

Aberystwyth University

Identification of Stipules reduced, a leaf morphology gene in pea (Pisum sativum)

Moreau, Carol; Hofer, Julie Margareth; Eléouët, Morgane ; Sinjushin, Andrey; Ambrose, Mike ; Skøt, Kirsten; Blackmore, Tina; Swain, Martin; Hegarty, Matthew; Balanza, Vincent; Ferrándiz, Cristina; Ellis, T. H. Noel

Published in:
New Phytologist

DOI:
[10.1111/nph.15286](https://doi.org/10.1111/nph.15286)

Publication date:
2018

Citation for published version (APA):

Moreau, C., Hofer, J. M., Eléouët, M., Sinjushin, A., Ambrose, M., Skøt, K., Blackmore, T., Swain, M., Hegarty, M., Balanza, V., Ferrándiz, C., & Ellis, T. H. N. (2018). Identification of Stipules reduced, a leaf morphology gene in pea (*Pisum sativum*). *New Phytologist*, 220(1), 288-299. <https://doi.org/10.1111/nph.15286>

General rights

Copyright and moral rights for the publications made accessible in the Aberystwyth Research Portal (the Institutional Repository) are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the Aberystwyth Research Portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the Aberystwyth Research Portal

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

tel: +44 1970 62 2400
email: is@aber.ac.uk

1 **Identification of *Stipules reduced*, a leaf morphology gene in pea (*Pisum sativum*).**

2

3

4 Carol Moreau², Julie M.I Hofer^{1,5}, Morgane Eléouët¹, Andrey Sinjushin³, Mike Ambrose²,
5 Kirsten Skøt¹, Tina Blackmore¹, Martin Swain¹, Matthew Hegarty¹, Vicente Balanzà⁶,
6 Cristina Ferrándiz⁶, and T.H. Noel Ellis^{1,4}

7

8 ¹ IBERS, Aberystwyth University, Plas Gogerddan, Aberystwyth, SY23 3EE, UK

9 ² John Innes Centre, Norwich, NR4 7UH, UK

10 ³ Genetics Department M.V. Lomonosov Moscow State University, 119991, Moscow, Russia

11 ⁴ School of Biological Sciences, University of Auckland, 1142, New Zealand

12 ⁵ Faculty of Education and Social Work, University of Auckland, 1023, New Zealand

13 ⁶ Instituto de Biología Molecular y Celular de Plantas, CSIC, 46022, Valencia, Spain

14

15 Corresponding Author Noel Ellis

16 e-mail (preferred) thnoeellis@gmail.com

17 alternative e-mail tell822@aucklanduni.ac.nz

18 Telephone +64 27 636 7531

19 Word count Total 6,467

20 Word count by section: Introduction (498) Materials and
21 Methods (1,101), Results (2,644), Discussion (2,187), and
22 Acknowledgements (37)

23 Figures 7 Figures, 3 colour

24 Tables 1

25 Supporting information Four files in total. File 1 has 10 supplementary figures Fig. S1 -
26 Fig. S10. File 2 is Methods S1 containing a Python script and
27 Files 3 and 4 are data files, Notes S1 and Notes S2, with
28 sequence related information.

29

30

31

32

33

34 **Summary**

- 35 ● Pea (*Pisum sativum* L.) is one of relatively few genetically amenable plant species with
36 compound leaves. Pea leaves have a variety of specialised organs: leaflets, tendrils, pulvini
37 and stipules which enable the identification of mutations that transform or affect distinct
38 parts of the leaf. Characterisation of these mutations offers insights into the development
39 and evolution of novel leaf traits. The previously characterised morphological gene
40 *Cochleata*, conferring stipule identity, was known to interact with *Stipules reduced*, which
41 conditions stipule size in pea, but the *Stipules reduced* gene remained unknown.
- 42 ● Here we analysed Fast Neutron irradiated pea mutants by Restriction site Associated DNA
43 sequencing.
- 44 ● We identified *Stipules reduced* as a gene encoding a C2H2 zinc finger transcription factor
45 that is regulated by *Cochleata*. *Stipules reduced* regulates both cell division and cell
46 expansion in the stipule.
- 47 ● Our approach shows how systematic genome-wide screens can be used successfully for
48 the analysis of traits in species for which whole genome sequence is not available.

49

50 **Key words**

51 Pea (*Pisum sativum*) - leaf - stipule – RAD sequencing - Fast Neutron – mutant - C2H2 zinc
52 finger

53

54 **Introduction**

55 Leaves have a variety of functions, notably photosynthesis, transpiration and canopy
56 shading. The shape of the leaf impacts the energy efficiency and water economy of the plant,
57 thus the genetics underlying variation in leaf form is of interest. In pea, a suite of leaf
58 development mutants affecting pattern formation is known (Marx, 1987); these mutations
59 affect the arrangement or identity of organs on the compound pea leaf. Mutations in six of
60 these the genes have been characterised at the molecular level (Chen *et al.*, 2012, Couzigou *et*
61 *al.*, 2012, Sainsbury *et al.*, 2006, Zhuang *et al.*, 2012 Hofer *et al.*, 2009, 1997) and for each,
62 at least one null allele has been generated by Fast Neutron (FN) bombardment in the line
63 JI2822 (Domoney *et al.*, 2013). These mutants offer a convenient opportunity for studying
64 gene interactions in a single genetic background.

65 Most of the 28 mutant alleles characterised so far in this population are deletions of
66 the entire gene; the deletion end-points are uncharacterised because they are far from the
67 known sequence. Exceptions are two small deletions, one (of *a2*) is 22bp (Hellens *et al.*,
68 2010) and the other (of *apu*) is 1.4kb (Chen *et al.*, 2012) and one *b* allele is known to be a
69 structural rearrangement (Moreau *et al.*, 2012). These data suggest that the FN deletions are
70 often large with respect to the size of a gene and are therefore amenable to systematic
71 searches for deleted sequences. Deletion mutant alleles in this genetic background have been
72 identified using AFLP markers (Hofer *et al.*, 2009); here we investigated whether AFLP
73 markers could be replaced by restriction-site-associated (RAD) sequence markers (Miller *et*
74 *al.*, 2007, Baird *et al.*, 2008).

75 We focussed attention on *Stipules reduced* (*St*; Pellew & Sverdrup 1923), a classical
76 leaf morphology gene not yet cloned. The unusually large stipules of pea are replaced by
77 smaller organs in the *st* mutant, more typical of close legume relatives in the genera *Lathyrus*,
78 *Vicia* and *Lens*. The *st* stipules are also simpler in form than the wild type, lacking the
79 serrated basal frill or mantle. In contrast to *Coch* (Couzigou *et al.*, 2012), the *St* gene is not
80 required for stipule identity, rather it is a determinant of organ size, due to the early loss of
81 marginal meristem activity (Meicenheimer *et al.*, 1983). Large stipules are rare in legumes,
82 but are found in *Pisum* and a few closely related *Lathyrus* species, notably *L.aphaca*, and in
83 several other more distantly related taxa (Lewis *et al.* 2005). Whether stipule size has any
84 adaptive significance is unknown, but the large stipules of pea enclose the developing shoot
85 apical meristem and may protect it from frost damage or insect herbivory, or create a
86 microclimate buffering the apex against changes in humidity or temperature. If there is some
87 adaptive significance this could be reflected in sequence divergence parameters associated

88 with genes regulating stipule size. The isolation and characterisation of *St* as a C2H2 zinc
89 finger gene will enable such studies in future.

90

91 **Materials and Methods**

92 **Plant material**

93 The line JI2822, a Recombinant Inbred Line (RIL) derived from a cross between JI15
94 and JI399, which has been described elsewhere (Lewis *et al.*, 2005, Hofer *et al.*, 2009), was
95 mutagenised using Fast Neutrons from the ²⁵²Cf facility at Oak Ridge National Laboratory,
96 USA (Domoney *et al.*, 2013). Among the M2 progeny from these lines one mutant
97 (FN2122/2) had a phenotype resembling *st* and a complementation test showed that it was
98 allelic to *st*. Two additional mutant lines (FN1889/3 and FN2002/7) resembling, and allelic
99 to, *creep* (Sidorova, 1975) were used as controls. Individual plants here designated FN1889-
100 BC3, FN2002-BC3 and FN2122-BC4 were obtained by back crossing to JI2822 selections
101 from M2 families that exhibited either the *creep* or *st* mutant phenotypes, followed by selfing
102 and repeating this for three or four backcross cycles (as indicated by BC3 or BC4). Plants
103 were finally selfed to extract homozygous mutants. M2 lineages have been estimated to carry
104 an average of seven independent deletions (Domoney *et al.*, 2013). After three cycles of
105 backcrossing this is expected to be reduced to a single deletion.

106 The cross between the cultivars Flagman and Filby was generated at Lomonosov Moscow
107 State University. The *st* mutant lines; JI17, JI132, JI143, JI924, JI1201, JI2160, JI2653,
108 JI3528, JI3529, JI3530 JI3531 and JI3537 were obtained from the John Innes Pisum
109 Germplasm collection, as were the wild type lines JI813 (cv Vinco), JI2822, JI3132 (cv
110 Auralia), JI3538 (cv Paloma) and JI3539 (cv Virtus). The mutants FN2122 (*st*) and FN3185
111 (*coch*) are available from the John Innes Pisum Germplasm collection as JI3604 and JI3596
112 respectively.

113 **Nucleic acid preparation**

114 DNA was prepared as described by Ellis *et al.*, (1984), and RNA was prepared from
115 seedling apices as described by Hofer *et al.*, (2009).

116 **Genetic Mapping**

117 Genetic markers were analysed in the JI281xJI399 recombinant inbred population by
118 simple matching with respect to existing markers. The identity of individual DNA samples
119 prepared from this population was verified with PDR1 SSAP markers with the +TT primer
120 combination (Ellis *et al.*, 1998), using fluorescent primers essentially as described by Knox *et*
121 *al.*, (2009).

122 **RAD tags and sequencing**

123 PstI digested DNA from four single plants (an FN2122-BC4 individual representing
124 the mutant *st* allele; individuals of the two *creep* lines and JI2822, all representing the wild
125 type *St* allele) was ligated to a RAD adaptor, sheared to the size range 100 - 600 nt and then
126 ligated to the second adaptor prior to selective amplification as previously described
127 (Domoney *et al.*, 2013). We used the method of Baird *et al.*, (2008) with PstI instead of SbfI
128 (or EcoRI) digestion; the PstI recognition site is internal to that of SbfI and the two enzymes
129 leave the same overhang, so standard RAD sequencing primers were used for the sequencing
130 reactions (Miller *et al.*, 2007). Sequence reads were compiled into unique tags using FASTX
131 Collapser (downloaded from http://hannonlab.cshl.edu/fastx_toolkit/) with the read depth per
132 tag noted in the tag identifier. JI2822 was used as a reference and a Python script (Methods
133 S1) was used to collate identical tags. This gave lists of tags of known read depth from each
134 individual. Tags present in JI2822 but absent from the mutants were identified using Excel.

135 **DNA sequence analysis**

136 DNA sequence was obtained directly from an Illumina HiSeq 2500. Sequence
137 characterisation of specific loci in pea and *Lathyrus* samples was performed using a BigDye
138 Terminator v3.1 cycle sequencing kit (Applied Biosystems) using the primers listed in Notes
139 S1 (for *Rca*) and S2 (for *St*). Pea and *Lathyrus* DNA sequences are deposited in Genbank
140 with the accession numbers MF033127-MF033135.

141 Sequences related to *St* were obtained by BLAST analysis in the Legume Information
142 System database (Dash *et al.*, 2016, <https://legumeinfo.org>) and the pea gene atlas (Alves-
143 Carvalho *et al.*, 2015, <http://bios.dijon.inra.fr/FATAL/cgi/pscam.cgi>). For phylogenetic
144 analysis those predicted amino acid sequences with an E value $< 10^{-20}$ over at least 40% of
145 the sequence were selected for alignment using MUSCLE and the phylogenetic tree was
146 constructed using PROML of the PHYLIP package with 1000 bootstraps. *RBE* was obtained
147 from The Arabidopsis Information Resource (<https://www.arabidopsis.org/index.jsp>). The
148 tree was drawn using DarWin5 (Perrier *et al.*, 2003).

149 **Quantitative PCR**

150 cDNA was synthesized from total RNA prepared as described by Hofer *et al.*, (2009)
151 using primers described in Notes S2. A Roche LightCycler 480 was used for qPCR
152 experiments and amplicons were detected with SYBR green. Three biological and three
153 technical replicates were used for each genotype. Reaction volumes were 15 μ l with 0.25 μ M
154 primer. The cycling regime was 95°C for 5min, followed by 45 cycles of 95°C for 20s, 62°C
155 for 15s, and 72°C for 15s. Analysis was done using the $\Delta\Delta$ Ct method (Livak & Schmittgen,

156 2001) and corrected for primer efficiencies (Pfaffl, 2004). The average Cp value of the nine
157 reactions was normalized relative to Actin.

158 **Phloroglucinol staining**

159 The nodal vasculature of *St* vs *st* plants was investigated using phloroglucinol
160 staining. F2 progeny from a cross between cultivars Flagman (*St*) and Filby (*st*) were sown in
161 a glasshouse. Nodes (4th and above) with stipules and short portions of petiole were excised
162 from individual plants and fixed in 70% ethanol. After storing in ethanol for 2 months, nodes
163 were soaked in 0.1 M phloroglucinol in 96% (w/v) ethanol for 12 h and then transferred to
164 concentrated HCl for 1 h. After washing specimens in distilled water, the acid-macerated
165 epidermis was partially removed. Images were captured from an Olympus SZ61
166 stereomicroscope (Olympus Corporation, Japan) using an ES-Experts 6600 digital camera
167 (ES-Experts, Russia).

168 **Epidermal cell area estimations**

169 Epidermal cell replicas were made from the abaxial (lower) surface of *St* and *st*
170 segregants of the Flagman x Filby cross using nail polish. These were examined
171 microscopically (Nikon Eclipse Ci - Nikon, Japan) and photographed with a digital camera
172 (Nikon DS-Vi1). Measurements were carried out on digital images using ImageJ 1.45m
173 software (National Institute of Health, USA). Four zones were selected on the stipule lamina
174 with respect to position on the proximodistal axis (proximal or distal) and the mediolateral
175 axis of the leaf as a whole, where medial is closer to the petiole and lateral is away from the
176 petiole. Thirty cells were measured in each zone. Statistical analysis was carried out using
177 Statistica 8 (Statsoft, USA).

178 ***In situ* hybridisation**

179 An RNA *in situ* hybridisation probe was derived from an *St* PCR product using primers p5 &
180 p6 (Notes S2) using a T7 promoter in the reverse primer. *St* RNA was labelled with
181 digoxigenin. Wax embedded sections were prepared from apices of JI2822 and the isogenic
182 lines FN3185 (*coch* mutant) and FN2122 (*st* mutant). *In situ* hybridisations were carried out
183 according to Balanzà *et al.*, (2018).

184

185 **Results**

186 **Identification of a candidate gene.**

187 Four genotypes were analysed; JI2822 (wild type *St*), FN2122/2 (*st*) as illustrated in Fig. 1
188 and Fig. S1, and two *creep* mutants FN1889/3 and FN2002/7 (both wild type *St*), the latter

189 used as independent controls. The *creep* mutant phenotype is not relevant and will not be
190 discussed further.

191 A preliminary AFLP screen, following the method of Hofer *et al.*, (2009), comparing
192 JI2822 (wild type *St*) with FN2122/2 (*st*) identified a PCR product presence/absence
193 polymorphism that distinguished these two genotypes (Fig. S2). Sequencing this amplicon
194 showed that it corresponded to a gene encoding Ribulose-1,5-bisphosphate
195 carboxylase/oxygenase (*Rca*). Further analysis confirmed that this sequence was missing
196 from FN2122/2 (Notes S1). The gene *Rca1* was mapped to the middle of pea linkage group
197 III on the genetic map of the JI281xJI399 RIL population, consistent with a location close to
198 the *St* locus (Pellew & Sverdrup 1923, de Winton 1928). The *Rca1* gene was sequenced from
199 JI2822 and JI1201 (an *st* mutant), but no lesion was found in the JI1201 sequence (Notes S1),
200 suggesting that *Rca1* is unlikely to correspond to *Stipules reduced*.

201 No other AFLP fragment differences were observed, so we presumed that some
202 fragments were masked by similar sized amplicons. We reasoned that any hidden
203 polymorphisms would be revealed by sequencing RAD tags associated with PstI/MseI
204 genomic DNA fragments. RAD libraries from the four genotypes were produced and 42bp
205 sequence reads adjacent to the PstI site were generated (including the 3' terminal G of the
206 PstI site). Sequences that did not begin with a G were ignored. This generated a total of ca.
207 25, 21.8, 22.9 and 42.8 million reads from FN1889-BC3, FN2002-BC3, FN2122-BC4 and
208 JI2822 respectively. These sequences were collapsed, using the programme FASTX
209 Collapser, into 480,671 individual unique RAD sequence tags shared with JI2822; the
210 number of reads was recorded in the tag name. On average there were approximately 90 reads
211 per tag, but there was a considerable range of read depths (Fig. S3). 331,668 tags occurred
212 once, 55,481 had a read depth <10 and >1 and a smaller number of sequences were very
213 common; 27 had a read depth >50,000 and were derived from chloroplast DNA or repetitive
214 elements of the nuclear genome. Although most tags had a low read depth, most reads (ca. 40
215 million) were for tags with a read depth greater than 100, and fewer reads (ca. 2 million) were
216 for tags with a lower read depth. Rare sequences were considered to be sequencing errors
217 (Domoney *et al.*, 2013) and were not investigated further.

218 Identical sequence tags in each of the four genotypes were identified with a simple
219 Python script (Methods S1) and collated in an Excel sheet that recorded the sequence, its
220 numerical identifier and read depth. For a given read depth from one genotype the read depth
221 in the other three genotypes was over-dispersed with respect to a Poisson distribution: the
222 variance was between 110 and 11,365 times greater than the mean. This suggested that, for

223 tags with low read depths, the absence of a tag from one genotype could be due to sampling
224 variation. We therefore needed to identify a read depth where the signal for a missing
225 sequence could be found among the noise of those absent by chance alone, as discussed by
226 Domoney *et al.*, (2013).

227 In order to screen for tags unlikely to be missing from FN2122/2 by chance alone a
228 cut-off read depth of the tags needed to be determined. The frequency distribution of read
229 depths (Fig. S3a) reflects the genomic copy number of the corresponding sequences; those
230 present once per genome should be a common class. In a log-log plot of read depth vs the
231 number of tags with that read depth, there is a shoulder in the plot between a read depth of ca.
232 100 and ca. 1000. A sample of sequences known to correspond to single copy genes that
233 carry at least one PstI site was found to have tag read depths of 433 ± 272 (mean \pm stdev, $n =$
234 34; Fig. S3b), consistent with these being in the shoulder between the read depths of 100 and
235 1000. There were 31,879 of these tags and a total of 9,913,708 reads in this group, giving an
236 average read depth of 310 for single copy sequences. Analysis of JI2822 tags showed that for
237 read depths less than 150, a high proportion were unique to that genotype (Domoney *et al.*,
238 2013). On average, sequence tags with a read depth of 100 for the *st* mutant (FN2122-BC4)
239 corresponded to tags with a read depth of approximately 200 in JI2822, so a read depth of
240 150 was chosen as a compromise cut-off value between an excessive false discovery rate and
241 a failure to detect genuinely missing sequences.

242 A total of 43,342 sequence tags were examined. These had a read depth of 150 or
243 more in at least one of the samples representing the wild type *St* allele. Of these, 40,722 had a
244 read depth of at least 150 in JI2822 and 40,020 were present in all samples (Fig. S4). These
245 tags had a read depth of 92 ± 41 (mean \pm stdev) in JI2822. 1,625 of these sequence tags were
246 identified as being absent from the *st* mutant FN2122/2 and having at least 150 reads in at
247 least one of the other three genotypes. Of these, 171 were missing from all the FN genotypes
248 and 460 were absent from either FN1889/3 or FN2002/7. These 631 tags were eliminated
249 from further analysis because both FN1889/3 and FN2002/7 carry a wild type *St* allele (Fig.
250 S4). The remaining 994 tags had average read depths of 209, 95 and 111 reads in JI2822,
251 FN1889/3 and FN2002/7 respectively. Although the number of tags identified in this way
252 suggests a high false discovery rate, the probability that tags from both sides of a single PstI
253 site are missing by chance alone is lower: the square of the false discovery rate. The
254 identification of paired tags was therefore of interest.

255 The 994 tags absent from FN2122/2 *st* mutant reads, but present in all other
256 genotypes, were used in a BLASTn search of transcript sequences downloaded from the

257 USDA pea unigene database (now available at
258 https://www.coolseasonfoodlegume.org/sativum_unigene_v2). A set of 498 unigene contigs
259 were identified that corresponded to these tags. This set was then used as the subject of a
260 BLASTn query with all (including read depths <100) FN2122/2 sequence tags. Unigenes that
261 matched one or more tags were eliminated from further investigation, because at least part of
262 these genes is present in the *st* mutant FN2122/2. This eliminated 455 unigenes, leaving 43
263 for which no tag was found. These 43 unigenes were therefore potential candidates for *St* and
264 were aligned against the medicago (*Medicago truncatula*) genome sequence (v3.5.1; Fig. S5).

265 Among these unigenes was *Rca* (contig19432), and a C2H2 zinc finger transcription
266 factor (contig27619). In the medicago genome, the corresponding sequences were closely
267 linked. The medicago C2H2 gene (Medtr5g080660) was annotated as *RABBIT EARS (RBE)-*
268 *like* (Takeda *et al.*, 2004; Krizek *et al.*, 2006). Since the pea and medicago genomes align
269 well (Duarte *et al.*, 2014), the position of *St* on pea linkage group III was expected to
270 correspond to the middle of medicago pseudomolecule 3. At first, this *Rca*-C2H2 region of
271 the medicago genome appeared to correspond to a break in collinearity with the pea genome,
272 however, a subsequent release of the medicago genome sequence (version 4.0
273 <https://legumeinfo.org/genomes/gbrowse/Mt4.0>) contains the sequence Medtr3g068095,
274 which is a better match to contig27619. An *Rca* gene (Medtr3g068030) is located ca. 50kb
275 distant from Medtr3g068095 and both lie in the expected syntenic region.

276 As described above, a presence/absence polymorphism for contig19432 (*Rca1*)
277 existed, but there was no sequence polymorphism associated with *Rca* that distinguished *St*
278 and *st* genotypes (Notes S1). A presence/absence polymorphism in wild type versus mutant
279 was detected by PCR primers for contig27619, suggesting it was a better candidate for the *St*
280 gene.

281 **Confirmation of a candidate gene for *Stipules reduced***

282 Genomic sequences corresponding to contig27619 from JI2822 (*St*), JI813 (*St*) and
283 JI143 (*st*), identified a SNP corresponding to an in-frame stop codon in the JI143 coding
284 sequence. This was the only observed sequence difference between JI143 (with reduced
285 stipules) and the other two genotypes (with normal stipules). Allele-specific PCR primers
286 were designed to detect this SNP in a JI813xJI143 F2 population segregating for *st*. No
287 recombination between the phenotype and SNP genotype was observed (Fig. S6).

288 Genomic sequences corresponding to contig27619 were obtained from a set of wild
289 type and *st* mutant *Pisum* lines. All of the sequences from the mutant lines carried lesions in
290 the predicted gene sequence as illustrated in Fig. 2 (see also Fig. S1 and Notes S2). Four

291 alleles resulted in a premature stop codon, two alleles had missense mutations at the 3' end of
292 the coding sequence, and two alleles which failed to amplify any sequence from the gene
293 were consistent with deletions.

294 **The structure of the *St* Gene**

295 The *St* gene is predicted to have a short upstream open reading frame (uORF) that is
296 in frame with the main ORF. Of the two start ATGs, the main ORF ATG has a better fit to
297 the Kozak consensus sequence for plants (Lütcke *et al.*, 1987). There is a short intron
298 predicted with respect to the unigene sequence (Notes S2), which was confirmed by PCR
299 (Fig. S7). The predicted amino acid sequence (Notes S2) carries two recognisable domains.
300 Towards the N terminus, beginning at C62, the sequence CxxCx₁₂HxxxH corresponds to a
301 C1–liG family C2H2 zinc finger domain (Englbrecht *et al.*, 2004), while at the N terminus
302 the sequence LDLELRL (beginning at L233) resembles an ERF-associated amphiphilic
303 repression motif (EAR domain, Ohta *et al.*, 2001).

304 *Pisum* is embedded in the genus *Lathyrus* (Schaefer *et al.*, 2012) so three *Lathyrus*
305 sequences were investigated; two were *L. odoratus* varieties (Lucy Hawthorne and Dorothy
306 Eckford, with small stipules) and the third was *L. aphaca* (which has large stipules). The *L.*
307 *odoratus* and *L. aphaca* nucleotide sequences were 95% and 94% identical to *St* and all three
308 *Lathyrus* sequences contained an intron with a 4 bp insertion with respect to *Pisum*,
309 furthermore *L. aphaca* carried a 3bp deletion in the coding sequence (Notes S2). The amino
310 acid sequence of *St* is 93% identical to all three *Lathyrus* sequences, the two *L. odoratus*
311 sequences are 99% identical to each other and 91% identical to *L. aphaca*. There were 5
312 residues that distinguished the large-stipuled species, pea and *L. aphaca*, from the small-
313 stipuled species, *L. odoratus* and *M. truncatula* (Notes S2).

314 A BLASTp search using the predicted *St* amino acid sequence identified homologues
315 in several sequenced legume species (Notes S2), and a closely related pea sequence
316 (PsCam039889) was identified in the pea gene atlas (Alves-Carvalho *et al.*, 2015,
317 <http://bios.dijon.inra.fr/FATAL/cgi/pscam.cgi>). These sequences, together with the
318 *Arabidopsis thaliana* RABBIT EARS were aligned and compared using MUSCLE and
319 PHYLIP, with the purpose of determining the most likely orthologue of *Stipules reduced* in
320 medicago, The zinc finger domains could be aligned unambiguously, permitting the
321 construction of a phylogenetic tree (Fig. 3) which supports *St* as the orthologue of
322 Medtr3g068095, and Medtr5g080660 as the orthologue of PsCam039889. A comparison
323 between *St* and Medtr3g068095, the most closely related medicago nucleotide sequence, is
324 shown in Fig. 4. There are regions of sequence conservation, notably including the zinc

325 finger and EAR domain coding sequences, but there is also an abundance of non-synonymous
326 changes, in addition to the difference in intron structure. The medicago intron is 72 bp,
327 compared to the 83 bp pea intron. The K_a/K_s ratio for the whole alignment is 1.29 (36/28)
328 which is not significantly different from 1 ($\chi^2 = 1$, $p=0.32$). For the sequence following the
329 zinc finger domain this value rises, ($K_a/K_s = 28/14 = 2$, $\chi^2 = 4.67$, $p= 0.035$) and at some
330 locations K_a/K_s is in considerable excess of the mean for the whole sequence, indicative of
331 diversifying selection (Fig. 4).

332 Considering the alignment as a whole (Fig. 4), there is no significant excess of amino
333 acid differences in either of these two sequences, however the contribution from extra amino
334 acids predicted in the Medtr3g068095 sequence is ignored. Both sequence conservation and
335 diversification are therefore manifest in *St*.

336 **Phenotypic description**

337 The *st* mutation conditions a reduction in the size of the stipule (Fig. 1) and there are
338 two mutant phenotypic classes (Pellew and Sverdrup, 1923; Apisitwanich and Swiecicki,
339 1992). With respect to wild type, the *st* stipule lamina is reduced by about 90% and *butterfly*
340 *stipules* (*st^{bs}*) lamina by about 70% (Fig. S8). The least severe phenotypes are conditioned by
341 mutations that affect the EAR domain in JI2653 and JI3521 (D234N) and JI3530 (L235P),
342 with the D234N missense mutation in the *st^{bs}* alleles being the least severely affected.

343 Meicenheimer *et al.*, (1983) concluded that the *st* mutant stipule phenotype derives
344 from an early cessation in marginal meristematic activity. We were interested in whether this
345 early cessation of cell division fully accounts for the reduction in organ size or whether there
346 were also cell size differences. Accordingly, we measured cell size on the abaxial epidermis
347 in four positions. The results (Table 1) show that cells are smaller in *st* mutants in all zones
348 except for the proximal lateral sector, corresponding to the basal frill (mantle) in wild type,
349 but only differences in the medial position were statistically significant. The difference in cell
350 size between the medial and lateral positions in the wild type is significantly different (Table
351 1, Fig. S9), while there is no significant difference between these values for the *st* mutant.
352 Therefore, reduction of cell size, particularly in the proximal medial region of the stipule,
353 also contributes to the *st* phenotype.

354 A comparison of the vascularization of the *st* type allele (Fig. 5) shows that, while the
355 vascular strands in the stem and the leaf of the *st* mutant have the configuration originally
356 described for *Pisum* (Kupicha, 1975), the pattern of vascularization of the *st* stipule is less
357 complex. For the *st* mutant, the main disturbance to vascularisation is within the basal

358 elaboration or mantle of the stipule (strand 7, Fig. 5). Apart from its effect on overall stipule
359 size, the action of *St* is thus most apparent in the proximal part of the stipule.

360 **Expression of *St***

361 In order to understand the process of stipule development further, we decided to
362 ascertain whether the identity of the stipule affected *St* expression. The *coch* mutant, which
363 replaces the stipule with a leaf-like structure, thus changing the identity of the organ at that
364 position, allowed us to address this question. We analysed *St* expression of in a *coch* mutant
365 and *Coch* expression in an *st* mutant by q-PCR (Fig. 6). This analysis shows that *St* transcript
366 abundance is dependent on *Coch*, whereas *Coch* expression is unaffected by *St*, consistent
367 with *St* expression being dependent on organ identity.

368 We further investigated the expression of *St* by *in situ* hybridisation (Fig. 7 and Fig.
369 S10) in wild type and isogenic *coch* and *st* mutants. These results show that *St* is expressed in
370 stipule primordia and developing stipules of wild-type plants. *St* expression was detected at
371 plastochron 1 until plastochron 8 and was strongest on the adaxial side of the stipule (Fig.
372 7a), but weak in stipule vascular tissue (Fig. 7b). In young primordia the transcript appears in
373 two symmetrically placed regions, presumably either side of the developing vasculature (Fig.
374 S10c). The *St* transcript was absent from floral tissues (Fig. 7a, 7b), however a signal was
375 detected on the flank of the inflorescence (Fig. 7a, b and d), consistent with expression in
376 bracts.

377 No signal was detected in the *st* deletion mutant (Fig. 7e, 7f), which shows that the
378 hybridization probe did not identify another *St*-related transcript. The *coch* mutant (Fig 7c,
379 7d) showed very weak *St* expression in stipules and bracts. This weak signal is consistent
380 with the results of the q-PCR (Fig. 6).

381

382 **Discussion**

383 **Methodology**

384 Genome wide sequencing can detect mutations in mutant populations and so identify
385 candidate genes in forward genetic screens (Tsai *et al.*, 2011), but this depends on the
386 availability of a reference genome sequence (Hwang *et al.*, 2015, Campbell *et al.*, 2016)
387 which is not available for pea. Insertion mutagenesis can also tag genes facilitating their
388 isolation (Schäuser *et al.*, 1999, Tadege *et al.*, 2008, Urbański *et al.*, 2012), but in pea
389 insertion mutagenesis is not available. Here we investigated an alternative approach in pea
390 and demonstrated that RAD sequencing can identify sequences deleted from FN mutants.

391 The nature of mutations induced by ionising radiation depends on several factors,
392 including the type and energy of the radiation and the cellular response to the free radical
393 induced damage. When considering FN mutagenesis as a methodology for gene identification
394 these factors need to be taken into account. The studies of Belfield *et al.* (2012) and Li *et al.*
395 (2016) describe sequence variation associated with FN mutagenesis in Arabidopsis and rice
396 respectively. Both studies attribute many types of mutation to FN mutagenesis, of which 36%
397 were deletion mutations and 50 - 60% were single base substitutions. In Arabidopsis the
398 deletions were small with only one greater than 55bp, while in rice 10% of the deletions were
399 greater than 1kb and two (out of 873) were greater than 1Mb. These results contrast with our
400 observations in pea, where no FN-induced allele (of 28 alleles distributed over 10 loci,
401 Domoney *et al.* 2013, McAdam *et al.* 2017), was a single base change, suggesting that single
402 base changes were relatively rarer in pea than in rice or Arabidopsis. It is notable that in these
403 three examples the proportion and size of deletions increases with increasing genome size.
404 The number of ways in which a deletion of x bp can occur, such that it that disrupts fewer
405 than y genes, is a combinatorial function of intergenic distance, so it is perhaps not surprising
406 that in pea, with a large genome and low gene density, that large deletions are more common.

407 The successful detection of the presence/absence of *St* depended on the large size of
408 the FN-generated deletion. Previous studies in this population had shown that large deletions
409 were common (Sainsbury *et al.*, 2006, Wang *et al.*, 2008, Hofer *et al.*, 2009, Hellens *et al.*,
410 2010, Moreau *et al.*, 2012, Chen *et al.*, 2012, Couzigou *et al.*, 2012, Domoney *et al.*, 2013).
411 The absence of at least two adjacent genes in FN2122/2 suggests a single large deletion has
412 occurred in this line in the region encompassing both the *Rca* and *St* loci. Precedent for such a
413 scale of deletion induced by FN in pea is the joint deletion of *alae keel-like (k)* and
414 *Convicilin (Cvc)* (Domoney *et al.*, 2013).

415 Our approach depended on reliable detection of a tag corresponding to a PstI site
416 when it was actually present, so that any tag missing in a mutant would warrant further
417 investigation. The variation in read depth of single copy sequences was very high and we
418 found that a cut-off value of 150 reads was an adequate compromise between sensitivity and
419 reliability. In JI2822, the *St* tags had read depths of 432 and 323 (Notes S2), while the *Rca*
420 tags were 327 and 693 (Notes S1) consistent with the expected read depth of single copy
421 genes (Fig. S3).

422 RNA-seq is an alternative approach (McAdam *et al.*, 2017) which may be
423 advantageous for large genome species such as pea, where many of the RAD-seq reads are
424 effectively wasted because they derive from repetitive sequences not represented in the

425 transcriptome. However, genes involved in developmental patterning may be expressed in
426 very few cells and therefore would be unusually rare in the transcriptome, so for these types
427 of gene, the advantage of RNA-seq may fail to materialise. Furthermore, low abundance
428 sequences would be most susceptible to stochastic loss.

429 The current lack of a genome sequence for JI2822 (the mutagenized line) hindered the
430 identification of paired RAD tags flanking the same PstI site. For this reason, *M. truncatula*,
431 the closest relative to pea for which genome sequence is available, was used in conjunction
432 with pea transcriptome sequence data. The sequences of many of the RAD tags presumed
433 missing from FN2122/2 corresponded to sequences distributed throughout the medicago
434 genome (Fig. S5), as would be expected of tags missing by chance.

435 It has been estimated that each M2 from this FN population has, on average, 7 independent
436 deletions (Domoney *et al.*, 2013), suggesting that the BC4S1 individual studied here would
437 not carry more than one deletion.

438 Publicly available transcriptome data were available for pea (Fransen *et al.*, 2012,
439 Kaur *et al.*, 2012 and the USDA database at
440 https://www.coolseasonfoodlegume.org/sativum_unigene_v2); but the work of Alves-
441 Carvalho *et al.*, (2015) was not available at the time this analysis was initially performed.
442 Alignment of the RAD tags to the transcriptome sequences from the USDA database enabled
443 the identification of paired sequences corresponding to the two sides of a PstI site, providing
444 independent evidence for a deletion of the PstI site. This permitted the identification of a
445 C2H2 zinc finger sequence as a candidate for the *St* gene, which was confirmed by the
446 sequence analysis of independently obtained mutant alleles.

447 Analysis of soybean FN mutant populations has highlighted the advantages of other
448 genome wide approaches such as resequencing or array hybridization when a reference
449 genome sequence is available (Hwang *et al.*, 2015, Campbell *et al.*, 2016); these two studies
450 also emphasise that in some cases simple deletions may not be the most frequent type of
451 mutation. In both these cases genomic rearrangements rather than deletions were detected. It
452 may be that the larger genome of pea, with interspersed repetitive elements, permits large
453 deletions that are non-lethal. The approach we took, in this and previous studies (Chen *et al.*,
454 2012, Hofer *et al.*, 2009), screened for loss of PstI sites, so we may have missed
455 rearrangements. Although our results demonstrate that a complete genome sequence of the
456 target species is not required for this method of gene identification, RAD based deletion
457 screens would be easier if extensive sequence were available.

458 **The role of *Stipules reduced* in the pea compound leaf.**

459 *St* is required for stipule enlargement (Meicenheimer *et al.*, 1983, Sinjushin *et al.*,
460 2011) rather than stipule identity, consistent with *St* being required for the elaboration of the
461 basal frilled mantle. The reduced vascularisation of the *st* stipule may be a consequence of
462 reduced stipule elaboration (Fig. 5) reminiscent of the reduced petal phenotype of the *rbe*
463 mutant in *A. thaliana* (Takeda *et al.*, 2004), *RBE* being the most closely related Arabidopsis
464 sequence to *St* (over the whole length of the predicted amino acid sequence). In the *st* mutant
465 there is no difference in cell size in the medial vs lateral position of the stipule, whereas there
466 is a significant difference in wild type (Table 1, Fig. S9) showing that differences in cell
467 expansion between zones of the stipule is dependent on *St*. Within the stipule, these *St*-
468 dependent cell sizes may reflect medial vs lateral identity.

469 The *st^{bs}* mutant phenotype is weaker than the other *st* mutants (Fig. 1, Fig. S8) and
470 results from a mis-sense (D234N) mutation within the C terminal EAR domain rather than
471 non-sense mutation. The L235P substitution in JI3530 also occurs in the EAR domain, but
472 the phenotype of this mutant is more severe than *st^{bs}* (Fig. 1) suggesting that this
473 transcriptional repressor domain (Ohta *et al.*, 2001) is required for *St* function. Like *rbe*
474 (Huang *et al.*, 2012, Huang & Irish, 2015, Li *et al.*, 2016), all of the *st* alleles examined have
475 reduced lamina growth at the proximal position of an organ.

476 *St* transcripts appear to be confined to stipules and bracts in pea but are not found in
477 flowers, consistent with the lack of alterations to floral morphology in the *st* mutant. There
478 has been no previous comment in the literature on an altered bract morphology in *st* mutants;
479 this would be hard to detect because bracts are variable in size and frequency of appearance
480 in pea. However, it has been noted previously that bracts are altered in *coch* mutants
481 (Couzigou *et al.*, 2012), so *Coch* is likely to be expressed in bract primordia, where it could
482 up-regulate *St* expression and so determine the final size of bracts. A high level of *St*
483 expression in the developing stipule (and bract) appears to be dependent on *Coch* (Figs 6
484 &7).

485 The *st* mutation in combination with *uni*, but neither mutant alone, completely
486 abolishes stipule formation at upper nodes (Hofer *et al.*, 2001, Kumar *et al.*, 2009; 2013). The
487 precise evolutionary relationship between the Arabidopsis C1–1iG family C2H2 zinc finger
488 domain proteins and *St* is not clear, due to sequence duplications in Arabidopsis and possible
489 recent diversifying selection acting on *St* (Fig. 4). *RBE*, through its regulation of TCP5 and
490 microRNA164 (Huang & Irish, 2015) appears to be involved in regulating the switch
491 between cell division and differentiation. *Uni* in pea leaves is responsible for a 'transient

492 phase of indeterminacy' (Hofer *et al.*, 1997) which is manifest as continued meristematic
493 activity in the leaf primordium while the *st* mutant has reduced stipule marginal meristem
494 activity (Meicenheimer *et al.*, 1983) so *St* promotes this marginal meristem activity. The
495 complete loss of stipules, late in shoot development of the *st uni* double mutant, may reflect
496 the roles of *Uni* in promoting primordial growth and *St* in promoting marginal growth.

497 The more distantly related Arabidopsis protein JAGGED (C1-1iA group, Englbrecht
498 *et al.*, 2004), like *St*, regulates cell growth and division (Dinney *et al.*, 2004) and is involved
499 in both bract and petal development. *St* regulates cell division to a greater extent than cell
500 size, similar to JAGGED (Dinney *et al.*, 2004) and to RABBIT EARS (Huang & Irish, 2015)
501 more generally.

502 The very low level of *St* transcript in the *coch* mutant predicts that the *coch st* double
503 mutant would be indistinguishable from *coch*. Yaxley *et al.*, (2001) reported that *coch st* and
504 *coch* were indistinguishable, in disagreement with Blixt (1967), Marx (1987), Gourlay *et al.*,
505 (2000) and Kumar *et al.*, (2009; 2013). Our transcript abundance results seem to be consistent
506 with Yaxley *et al.*, (2001), unless the small amount of *St* expression in the *coch* mutant can,
507 under some circumstances, have consequences different from the null *st* mutant. The uORF
508 may be relevant to these observations if it mediates posttranscriptional regulation (Laing *et al.*
509 *et al.*, 2015), so the lower amount of *St* transcript in the *coch* mutant vs *Coch* (Fig. 6) may not
510 necessarily result in a difference in the amount of *St* protein. Such regulation may be
511 dependent on additional genetic or environmental factors and therefore explain the
512 differences in the reported phenotypes of the *coch st* double mutant.

513 The *in situ* hybridisation and q-PCR results are in agreement. The *in situ* analysis
514 additionally shows that the *St* transcript is limited to stipules and bracts, and is absent from
515 floral meristems and other parts of the leaf primordium. The weak expression of *St* in the
516 *coch* mutant is not associated with mis-location or mis-timing. It therefore appears that *Coch*
517 is epistatic to *St*, consistent with *Coch* determining stipule identity. However, we cannot
518 therefore completely rule out a role for *St* in determining stipule identity in certain genetic
519 backgrounds because a leaf-like stipule structure was reported in an *af tl st* triple mutant
520 (Gourlay *et al.*, 2000), but this phenotype was noted to occur sporadically and only in the
521 triple mutant. There is no evidence from the q-PCR result of a feedback between *St* and *Coch*
522 whereby *St* would maintain *Coch* expression and indirectly stipule identity.

523 ***Stipules reduced in legume species.***

524 Coding sequence and structural differences between *St* and corresponding sequences
525 in medicago and other legumes raises the possibility that *St* may have diverged in *Pisum*, in

526 association with the occurrence of large stipules. *L. aphaca* and *L. odoratus* differ in stipule
527 size, the *L. aphaca* stipules being notably large, however sequence alignment (Notes S2) does
528 not support a closer relationship between *Pisum* and *L. aphaca St* genes than *Pisum* and *L.*
529 *odoratus St* genes. Nevertheless, one position (A199 see Notes S2) distinguishes the *L.*
530 *aphaca* and *P. sativum* sequences from all the other sequences that were aligned in Fig. 3 and
531 this could be targeted in future functional studies. Whether there is any association between
532 stipule size and variation in the *St* gene more broadly in these taxa remains to be determined.

533 If *St* has undergone neo-functionalisation this may explain the elaboration of the pea
534 stipule. There are strong signals of purifying selection acting on parts of the gene, yet in
535 comparison to Medtr3g068095, some regions of *St* (and the gene as a whole) have an excess
536 of amino acid substitutions given the nucleotide divergence (Fig. 4), suggestive of
537 diversification (in one or other or both sequences). It should be noted that the vascularization
538 of the stipules of these two *Lathyrus* taxa is different from each other and from pea (Kupicha,
539 1975), so these may represent three different consequences of *St* gene variants, or, stipule
540 development in *Lathyrus* taxa may be independent of *St*.

541

542 **Acknowledgements:**

543 We thank Claire Domoney for her help and support, Clare Coyne for her advice on
544 transcriptome sequence data, Wayne Powell for enabling these experiments, BBSRC the
545 Welsh Government and the University of Aberystwyth for financial support.

546

547 **Author contribution**

548 CM, JH, ME, AS, MA, KS, TB, MH, VB, CF and NE planned and undertook
549 experimental work and analysed data. MS wrote the Python script, advised on bioinformatics
550 and edited the manuscript. NE, JH and AS wrote and edited the manuscript. All authors have
551 read and accepted the manuscript.

552

553 **References**

- 554 **Alves-Carvalho S, Aubert G, Carrère S, Cruaud C, Brochot A-L, Jacquin F, Klein A,**
555 **Martin C, Boucherot K, Kreplak J, et al., 2015.** Full-length de novo assembly of RNA-seq
556 data in pea (*Pisum sativum* L.) provides a gene expression atlas and gives insights into root
557 nodulation in this species. *The Plant Journal* **84**: 1–19.
- 558 **Apisitwanich S, Swiecicki WK. 1992.** A new allele, *st^{bs}*, at the *St* locus. *Pisum Genetics* **24**:
559 13.
- 560 **Baird NA, Etter PD, Atwood TS, Currey MC, Shiver AL, Lewis ZA, Selker EU, Cresko**
561 **WA, Johnson EA. 2008.** Rapid SNP Discovery and Genetic Mapping Using Sequenced
562 RAD Markers. *PLoS ONE* **3**: e3376.
- 563 **Balanzà V, Martínez-Fernández I, Sato S, Yanofsky MF, Kaufmann K, Angenent GC,**
564 **Bemer M, Ferrándiz C. 2018.** Genetic control of meristem arrest and life span in
565 Arabidopsis by a *FRUITFULL-APETALA2* pathway. *Nature Communications* **9**: 565.
- 566 **Belfield EJ, Gan X, Mithani A, Brown C, Jiang C, Franklin K, Alvey E, Wibowo A,**
567 **Jung M, Bailey K, Kalwani S, Ragoussis J, Mott R, and Harberd NP. 2012.** Genome-
568 wide analysis of mutations in mutant lineages selected following fast-neutron irradiation
569 mutagenesis of *Arabidopsis thaliana*. *Genome Research* **22**: 1306–1315
- 570 **Blixt S. 1967.** Linkage studies in Pisum VII. The manifestation of the genes *cri*, and *coch*,
571 and the double-recessive in *Pisum*. *Agric. Hort. Genet.* **25**: 131–144.
- 572 **Campbell BW, Hofstad AN, Sreekanta S, Fu F, Kono TJY, O'Rourke JA, Vance CP,**
573 **Muehlbauer GJ, Stupar RM. 2016.** Fast neutron-induced structural rearrangements at a
574 soybean *NAPI* locus result in *gnarled* trichomes. *Theor. Appl. Genet.* **129**:1725–1738.
- 575 **Chen J, Moreau C, Liu Y, Kawaguchi M, Hofer J, Ellis N, Chen R. 2012.** Conserved
576 genetic determinant of motor organ identity in *Medicago truncatula* and related legumes.
577 *Proc. Natl. Acad. Sci. USA* **109**: 11723 - 11728.
- 578 **Couzigou J-M, Zhukov V, Mondy S, el Heba GA, Cosson V, Ellis THN, Ambrose M,**
579 **Wen J, Tadege M, Tikhonovich I, et al. 2012.** *NODULE ROOT* and *COCHLEATA*
580 Maintain Nodule Development and Are Legume Orthologs of Arabidopsis *BLADE-ON-*
581 *PETIOLE* Genes. *Plant Cell* **24**: 4498-4510.
- 582 **Dash S, Campbell JD, Cannon EK, Cleary AM, Huang W, Kalberer SR, Karingula V,**
583 **Rice AG, Singh J, Umale PE, Weeks NT, Wilkey AP, Farmer AD, Cannon SB. 2016.**
584 Legume information system (LegumeInfo. org): a key component of a set of federated data
585 resources for the legume family. *Nucl. Acids Res.* **44**: D1181-D1188.

586 **Domoney C, Knox M, Moreau C, Ambrose M, Palmer S, Smith P, Christodoulou V,**
587 **Isaac PG, Hegarty M, Blackmore T et al. 2013.** Exploiting a fast neutron mutant genetic
588 resource in *Pisum sativum* (pea) for functional genomics. *Functional Plant Biology* **40**: 1261-
589 1270.

590 **Duarte J, Rivière N, Baranger A, Aubert G, Burstin J., Cornet L, Lavaud C, Lejeune-**
591 **Hénaut I, Martinant J-P, Pichon J-P, et al. 2014.** Transcriptome sequencing for high
592 throughput SNP development and genetic mapping in Pea. *BMC Genomics* **15**:126.

593 **Ellis THN, Davies DR, Castleton JA, Bedford ID. 1984** The Organization and Genetics of
594 rDNA Length Variants in Peas. *Chromosoma* **91**: 74-81.

595 **Ellis THN, Poyser SJ, Knox MR, Vershinin AV, Ambrose MJ. 1998.** Polymorphism of
596 insertion sites of *Ty1-copia* class retrotransposons and its use for linkage and diversity
597 analysis in pea. *Mol. Gen. Genet* **260**: 9-19.

598 **Englbrecht CC, Schoof H, Böhm S. 2004.** Conservation, diversification and expansion of
599 C2H2 zinc finger proteins in the *Arabidopsis thaliana* genome. *BMC Genomics* **5**: 39.

600 **Franssen SU, Shrestha RP, Bräutigam A, Bornberg-Bauer E, Weber APM. 2011.**
601 Comprehensive transcriptome analysis of the highly complex *Pisum sativum* genome using
602 next generation sequencing. *BMC Genomics* **12**: 227.

603 **Gourlay CW, Hofer JMI, Ellis THN. 2000.** Pea Compound Leaf Architecture Is Regulated
604 by Interactions among the Genes *UNIFOLIATA*, *COCHLEATA*, *AFILA*, and *TENDRIL-LESS*.
605 *Plant Cell* **12**: 1279-1294.

606 **Hellens R, Moreau C, Lin-Wang K, Schwinn KE, Thomson SJ, Fiers MWEJ, Frew TJ,**
607 **Murray SR, Hofer JMI, Jacobs JME, et al. 2010.** Identification of Mendel's White Flower
608 Character *PLoS ONE* **10**: e13230.

609 **Hofer JMI, Gourlay CW, Ellis THN. 2001.** Genetic control of leaf morphology: A partial
610 view. *Annals of Botany* **88**: 1129-1139.

611 **Hofer J, Turner L, Hellens R, Ambrose M, Matthews P, Michael A, Ellis N. 1997.**
612 *Unifoliata* regulates leaf and flower morphogenesis in pea. *Curr. Biol.* **7**: 581-587.

613 **Hofer J, Turner L, Moreau C, Ambrose M, Isaac P, Butcher S, Weller J, Dupin A,**
614 **Dalmis M, Le Signor C, et al. 2009** *Tendrill-less* regulates tendrill formation in pea leaves.
615 *Plant Cell* **21**:420-428.

616 **Huang T, Irish VF. 2015.** Temporal Control of Plant Organ Growth by TCP Transcription
617 Factors. *Curr. Biol.* **25**: 1765-1770.

618 **Huang T, López-Giráldez F, Townsend JP, Irish VF. 2012.** RBE controls microRNA164
619 expression to effect floral organogenesis. *Development* **139**: 2161-2169.

620 **Hwang WJ, Kim MY, Kang YJ, Shim S, Stacey MG, Stacey G, Lee S-H. 2015.** Genome-
621 wide analysis of mutations in a dwarf soybean mutant induced by fast neutron bombardment
622 *Euphytica* **203**: 399–408.

623 **Kaur S, Pembleton LW, Cogan NOI, Savin KW, Leonforte T, Paull J, Materne M,**
624 **Forster JW. 2012.** Transcriptome sequencing of field pea and faba bean for discovery and
625 validation of SSR genetic markers *BMC Genomics* **13**:104.

626 **Knox M, Moreau C, Lipscombe J, Baker D, Ellis N. 2009.** High-throughput
627 retrotransposon-based fluorescent markers: improved information content and allele
628 discrimination. *Plant Methods* **5**: 10.

629 **Krizek BA, Lewis MW, Fletcher JC. 2006.** *RABBIT EARS* is a second-whorl repressor of
630 *AGAMOUS* that maintains spatial boundaries in Arabidopsis flowers. *Plant J.* **45**: 369-383.

631 **Kumar S, Mishra RK, Kumar A, Srivastava S, Chaudhary S. 2009.** Regulation of stipule
632 development by *COCHLEATA* and *STIPULE-REDUCED* genes in pea *Pisum sativum*.
633 *Planta* **230**: 449–458.

634 **Kumar A, Sharma V, Kumar S. 2013.** Interaction between *cochleata* and *stipule-reduced*
635 mutations results in exstipulate hypertrophied leaves in *Pisum sativum* L. *Ind. J. Exptl. Biol.*
636 **51**: 492-501.

637 **Kupicha FK. 1975.** Observations on the vascular anatomy of the tribe Viciaeae
638 (Leguminosae) *Bot. J. Linn. Soc.* **70**: 231-242.

639 **Laing WA, Martínez-Sánchez M, Wright MA, Bulley SM, Brewster D, Dare AP,**
640 **Rassam M, Wang D, Storey R, Macknight RC et al. 2015.** An Upstream Open Reading
641 Frame Is Essential for Feedback Regulation of Ascorbate Biosynthesis in Arabidopsis. *Plant*
642 *Cell.* **27**: 772-786.

643 **Lamprecht H. 1960.** Eine neue Stipel-Mutante bei *Pisum*. *Agr. Hort. Genet.* **18**: 209-213

644 **Lewis GP, Schrire B., Mackinder B, Lock M. 2005.** *Legumes of the World*. Kew
645 Publishing.

646 **Li G, Chern M, Jain R, Martin JA, Schackwitz WS, Jiang L, Vega-Sánchez ME, Lipzen**
647 **AM, Barry KW, Schmutz J and Ronald PC. 2016.** Genome-wide sequencing of 41 rice
648 (*Oryza sativa* L.) mutated lines reveals diverse mutations induced by fast-neutron irradiation.
649 *Molecular Plant* **9**: 1078–1081

650 **Li J, Wang Y, Zhang Y, Wang W, Irish VF, Huang T. 2016.** *RABBIT EARS* regulates the
651 transcription of TCP4 during petal development in Arabidopsis. *J. Exp. Bot.* **67**: 6473-6480.

652 **Lütcke HA, Chow KC, Mickel FS, Moss KA, Kern HF, Scheele GA. 1987.** Selection of
653 AUG initiation codons differs in plants and animals. *EMBO J.* **6**: 43–48.

654 **McAdam SAM, Eléouët MP, Best M, Brodribb TJ, Murphy MC, Cook SD, Dalmais M,**
655 **Dimitriou T, Gélinas-Marion A, Gill WM, Hegarty M, Hofer JMI,**
656 **Maconochie M, McAdam EL, McGuinness P, Nichols DS, Ross JJ, Susmilch FC, Shelley**
657 **Urquhart S. 2017.** Linking Auxin with Photosynthetic Rate via Leaf Venation. *Plant*
658 *Physiol.* **175:** 351-360.

659 **Marx GA. 1987.** A suite of mutants that modify pattern formation in pea leaves. *Plant Mol.*
660 *Biol. Rep.* **5:** 311–335.

661 **Meicenheimer RD, Muehlbauer FJ, Hindman JL, Gritton ET. 1983.** Meristem
662 characteristics of genetically modified pea (*Pisum sativum*) leaf primordia. *Can. J. Bot.* **61:**
663 3430-3437.

664 **Miller MR, Dunham JP, Amores A, Cresko WA, Johnson EA. 2007.** Rapid and cost-
665 effective polymorphism identification and genotyping using restriction site associated DNA
666 (RAD) markers. *Genome Research.* **17:** 240-248.

667 **Moreau C, Ambrose MJ, Turner L, Hill L, Ellis THN, Hofer JMI. 2012.** The *b* gene of
668 pea encodes a defective flavonoid 3',5'-hydroxylase, and confers pink flower color. *Plant*
669 *Physiol.* **159:** 759-68.

670 **Ohta M, Matsui K, Hiratsu K, Shinshi H, Ohme-Takagi M. 2001.** Repression Domains of
671 Class II ERF Transcriptional Repressors Share an Essential Motif for Active Repression
672 *Plant Cell* **13:** 1959–1968.

673 **Pellew C, Sverdrup A. 1923.** New observations on the genetics of peas. *J. Genetics* **13:** 125-
674 131.

675 **Perrier X, Flori A, Bonnot F. 2003.** Data analysis methods. *in: Hamon, P., Seguin, M.,*
676 **Perrier X, Glaszmann JC. eds.,** Genetic diversity of cultivated tropical plants. Enfield,
677 Science Publishers. Montpellier. pp 43-76.

678 **Sainsbury F, Tattersall AD, Ambrose MJ, Turner L, Ellis THN, Hofer JMI. 2006.** A
679 *crispa* null mutant facilitates identification of a *crispa*-like pseudogene in pea. *Functional*
680 *Plant Biology* **33:** 757–763.

681 **Schaefer H, Hechenleitner P, Santos-Guerra A, Sequeira MM de, Pennington RT,**
682 **Kenicer G, Carine MA. 2012.** Systematics, biogeography, and character evolution of the
683 legume tribe Fabeae with special focus on the middle-Atlantic island lineages. *BMC Evol.*
684 *Biol.* **12:** 250.

685 **Schauser L, Roussis A, Stiller J, Stougaard J. 1999.** A plant regulator controlling
686 development of symbiotic root nodules *Nature* **402:** 191-195.

687 **Sidorova KK. 1975.** Induced pea mutant with creeping stem. *Pisum Newsletter* **7:** 57–58

688 **Sinjushin AA, Khartina GA, Gostimskii SA. 2011.** New allele of the *COCHLEATA* gene in
689 pea *Pisum sativum* L. *Russ. J. Genet.* **47**: 1422-1427.

690 **Tadege M, Wen J, He J, Tu H, Kwak Y, Eschstruth A, Cayrel A, Endre G, Zhao PX,**
691 **Chabaud M, et al. 2008.** Large-scale insertional mutagenesis using the *Tnt1* retrotransposon
692 in the model legume *Medicago truncatula*. *Plant J.* **54**: 335–347.

693 **Takeda S, Matsumoto N, Okada K. 2004.** *RABBIT EARS*, encoding a SUPERMAN-like
694 zinc finger protein, regulates petal development in *Arabidopsis thaliana*. *Development* **131**:
695 425-434.

696 **Tsai H, Howell T, Nitcher R, Missirian V, Watson B, Ngo KJ, Lieberman M, Fass J,**
697 **Uauy C, Tran RK, et al. 2011.** Discovery of Rare Mutations in Populations: TILLING by
698 Sequencing *Plant Physiology* **156**: 1257–1268.

699 **Urbański DF, Malolepszy A, Stougaard J, Andersen SU. 2012.** Genome-wide *LOREI*
700 retrotransposon mutagenesis and high-throughput insertion detection in *Lotus japonicus*.
701 *Plant J.* **69**: 731–741.

702 **Vassileva M. 1979.** Induced mutagenesis in *Pisum*. I. Genetic studies on the *acacia* mutant.
703 *Genet. Sel.* **12**: 396-408.

704 **Wang Z, Luo Y, Li X, Wang L, Xu S, Yang J, Weng L, Sato S, Tabata S, Ambrose M,**
705 **Rameau C, et al. 2008.** Genetic control of floral zygomorphy in pea (*Pisum sativum* L.)
706 *Proc. Natl. Acad. Sci. U.S.A* **105**: 10414-10419.

707 **de Winton D. 1928** Further linkage work in *Pisum sativum* and *Peimula sinensis* Z. *Ind.*
708 *Abst. -u Vererb. -lehre, Suppl.* **2**: 1594-1600.

709 **Yaxley JL, Jablonski W, Reid JB. 2001.** Leaf and Flower Development in Pea (*Pisum*
710 *sativum* L.): Mutants *cochleata* and *unifoliata*. *Annals of Botany* **88**: 225-234.

711 **Zhuang L, Ambrose M, Rameau C, Weng L, Yang J, Hu X, Luo D, Li X. 2012.**
712 *LATHYROIDES*, encoding a *WUSCHEL*-related homeobox1 transcriptional factor, controls
713 organ lateral growth and regulates tendril and dorsal petal identities in garden pea (*Pisum*
714 *sativum* L.). *Molecular Plant.* **5**: 1333–1345.

715

716 **Supplementary Information Legends:**

717 **Fig. S1**

718 The stipule phenotypes of pea lines carrying *St* or *st* alleles is illustrated for corresponding
719 mutant and wild type lines.

720

721 **Fig. S2**

722 A screen-shot of AFLP profiles using the selective primers Pst+AG, and Mse+GTC that
723 identified a presence absence polymorphism of an amplicon approximately 212bp in size
724 present among the products of the pea line JI2822, but absent from the isogenic FN2122/2
725 products is shown.

726

727 **Fig. S3**

728 The frequency distribution of read depths of RAD-tags in the pea line JI2822 is presented.

729

730 **Fig. S4**

731 A Venn diagram presents the number and pattern of the distribution of RAD-tags among the
732 four pea lines examined.

733

734 **Fig. S5**

735 The positions of *M. truncatula* homologues of the 43 candidate pea genes on the 8 *M.*
736 *truncatula* pseudomolecules (Mt Assembly v3.5.1) is presented.

737

738 **Fig. S6**

739 A co-segregation analysis of *St* and its candidate gene in an F2 population of the cross
740 between the pea lines JI143 (*st*) and JI813 (*St*) as assessed with allele specific PCR primers is
741 presented.

742

743 **Fig. S7**

744 A PCR analysis confirms the presence of an intron in the pea *St* gene.

745

746 **Fig. S8**

747 The stipule area in selected mutant and wild type pea plants is presented.

748

749 **Fig. S9**

750 Stipule epidermal cell area is plotted for F2 segregants of the cross between the pea lines
751 Flagman (*St*) and Filby (*st*).

752

753 **Fig. S10**

754 The *St* transcript is localised by *in situ* hybridisation to thin sections and whole mount
755 preparations of pea tissue from wild type and *coch* mutant lines, using the *st* deletion mutant
756 as a control for cross hybridisation with related transcripts.

757

758 **Methods S1**

759 This file is the Python script used to collate, count and record identical short sequence tags.

760

761 **Notes S1**

762 This file contains information relevant to the sequence analysis and alleles of the pea *Rca*
763 gene.

764

765 **Notes S2**

766 This file contains information relevant to the sequence analysis, alleles, and phylogenetic
767 relationships of the pea *Stipules-reduced* gene.

768 **Table 1 Epidermal cell areas in wild type and *st* mutant pea plants**

769

Genotype	Proximal		Distal	
	<i>St</i>	<i>st</i>	<i>St</i>	<i>st</i>
medial	4.1 ± 0.9	3.0 ± 0.5**	4.1 ± 1.3	3.2 ± 0.8*
lateral	2.8 ± 0.6	3.1 ± 0.8	3.5 ± 0.6	3.0 ± 0.7
medial - lateral	1.3	0.1	0.6	0.2
<i>t</i>	9.15**	0.98	5.85**	1.45

770

771 The cell area of stipule epidermal pavement cells area was measured at four locations in *St*
 772 and *st* F2 segregants (n = 30 for each class) of the cross between the pea lines Flagman (*St*)
 773 and Filby (*st*). Epidermal cell areas (in units of 1,000 μm²) are given as Mean ± standard
 774 deviation n = 30. Measurements for *st* that are significantly different from wild type
 775 (Kruskal-Wallis test) are marked with asterisks (*, *p* < 0.05; **, *p* < 0.01). For the difference
 776 between the means Student's *t* values are given. See Fig. S9 for a graphical representation of
 777 these data.

778

779

780

781 **Figure Legends**

782

783 **Figure 1. The classes of *stipules reduced* mutant phenotypes**

784 **a:** The pea wild type (JI2822) and corresponding FN induced deletion mutant (FN2122/2) are
785 shown in two views; a single compound leaf, adaxial view on the left, a whole shoot tip in
786 side view on the right. The single leaves comprise a proximal pair of stipules at the base of
787 the leaf, a pair of leaflets and a distal tendril (obscured by the leaflets). These illustrate the
788 small stipule size of a *st* null allele. The shoot tips illustrate the difference in the way the
789 shoot apex is enclosed by stipules in *St* vs *st*.

790 **b:** Single compound leaves from the wild type progenitor (JI3538), left, and its corresponding
791 weak mutant allele (JI2653), designated *st^{bs}* (*stipules reduced - butterfly stipules*).

792 Plants were 1 month old. The scale bar indicates 5 centimetres. Additional alleles are shown
793 in Notes S2.

794

795 **Figure 2. Gene structure**

796 The structure of the pea *St* gene is illustrated. Open reading frames, including a predicted
797 short upstream open reading frame are boxed. The zinc finger and EAR domains are shaded
798 in black, and the intron is marked as a line. The nature and location of observed mutations is
799 given above with the corresponding JI accession numbers for the lines carrying each mutation
800 given below. The scale below is in nucleotides.

801

802 **Figure 3 Phylogenetic analysis**

803 Phylogenetic relationship between *Stipules reduced*-like amino acid sequences in selected
804 legume species. The tree was generated from the sequence alignment of the zinc finger
805 domain as shown in Notes S2. Bootstrap values in excess of 40% are given to the left of the
806 corresponding branch. Groupings of sequences are largely congruent with species phylogeny
807 as indicated by colour shading. Open arrows indicate the most closely related sequences
808 deduced from the *M. truncatula* genome sequence and filled arrows indicate the pea *Stipules*
809 *reduced* and its closest relative in *Pisum* (PsCam039889). Abbreviations are: Ardu *Arachis*
810 *duranensis*, Araip *Arachis ipaensis*, At *Arabidopsis thaliana*, Ca_D, *Cicer arietinum* var.
811 Desi, Ca_K *Cicer arietinum* var. Kabuli, Ccaj *Cajanus cajan*, Gm *Glycine max*, Lan *Lupinus*
812 *angustifolius*, Lj *Lotus japonicus*, Mt *Medicago truncatula*, Pv *Phaseolus vulgaris*, Tp
813 *Trifolium pratense*, Va *Vigna angularis*, Vr *Vigna radiata*

814

815 **Figure 4 Comparison between *Stipules reduced* and Medtr3g068095**

816 Analysis of pea *Stipules reduced* and *M.truncatula* Medtr3g068095 sequences.

817 **a:** The Ka/Ks ratio (y axis) is plotted for a sliding window of 25 residues of the alignment.

818 Where Ks is zero the line is broken. Dashed line, Ka=Ks. Dotted line represents mean + 3x

819 Std. Dev. of Ka/Ks.

820 **b:** The location of a gap caused by a difference in splice donor sites is marked and

821 highlighted with diagonal stripes. The conserved zinc finger and EAR domains are marked,

822 and, together with other conserved sequences, are highlighted with a grey background.

823 Note the x axis does not represent actual positions in either sequence because the

824 alignment includes indels.

825

826 **Figure 5 Stipule vascular supply**

827 Phloroglucinol stained vascular strands of pea (**a**) wild type and (**b**) *stipules reduced* stipules.

828 Diagrams to the left follow the vasculature numbering system of Kupicha (1975), these

829 numbers are shown as labels on acid-macerated samples, right.

830

831 **Figure 6 Expression of *Stipules reduced* and *Cochleata* in wild type and mutant plants**

832 Relative expression levels of *St* and *Coch* in wild type, single and double mutant pea seedling

833 shoot apices were measured by q-PCR. Relative expression levels and the experimental error

834 estimated from three independent plants measured in triplicate is shown. The wild type *St*

835 allele is from JI2822, the *coch* allele is the deletion mutation of FN3185 generated in the

836 JI2822 background (Couzigou *et al.*, 2012) and the *st* allele is the deletion allele of FN2122

837 in the JI2822 background, which serves as a negative control indicative of absence of the *St*

838 transcript.

839

840 **Figure 7 In situ hybridization of *Stipules reduced* to wild type and mutant sections.**

841 RNA *in situ* hybridisations with an *St* probe. All stained pea tissues are stipules except for the

842 three arrowed locations which are in the position of bracts. Apical meristems are adjacent to

843 the symbol x. A) Wild type (JI2822) longitudinal section. B) Wild type (JI2822) transverse

844 section. C) *coch* mutant (FN3185) longitudinal section. D) *coch* mutant (FN3185) transverse

845 section, the asterisk marks a weakly stained stipule. E) *st* mutant (FN2122) longitudinal

846 section. F) *st* mutant (FN2122) transverse section. The FN2122 *st* mutant acts as a negative

847 control because the *St* gene, and therefore its transcript, is missing from plants of this

848 genotype. A 200 μ m scale bar is given in each panel.