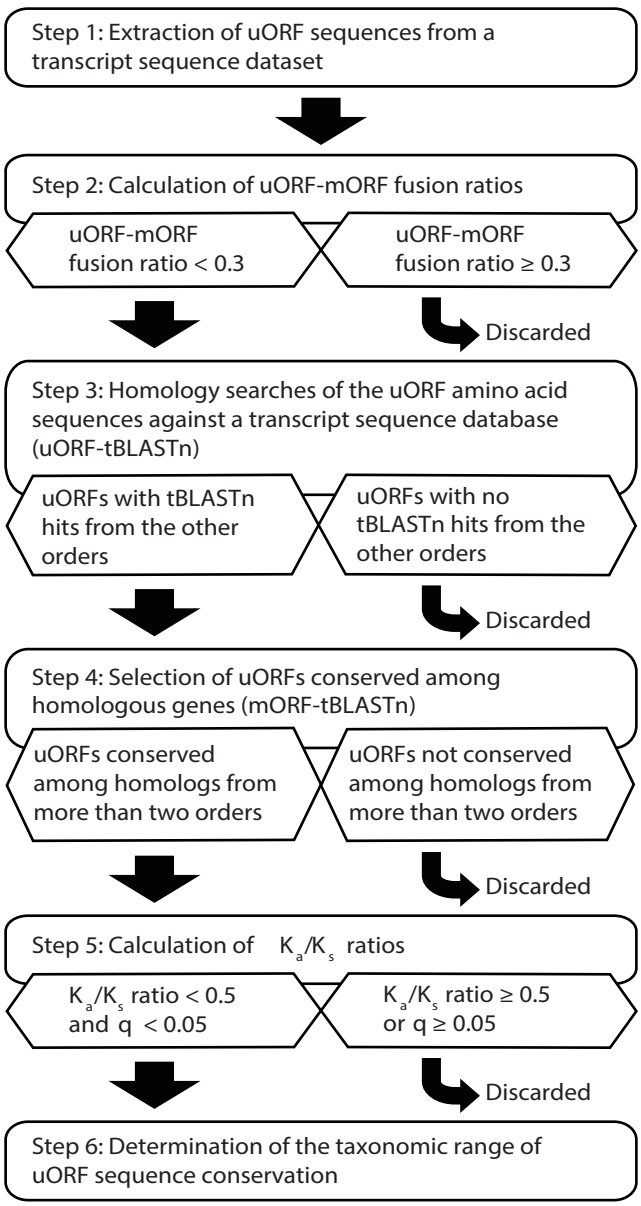

Step 1: Extraction of uORF sequences from a transcript sequence dataset
Step 2: Calculation of uORF-mORF fusion ratios
uORF-mORF fusion ratio < 0.3
uORF-mORF fusion ratio ≥ 0.3
Discarded
Step 3: Homology searches of the uORF amino acid sequences against a transcript sequence database (uORF-tBLASTn)
uORFs with tBLASTn hits from the other orders
uORFs with no tBLASTn hits from the other orders
Discarded
Step 4: Selection of uORFs conserved among homologous genes (mORF-tBLASTn)
uORFs conserved among homologs from more than two orders
uORFs not conserved among homologs from more than two orders
Discarded
Step 5: Calculation of K_a/K_s ratios
K_a/K_s ratio < 0.5 and q < 0.05
K_a/K_s ratio ≥ 0.5 or q ≥ 0.05
Discarded
Step 6: Determination of the taxonomic range of uORF sequence conservation

Figure 2

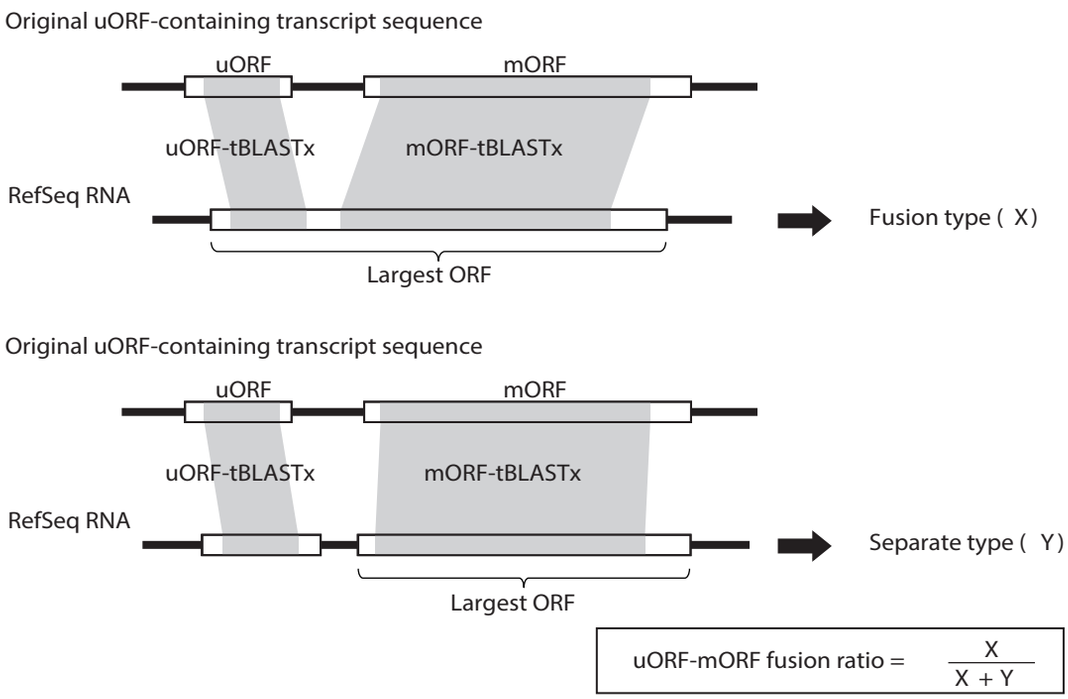

Original uORF-containing transcript sequence
uORF
mORF
uORF-tBLASTx
mORF-tBLASTx
RefSeq RNA
Fusion type (X)
Largest ORF
Original uORF-containing transcript sequence
uORF
mORF
uORF-tBLASTx
mORF-tBLASTx
RefSeq RNA
Separate type (Y)
Largest ORF
uORF-mORF fusion ratio = X / X + Y

Figure 3

Original uORF-containing transcript sequence

uORF-tBLASTn and mORF-tBLASTn hit sequence (Assembled EST/TSA and RefSeq RNA)

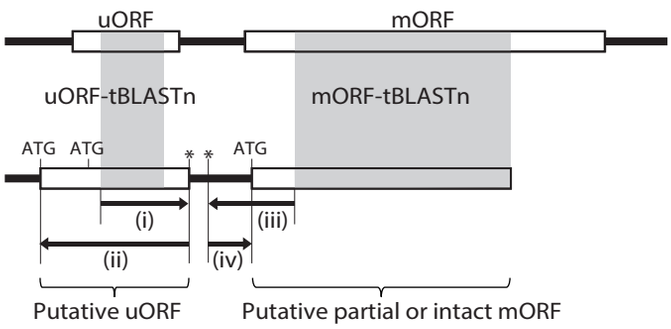


Figure 4

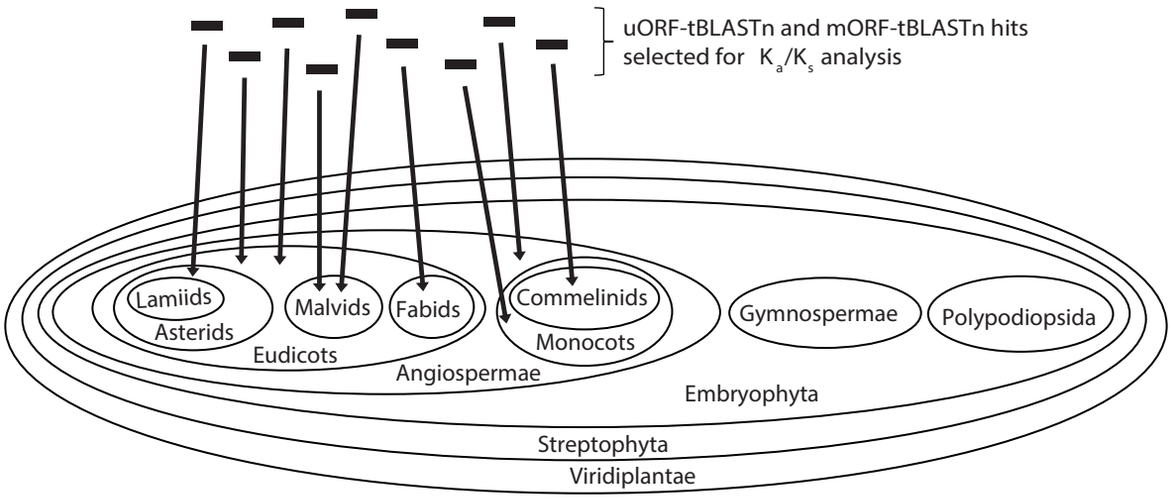
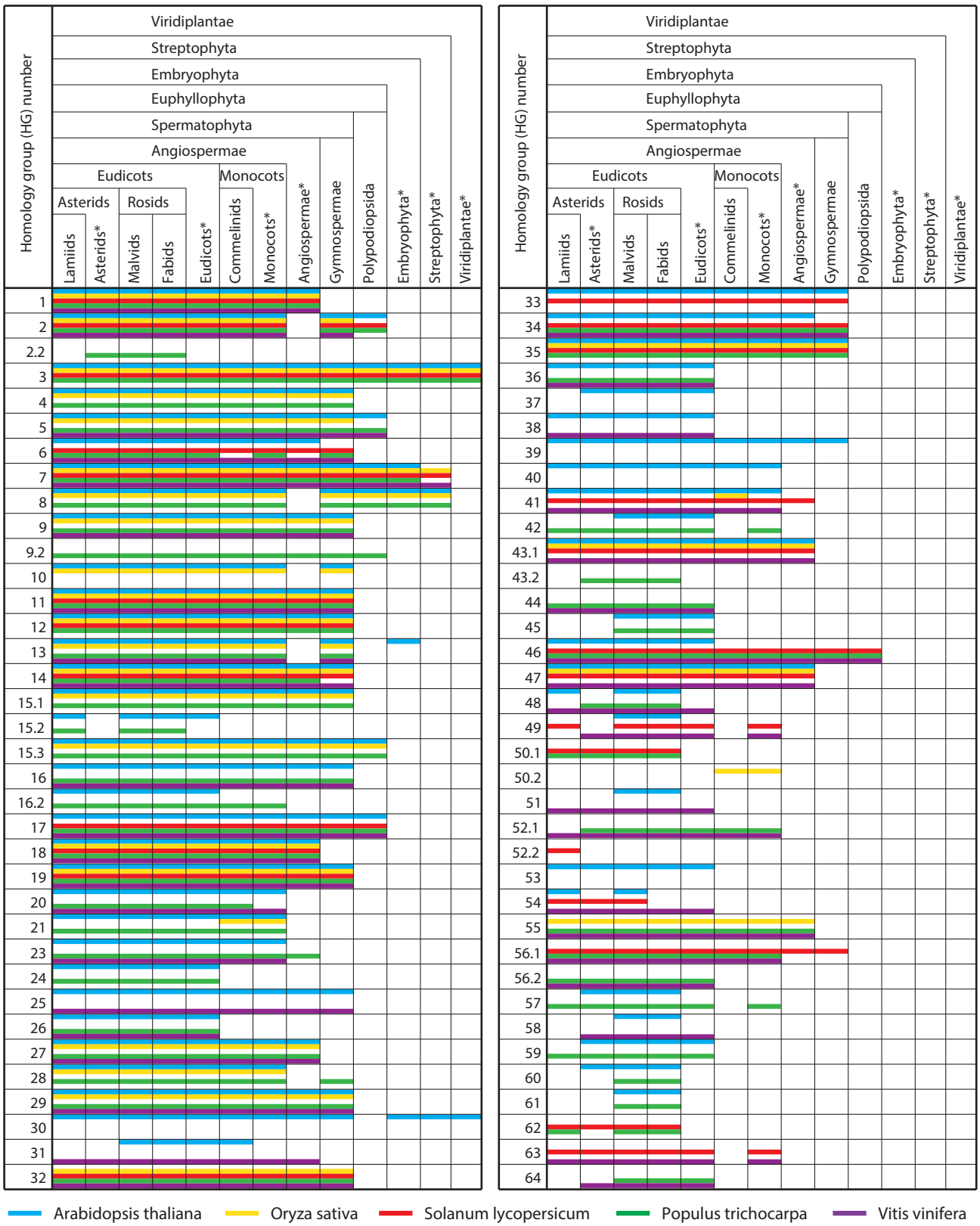

uORF-tBLASTn and mORF-tBLASTn hits
selected for K_a/K_s analysis
Lamiids
Asterids
Malvids
Fabids
Commelinids
Monocots
Gymnospermae
Polypodiopsida
Eudicots
Angiospermae
Embryophyta
Streptophyta
Viridiplantae

Figure 5


Homology group (HG) number
Viridiplantae
Streptophyta
Embryophyta
Euphyllophyta
Spermatophyta
Angiospermae
Eudicots
Monocots
Asterids
Rosids
Lamiids
Asterids*
Malvids
Fabids
Eudicots*
Commelinids
Monocots*
Angiospermae*
Gymnospermae
Polypodiopsida
Embryophyta*
Streptophyta*
Viridiplantae*
1
2
2.2
3
4
5
6
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8
9
9.2
10
11
12
13
14
15.1
15.2
15.3
16
16.2
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39
40
41
42
43.1
43.2
44
45
46
47
48
49
50.1
50.2
51
52.1
52.2
53
54
55
56.1
56.2
57
58
59
60
61
62
63
64
Arabidopsis thaliana
Oryza sativa
Solanum lycopersicum
Populus trichocarpa
Vitis vinifera

Figure 5 (continued)

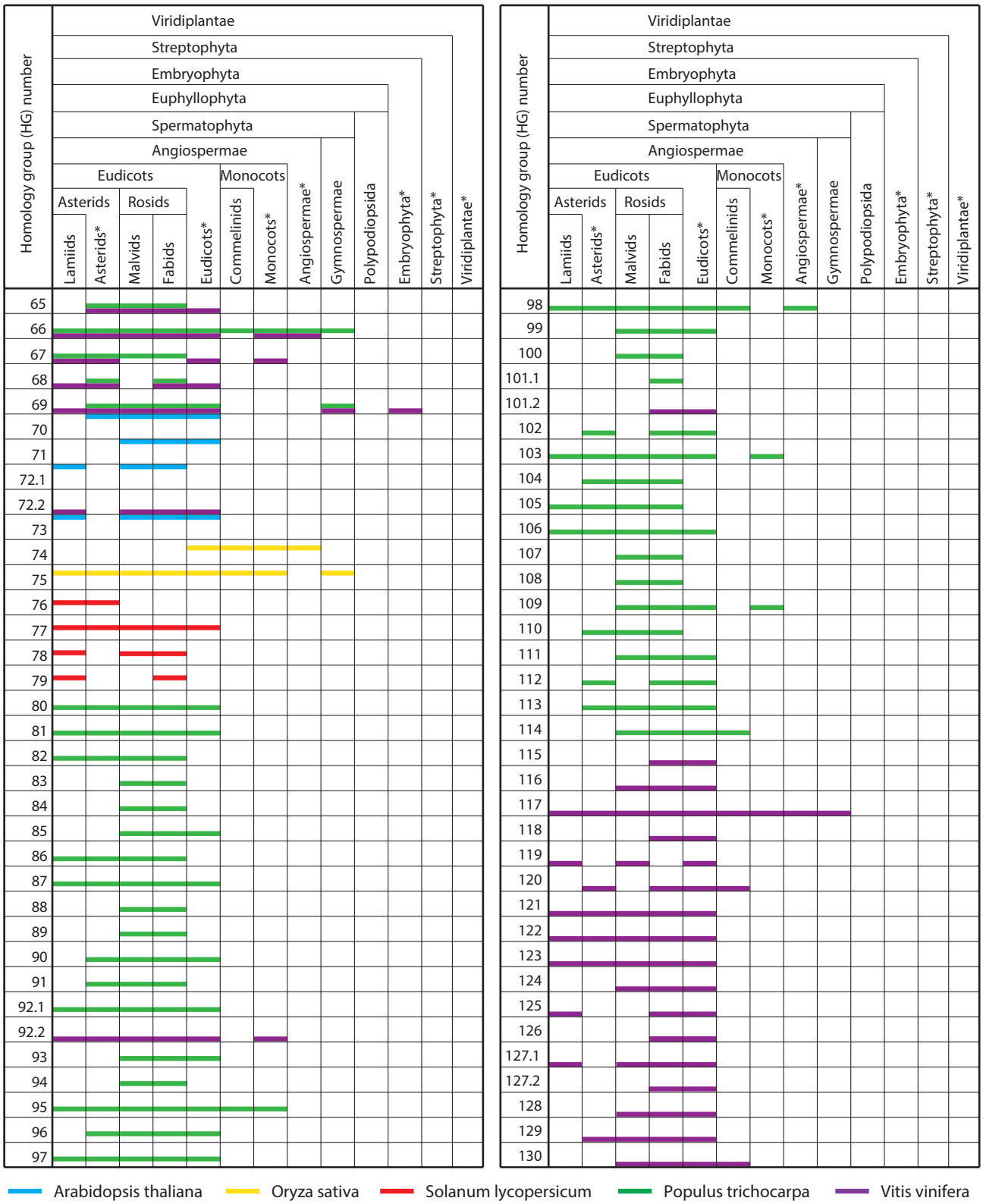
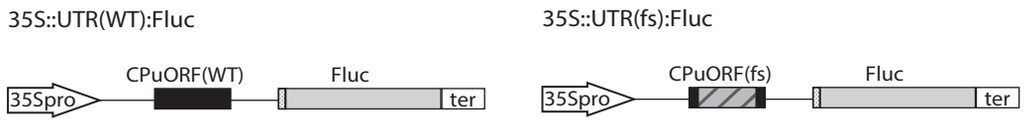
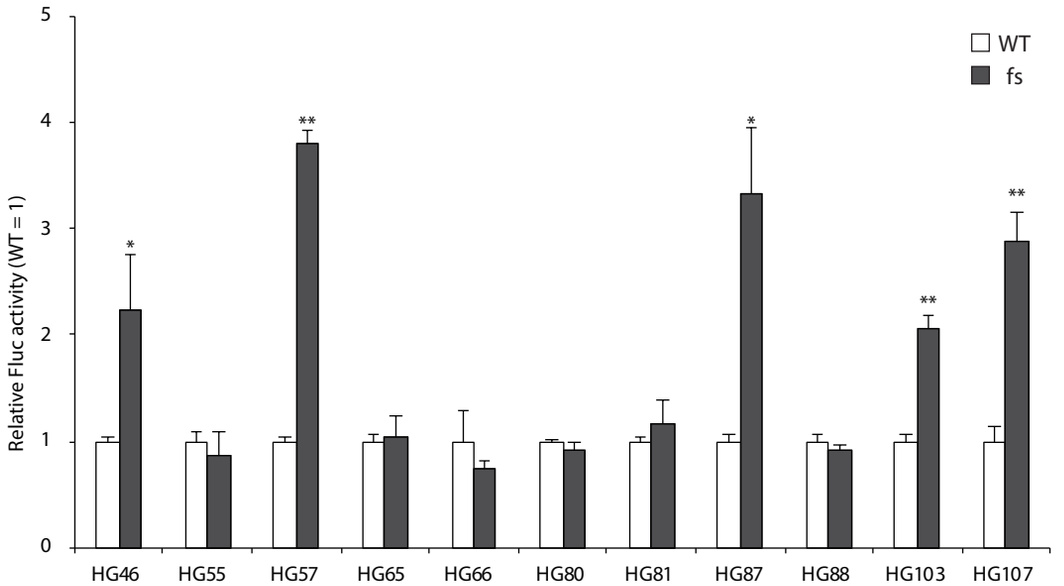


Figure 6

A


35S::UTR(WT):Fluc
35Spro
CPuORF(WT)
Fluc
ter
35S::UTR(fs):Fluc
35Spro
CPuORF(fs)
Fluc
ter

B


Relative Fluc activity (WT = 1)
0
1
2
3
4
5
WT
fs
*
**
*
**
**
HG46
HG55
HG57
HG65
HG66
HG80
HG81
HG87
HG88
HG103
HG107