1 **Two Y chromosome-encoded genes determine sex in kiwifruit**

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ABSTRACT

Dioecy, the presence of male and female individuals, has evolved independently in multiple flowering plant lineages. Although theoretical models for the evolution of dioecy, such as the "two-mutation" model, are well established, little is known about the specific genes determining sex and their evolutionary history. Kiwifruit, a major tree crop consumed worldwide, is a dioecious species. In kiwifruit, we had previously identified a Y-encoded sex-determinant candidate gene acting as the suppressor of feminization (SuF), named *Shy Girl* (*SyGI*). Here, we identified a second Y-encoded sex-determinant that we named *Friendly boy* (*FrBy*), which exhibits strong expression in tapetal cells. Gene-editing and complementation analyses in *Arabidopsis thaliana* and *Nicotiana tabacum* indicated that *FrBy* acts for the maintenance of male (M) functions, independently of *SyGI*, and that these functions are conserved across angiosperm species. We further characterized the genomic architecture of the small (< 1 Mb) male specific region of the Y-chromosome (MSY), which harbors only two genes significantly expressed in developing gynoecia and androecia, respectively: *SyGI* and *FrBy*. Resequencing of the genome of a natural hermaphrodite kiwifruit revealed that this individual is genetically male but carries deletion(s) of parts of the Y-chromosome, including *SyGI*. Additionally, expression of *FrBy* in female kiwifruit resulted in hermaphrodite plants. These results clearly indicate that Y-encoded *SyGI* and *FrBy* act independently as the SuF and M factors in kiwifruit, respectively, and provide insight into the evolutionary path leading to a two-factor sex determination system but also a new breeding approach for dioecious species.

MAIN TEXT

In flowering plants, hermaphroditism is ancestral and most common, but a minority of plant species have evolved separate sexes (dioecy), in a lineage-specific manner (1-3). Similar to mammals, sexuality in plants is often determined by a heterogametic male system with XY chromosomes, where the Y chromosome is thought to carry one or two male-determining factors

52   (1, 3, 4, 5). Previous analyses of Y chromosome evolution in plants, initially in *Silene latifolia*

53   and *Carica papaya*, have revealed long non-recombining male-specific regions, which encompass

54   many genes (6-8), although the sex-determining genes have not been fully characterized. Recently,

55   Y chromosome-encoded sex determinants have been identified in persimmons (*Diospyros* spp.)

56   and garden asparagus (*Asparagus officinalis*) (9, 10). In persimmons, the Y-encoded pseudogene

57   *OGI* encodes a small-RNA targeting its autosomal counterpart gene, *MeGI*. To the best of our

58   understanding, *OGI* is sufficient for expression of maleness and repression of female development

59   (3, 9, 11). In garden asparagus, two Y-encoded factors, named *SOFF* and *aspTDF*, act

60   independently to suppress gynoecium and promote androecium development, respectively (10).

61   The asparagus observation is consistent with a previously proposed theoretical framework, called

62   the "two-mutation model" (12, 13), while the persimmon case is not. This model proposes that

63   evolution from an ancestral hermaphrodite could occur if females carried a mutated (non-

64   functional) version of a male promoting factor (M) on the proto-X chromosome, resulting in

65   establishment of gynodioecy. A second mutation, a gain-of function suppressor of feminization

66   (SuF) on the proto-Y chromosome would then establish males. Together, these sex-determining

67   mutations may, if closely linked, define a genome region resembling an XY chromosome pair or

68   sex-linked genome region (13). Still, the evolutionary pathways governing the transitions into

69   dioecy are poorly understood because only a few examples have been characterized to date.

70   Kiwifruit, a major fruit crop consumed worldwide, belongs to the genus *Actinidia*, in

71   which most species are dioecious (14). The sexuality in kiwifruit is genetically controlled by a

72   heterogametic male system (i.e., XY system). A Y-encoded cytokinin response regulator, named

73   *Shy Girl* (*SyGI*), acts as one of the two putative sex determinants, the suppressor of female

74   development (Su^F) (15). The other sex determinant, the putative male promoting factor (M) has

75   not been identified. Although the establishment of SuF in *Actinidia* is estimated to have occurred

76   approximately 20-mya (15) and predated the divergence of the *Actinidia* species, kiwifruit still

77   carries incipient and homomorphic sex chromosomes, including a small sex-determining region

78   (15-17). Recent breeding has derived some hermaphroditic and neuter individuals in *A. deliciosa*,

79   which constitute an additional resource for the identification of a second sex determinant in

80   kiwifruit. Here, we attempted to identify the male promoting sex determinant (M factor), by fine

81   assessment of the genes located on the male-specific region of the Y-chromosome (MSY), and

82   identification of genes differentially expressed between male and female at an early stage of

83   tapetum differentiation. The function of the M factor was validated in model plants and in

84   kiwifruit, resulting in the first development of an artificial hermaphrodite crop from a dioecious

85   individual. Finally, we further assessed the evolution of the two sex determinants on the Y-

86   chromosome, unveiling transitions into and out of dioecy in kiwifruit.

87

88   Previously, genomic sequencing reads from F1 sibling trees derived from an

89   interspecific cross, *A. rufa* sel. Fuchu × *A. chinensis* sel. FCM1 were used to identify and assemble

90   the potential male specific region of the Y chromosome (MSY) of *A. chinensis* (15). The 249

91   resulting contigs, totaling approximately 0.5Mb in length, contained Y-specific sequences with

92   perfect co-segregation with the plants' sex, and included 61 hypothetical genes (15). In kiwifruit,

93   androecia differentiation between males and females is observed during tapetum degeneration

94   (stage 3-4 in Fig. S1) (15, 18, 19). To identify candidate male promoting (M) factors within the

95   MSY, we conducted mRNA-Seq analyses on developing anthers (5 males and 5 females) before

96   tapetum degeneration ("stage 1-2" in Fig. S1) from the F1 population described above. The

97   mRNA-Seq reads were mapped to the 61 hypothetical candidate genes. Only one of them

98   exhibited male-specific expression (RPKM > 1). This gene included a fasciclin domain, which

99   are typically involved in cell adhesion (Table S1). This fasciclin-like gene was named "*Friendly*

100   *Boy* (*FrBy*)", as a potential counterpart of the Su^F sex determinant, *Shy Girl* (*SyGI*). *FrBy* is nested

101   within the monophyletic MTR1 family (Fig. 1a). In rice, MTR1 contributes to tapetum

102   degradation via programmed cell death (PCD), resulting in male fertility (20). The kiwifruit *FrBy*

103   and its orthologs in *Nicotiana tabacum* (FAS1 domain protein), *Arabidopsis thaliana*

104   (AT1G30800) and rice (*Oriza sativa*, MTR1), showed no significant differentiation according to

105   site-branch-specific evolutionary rate analysis against the other branches (Fig. 1b), suggesting

106   conserved protein function. The presence of the *FrBy* gene was male-specific in a wide variety of

107   *Actinidia* species (Fig. 1c). The expression of *FrBy* was specific to early developing androecia

108   (Fig. 1d-e). Tapetum cell-specific qRT-PCR using laser capture microdissection (Supplemental

109   Figure S2) and *in situ* RNA hybridization analysis (Fig. 1f-h) both indicated that *FrBy* expression

110   in androecia was confined to tapetal cells in stage 1-2 and possibly to meiocyte or tetrads. This is

111   consistent with previous observations of MTR1 in rice (Tan et al. 2012) and with its putative

112   function to contribute to tapetum degradation following PCD in kiwifruit (Supplemental Fig. S1

113   and S3) (19). Differentially expressed genes (DEGs) in male and female anthers (Supplemental

114   Table S2, Figure S4) were also consistent with the potential function of *FrBy*. We identified 538

115   DEGs (FDR < 0.1) when analyzing transcriptome data from developing anthers (as described

116   above). In those 538 DEGs, GO terms involving PCD and phosphorylation signals were highly

117   enriched (Supplemental Table S3). Not only PCD, but abundant phosphorylation signals are

118   indispensable for proper tapetum maturing and degradation (21-23). Furthermore, an ortholog of

119   *Tapetal Development and Function 1* (*TDF1*) or *MYB35*, a key gene in tapetum maturation in

120   Arabidopsis (Zhu et al. 2008) and one of the two sex determinants in dioecious garden asparagus

121   (10, 24, 25), was detected as one of the male-biased DEGs in kiwifruit, although this gene

122   (Acc30672.1) was not located within the MSY (Supplementary Table S2, Figure S5).

123   To investigate the function of *FrBy*, we first used the CRISPR/Cas9 gene editing system

124   in two distantly related model plants, *Arabidopsis thaliana* and *Nicotiana tabacum* (Supplemental

125   Table S4). Although the Arabidopsis genome includes three paralogs of *FrBy*, only one,

126   AT1G30800, was in the cluster which was conserved across the *Brassicaceae* species (Fig. 1a).

127   In Arabidopsis, the AT1G30800-null lines (Supplemental Figure S6) were self-sterile, with low

128   pollen germination rates (Fig. 2g), but could successfully produce seed after being crossed to

129   control male plants (Fig. 2a-e). The null line showed substantial delay in tapetal layer degradation

130   (Supplemental Fig. S7), which is consistent with the development of female kiwifruit plants (Fig.

131   S1) (18, 19). On the other hand, the lack of AT1G30800 had no significant effect on female

132   reproductive function ($P > 0.1$, Supplemental Fig. S8). In *N. tabacum*, knock-out mutation of the

133   *FrBy* ortholog, *FAS1* (*fas1*) (Supplemental Figure S9) resulted in male sterility, with substantial

134   reduction in pollen germination rate, and was accompanied by a delay in tapetum degradation.

135   The other organs, including the gynoecium, showed no differentiation compared to the control

136   plants (Fig. 2h-o). The transgenic *N. tabacum* lines expressing the kiwifruit Su^F gene, *SyGI*, under

137   the control of native promoter (p*SyGI-SyGI*) exhibited female-sterility (15). Reciprocal crossing

138   using control plants, p*SyGI-SyGI*, and *fas1* indicated that *SyGI* and *FAS1* independently promote

139   gynoecium and androecium development, respectively (Fig. 2p). Importantly, male function in a

140   *fas1* null line could be complemented by introduction of the kiwifruit *FrBy* under the control of

141   its native promoter (Fig. 2q-r, Supplemental Figure S10), indicating that *FrBy* can act to maintain

142   male fertility via proper tapetum degradation in *N. tabacum*. These results all suggest that *FrBy*

143   is likely to be the male promoting factor, and that the two sex determining genes, *SyGI* and *FrBy*

144   work independently for female and male fertility, respectively, in kiwifruit. Furthermore, our

145   phylogenetic and evolutionary analyses indicated that the function of this fasciclin-like

146   monophyletic gene is highly conserved across angiosperm species.

147   Reference genome sequences for kiwifruit have been assembled from female (2A+XX)

148   cultivars (26, 27) but the Y chromosome of kiwifruit (or *Actinidia* spp.) has not been sequenced

149   to date. Here, we constructed the whole genome reference sequence of a male cultivar, Soyu,

150   which is one of the main pollinizers used in Japan. The sequences were assembled using 10X

151   Genomics Supernova v1.2.2, which is based on a long haploblocking method suitable for

152   assembly of highly heterozygous diploid genomes (28). Downstream genomic analysis for the

153   Soyu cultivar was conducted on the "pseudohaploid" version of the whole genome assembly. The

154   drafted genome sequence covered ca 710Mb which corresponds to 94% of the estimated size of

155   kiwifruit genome (758 Mb) (Hopping, 1994, Huang 2013), with N50 = *ca* 318kb for scaffolds

156　(Supplemental Table S5). The genomic short-read sequences were generated from large DNA

157　molecules partitioned and barcoded using the Gel Bead in Emulsion (GEM) microfluidic method

158　of 10X Genomics (28). Thus, the information present in the GEM barcodes anchored to the

159　assembled contigs reflect their physical distance, in which proximal contig pairs often share the

160　same GEM barcodes. GEM barcodes were extracted from read pairs and linkage information was

161　assigned using a custom script. To construct longer scaffolds within the MSY, we first identified

162　9 scaffolds, which together spanned 1.43 Mb, and contained ca 87% of the Y-specific contigs

163　previously assembled (15) (Supplemental Table S6). To organize these scaffolds relative to each

164　other, we applied DelMapper (6), an approach that employed the traveling salesman problem

165　(TSP), used in radiation hybrid mapping of mammalian chromosomes (29, 30) or deletion

166　mapping of Y-chromosome in dioecious *Silene latifolia* (6). Using this method, 8 of the 9

167　scaffolds were successfully organized. In this new assembled super-scaffold, the two sex

168　determinants, *SyGI* and *FrBy*, are located on adjacent scaffolds, at an estimated distance of ca

169　500kb (Fig. 3a, Supplemental Figure S11). Mapping of genomic reads from male and female

170　individuals in the KE population (15) indicated that a ~800-kb region enriched in male-specific

171　sequences (putative MSY) was located at the center of this assembled super-scaffold (Fig. 3a).

172　The putative MSY includes the two sex determinants and highly repetitive sequences, which is

173　consistent with the structure of MSY in other plants or animals (10, 31). The putative

174　pseudoautosomal region (PAR) appears to be single copy and exhibited no substantial gender-

175　bias, and mostly flanked the putative MSY, although some PAR-like sequences were also located

176　inside the putative MSY (Fig. 3a). In this super-scaffold, 145 genes were predicted using

177　AUGUSTUS (32), and 30 of these genes were fully male-specific (Fig. 3b-c, Supplemental Table

178　S7). Of the 30 male-specific genes, only *SyGI* and *FrBy* were substantially expressed (RPKM >

179　1) in carpel and anther, respectively (Fig. 3d-e, Supplemental Table S8), based on transcriptome

180　information from the KE population (Akagi et al. 2018 for carpel) (15). These data support the

181　hypothesis that they are the two factors determining sexuality in kiwifruit.

182   We further corroborated the role of these two sex determinants in two ways. Breeding

183   programs have generated a few hermaphrodite accessions in hexaploid *A. deliciosa* (33). We

184   sequenced one of them, the KH-line (Supplementary Table S9), which was thought to be a Y-

185   dependent (or possibly X-dependent) hermaphrodite (6A+XXXXXY^h or 6A+XXXXXX^h,

186   Supplementary Figure S12). Mapping of the KH and control *A. deliciosa* [male cv. Matua

187   (6A+XXXXXY)] genomic reads to the Y-chromosomal scaffolds described above (Fig. 3)

188   demonstrated that the KH-line carries the *FrBy* gene, but not the *SyGI* gene, either through one

189   or several long deletion(s), including the loss of *SyGI,* or through gain of *FrBy* on the X

190   chromosome from recombination with the Y chromosome (Figure 4a, Supplementary Figure S13).

191   Consistent with this result, *SyGI* could not be amplified in the KH-line, while *FrBy* could (Figure

192   4b). This suggested that loss of *SyGI* (the Su^F) in the Y, or gain of *FrBy* in the X, resulted in a

193   natural hermaphrodite line (Figure 4c). Next, we set out to develop hermaphrodite kiwifruit

194   artificially, as well as to further validate the *FrBy* function. We introduced the *FrBy* ORF under

195   the control of its native promoter (p*FrBy-FrBy*) into a "rapid flowering" *A. chinensis* female cv.

196   Hort16A. In this line, the *CENTRORADIALIS* (*CEN*) genes have been truncated by gene-editing,

197   resulting in lines that bypass the long juvenile phase (*ca* 3-4 years) and flower precociously (34).

198   As anticipated, the p*FrBy-FrBy* lines, which flowered 4 months after regeneration, were

199   hermaphroditic, exhibiting restored androecium function. These produced fruits including fertile

200   seeds after self-pollination (Fig. 4d-h, Supplementary Table S10). Pollen tubes from the p*FrBy-*

201   *FrBy* lines grew similarly to those from male accessions, in contrast to the control lines (Figure

202   4g-i, Supplementary Figure S14). These results clearly indicated that *FrBy* acts as the M factor in

203   kiwifruit sex determination. They also provide valuable insight into new breeding approaches for

204   dioecious species.

205

206   Taken together, our results are consistent with the following evolutionary path for the

207   transition from hermaphroditism to dioecy in *Actinidia*, based on two tightly linked genes within

208   a small MSY: loss-of-function of *FrBy* established a proto-X chromosome, while lineage-specific

209   gain-of-function in *SyGI* (15) derived a dominant suppressor of gynoecium development,

210   establishing a proto-Y chromosome (Figure 5). This evolutionary process and the predicted

211   function of the determinants are consistent with the "two-mutation model" (12, 13). This proposed

212   evolutionary history of *SyGI* and *FrBy* is also consistent with those of the two-locus type sex

213   determinants in *Asparagus* or *Phoenix* (10, 35), although the specific function of these sex-

214   determinants are different. The putative SuF genes, *SOFF* for *Asparagus* and *LOG1*-like for

215   *Phoenix*, were established by lineage-specific gene duplication/translocation on the Y

216   chromosome; while the putative M genes, *MYB35* (*TDF1*) for *Asparagus* and *CPY703/GPAT3*

217   for *Phoenix* were lost from the X chromosomes. Within the order Ericales, kiwifruit (*Actinidia*)

218   evolved these Y-encoded sex determinants while persimmons (*Diospyros*), evolved a single sex

219   determinant on the Y chromosome, *OGI*, which encodes small-RNAs repressing an autosomal

220   feminizing gene, *MeGI* (9, 36). Despite their different specific functions, *SyGI* in kiwifruit and

221   *OGI* in persimmon both act as dominant suppressors and were both derived from lineage-specific

222   duplications, suggesting evolutionary consistency in how the diverse sex determination systems

223   have evolved in angiosperms.

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301 **SUPPLEMENTARY INFORMATION**

302 **Materials and Methods (S1-S11)**

303 **Method S1**: Screening of the expressed candidate sex determinants

304 **Method S2**: Expression profiling in kiwifruit anthers

305 **Method S3**: *in situ* RNA hybridization

306 **Method S4**: Phylogenetic analysis and detection of positive selection

307 **Method S5**: 10X Genomics (Chromium) library construction and genome assembly with

308 Supernova

309 **Method S6**: Anchoring and characterization of the scaffolds surrounding the two sex

310 determinants

311 **Method S7**: Breeding of hermaphrodite kiwifruit

312 **Method S8**: Genomic analysis of hermaphrodite kiwifruit

313 **Method S9**: Construction of vectors

314 **Method S10**: Transformation

315 **Method S11**: Floral phenotyping in transformed plants

316

317 **Accession numbers**

318

319 **Supplemental Figures (S1-S14)**

320 **Supplemental Figure S1**: Transition of anther and definition of the developmental stage

321 **Supplemental Figure S2**: Tapetum cells-specific *FrBy* expression pattern with laser capture

322 microdissection (LCM).

323 **Supplemental Figure S3**: Visualization of programmed cell death in tapetum cells of male and

324 female kiwifruit

325 **Supplemental Figure S4**: Gene expression pattern in male and female anthers

326 **Supplemental Figure S5**: Phylogenetic analysis of TDF1/MYB35-like genes in *Actinidia*

327 **Supplemental Figure S6**: Gene disruption of *AT1G30800* in Arabidopsis using CIRISPR/Cas9

328 **Supplemental Figure S7**: Characterization of anther development in *AT1G30800* null line

329 **Supplemental Figure S8**: Silique and seed production in *AT1G30800* null line

330 **Supplemental Figure S9**: Gene disruption of *FAS1* in *Nicotiana tabacum* using CRISPR/Cas9

331 **Supplemental Figure S10**: Complementation of male function in *FAS1* null lines by kiwifruit

332 *FrBy*

333 **Supplemental Figure S11**: Correspondence between GEM barcodes and the anchored scaffolds

334 **Supplemental Figure S12**: Segregation of *FrBy*, in a KH-derived segregating populations.

335 **Supplemental Figure S13**: Characterization of the putative KH-specific deletion

336 **Supplemental Figure S14**: Cross pollination with p*FrBy*-*FrBy*-induced cv. Hort 16A

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338 **Supplemental Tables (S1-S12)**

339 **Supplemental Table S1**: 61 hypothetical genes identified in the MSY contigs and their

340 expression levels in anther

341 **Supplemental Table S2**: List of DEGs between male and female anthers

342 **Supplemental Table S3**: GO enrichment analysis in the DEGs between male and female anthers

343 **Supplemental Table S4**: Characterization of gene-edited *Arabidopsis* and *N. tabacum* lines

344 **Supplemental Table S5**: Summary of the draft genome assembly in cv. Soyu by 10X Genomics

345 data

346 **Supplemental Table S6**: List of the assembled scaffolds of cv. Soyu anchored by Y-specific

347 contigs

348 **Supplemental Table S7**: List of genes predicted in the 9 scaffolds anchored by the Y-specific

349 contigs

350 **Supplemental Table S8**: Expression patterns of the genes predicted in the 9 scaffolds anchored

351 by the Y-specific contigs

352 **Supplemental Table S9**: Pedigree of the KH line and the segregating population derived from

353   the KH line

354   **Supplemental Table S10**: Phenotypic characterization in p*FrBy*- *FrBy*-induced kiwifruit.

355   **Supplemental Table S11**: List of plant materials.

356   **Supplemental Table S12**: List of primers used in this study.

357

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369

370   **AUTHOR CONTRIBUTION**

371   TA, IK, and RT conceived the study. TA designed the experiments. TA, SMP, EV, SSS, MS, AF,

372   MJD, TW, RR and CV conducted the experiments. TA, SMP, EV, SSS, MS, IMH, and AF

373   analyzed the data, SMP, MAM, PD, ACA, KB, and IK initiated/bred and maintained the plant

374   materials. TA, SMP, EV, and IMH drafted the manuscript. All authors approved the manuscript.

375

376   **AUTHOR INFORMATION**

377   All sequence data generated in the context of this manuscript has been deposited in the appropriate

378   DDBJ database: Illumina reads for gDNAseq and mRNAseq in the Short Read Archives (SRA)

379   database (SRA IDs), the genomic contig sets constructed with 10X Genomics reads were

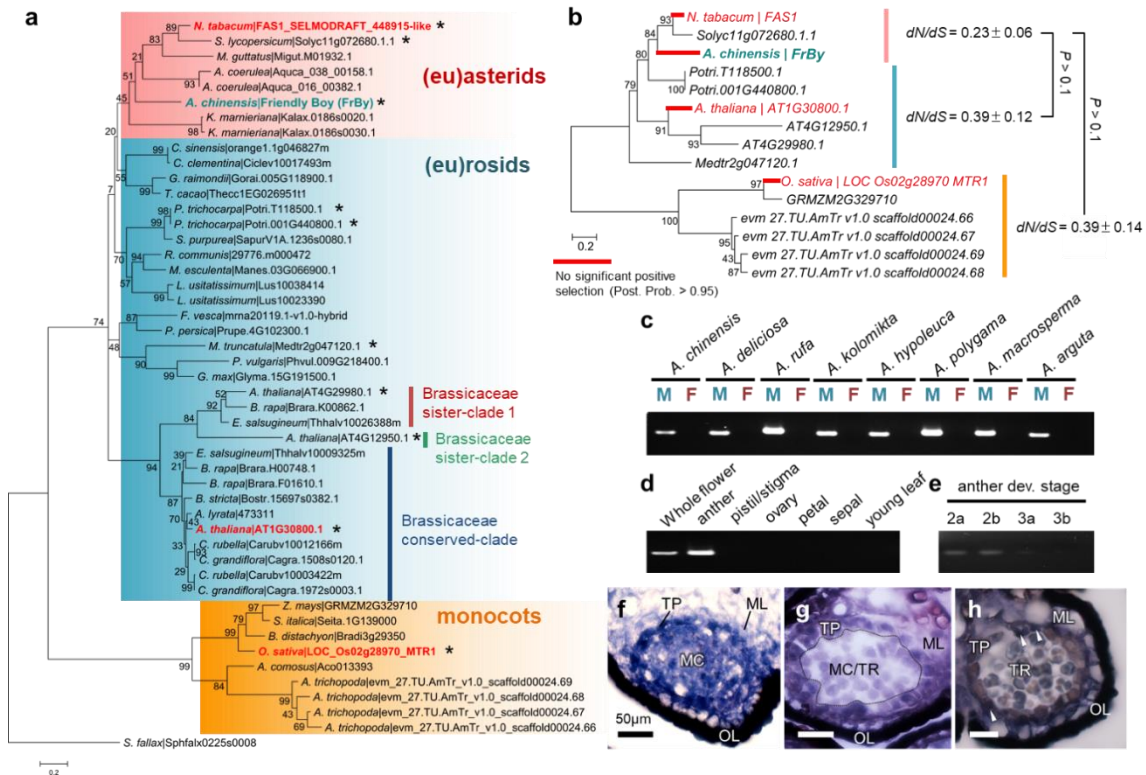380   submitted to Genbank (IDs).

381

382   Reprints and permissions information is available at www.nature.com/reprints.

383   The authors declare no competing financial interests.

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386

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Figure 1: Identification of the *fasciclin*-like *FrBy* as the candidate of the M factor

a, Phylogenetic tree of fasciclin-like genes from 32 angiosperm species, including the kiwifruit *FrBy* (light green). The orthologs from (eu)asterids, (eu)rosids, and monocots constitute three putatively monophyletic clades, shown in red, blue and yellow, respectively. The family Brassicaceae forms three subclades, although only one of them is conserved across the Brasicaceae species. The gene functions of the orthologs written in red have been validated previously (for MTR1 in rice) or in this study (for AT1G30800 for Arabidopsis, FAS1 for *Nicotiana tabacum*). The orthologs with asterisks were used for further evolutionary analysis in panel (**b**). **b**, pairwise evolutionary rates (*dN/dS*) in each clade suggested no protein functional differentiation between these orthologs. No site-branch specific positive selection was detected in red branches, suggesting that protein function and key domains were conserved in MTR1, AT1G30800, FAS1, and kiwifruit FrBy. **c**, PCR analysis specific to *FrBy*. Male-specificity of *FrBy* is conserved in a wide variety of *Actinidia* species. M: male, F: female. **d-e**, expression pattern of *FrBy*. (**d**), *FrBy* is expressed specifically in anthers and not on other organs. Within the anthers (**e**), *FrBy* is substantially expressed in stage 2a-b, and faintly at stage 3a. **f-h**, RNA *in situ* hybridization using antisense *FrBy* probe. *FrBy* is expressed highly in tapetum cells (TP) and meiocyte (MC) at stage 2a (**f**), in tapetum cells only at stage 2b (**g**), and faintly in tapetum cells (shown by arrows) at stage 3a (**h**). These results of RNA in situ hybridization were consistent with tapetum-specific expression analysis using laser capture microdissection (Supplemental Figure S2).
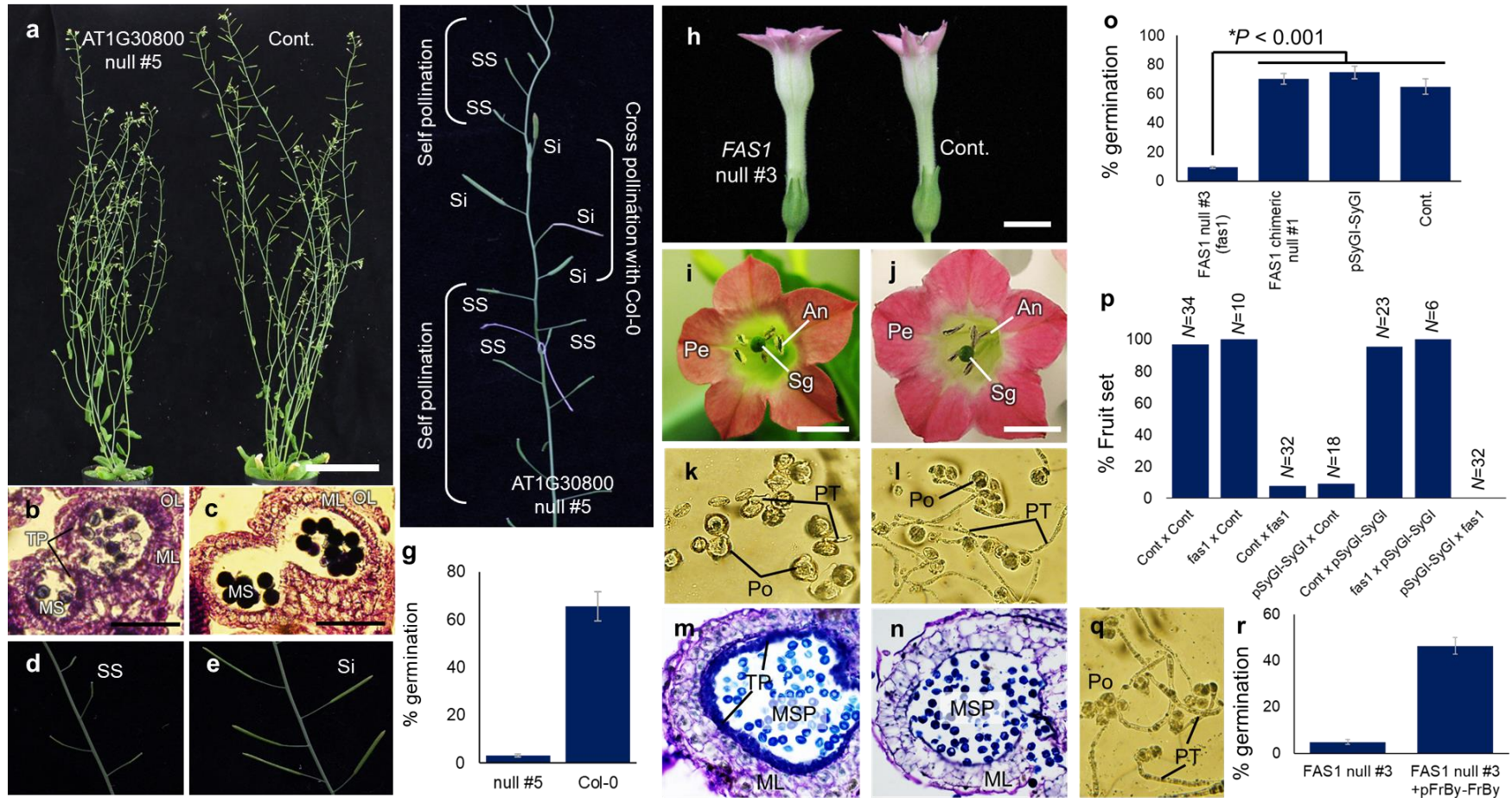
Figure 2: Functional validation of *FrBy* in two model plants.
a-g, A knock-out mutation in the *FrBy* ortholog, *AT1G30800*, resulted in male sterility in Arabidopsis. **a**, phenotype of the *AT1G30800* null mutant #5 and control lines. Developing anthers showed no tapetum degradation in the AT1G30800 null mutant (**b**), while controls showed complete degeneration of tapetum cells and matured microspore (MS) (**c**). TP: tapetum cells, OL: outer layer, ML: middle layer. **d-e**, reduced seed production

in the *AT1G30800* null mutant (**d**) and control (**e**). SS: sterile silique bearing no seeds, Si: silique. **f**, silique development after self- and cross-pollination of the *AT1G30800* null mutant line #5. Only cross pollination with Col-0 control plants resulted in seeds production. **g**, pollen germination ratios in *AT1G30800* null mutant #5 and #2, and control plants (Col-0). Null mutant #5 mostly lost the ability to produce fertile pollen, and chimeric null mutant #3 showed substantial reduction in pollen fertility. **h-p**, In *N. tabacum*, knock-out mutants of the *FrBy* ortholog, *FAS1*, showed male sterility. Whole plant (**h**) and flower organs (**i** for the *FAS1* mutant and **j** for control) phenotypes were not different between the *FAS1* null mutant and control lines. An: anther, Sg: stigma, Pe: petal. Pollen germination ability was substantially reduced in the *FAS1* null mutant (**k**), in comparison to the control lines (**l**). Po: pollen grain, PT: pollen tube. Thick tapetum layers were observed after anther dissection in the microspore stage of the *FAS1* null mutant (**m**), while control lines showed complete degradation of tapetum cells (**n**). In comparison to the *FAS1* null mutant, the SuF gene, *SyGI*, had no significant effect on pollen germination rate (**o**). Reciprocal crosses with control, *FAS1* null, and *SyGI*-expressing lines indicated that *FAS1* and *SyGI* act only for male and female functions, respectively (**p**). **q-r**, complementation of male function in *FAS1* null, using the kiwifruit *FrBy* gene under the control of its native promoter (p*FrBy-FrBy*). Observation of pollen germination (**q**) and germination ratio (**r**) in *FAS1* null mutants transformed with p*FrBy-FrBy*.
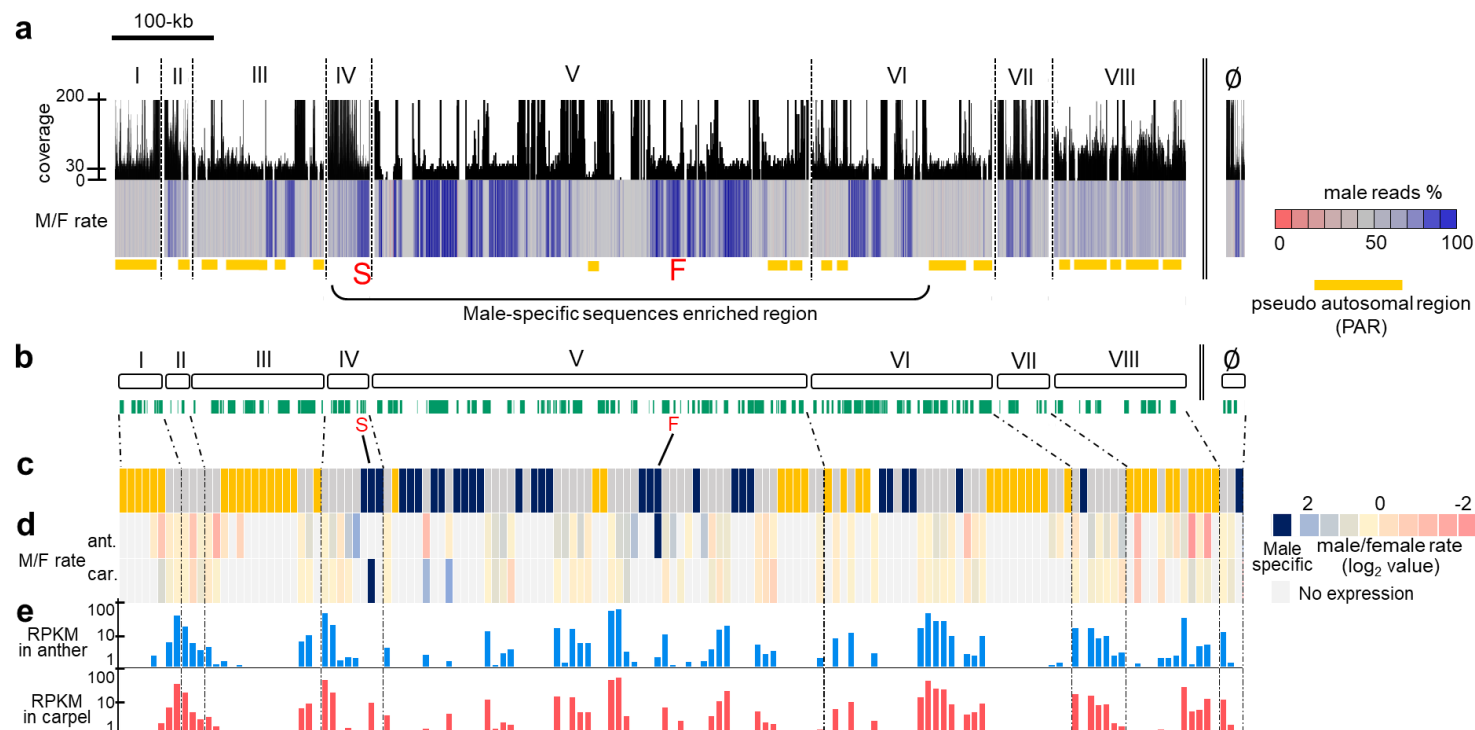
Figure 3. Sequence architecture of the kiwifruit Y chromosome, including the two sex determinants

a, Super-scaffold constituted of anchored 8 contigs (I-VIII) and unanchored one (∅), with coverage of male genomic read (bars) and percentage of male genomic reads (color chart). The coverage from simplex alleles at single sites (or male-specific region with no X-chromosome counterpart) is estimated at 20-23X. Repeated regions, such as transposable elements, were identified as those with high coverage (>50X). Putative pseudo-autosomal regions (PAR) were defined as non-repetitive regions with unbiased reads coverage (ca 25-75% male reads), and highlighted with orange bars. *SyGI* (S) and *FrBy* (F) were located on male-specific regions in the middle of the Y-chromosomal contigs. **b**, models of the nine contigs (white bars) and the genes predicted in these contigs (green bars). **c**, male-specificity in the predicted genes in each contig. Genes in male-specific, PAR, and repetitive regions, are shown in blue, orange, and gray, respectively. **d-e**, expression patterns of the predicted genes in each contig. **d**, expression bias between male and female individuals, in anthers (ant.) and carpels (car.). Complete male-specific expression was given in thick blue. **e**, expression levels (RPKM) in anther and carpel. Across the Y-specific genes as given in deep blue, only *FrBy* and *SyGI* were substantially expressed (RPKM > 1) in differentiating anther and carpel, respectively.
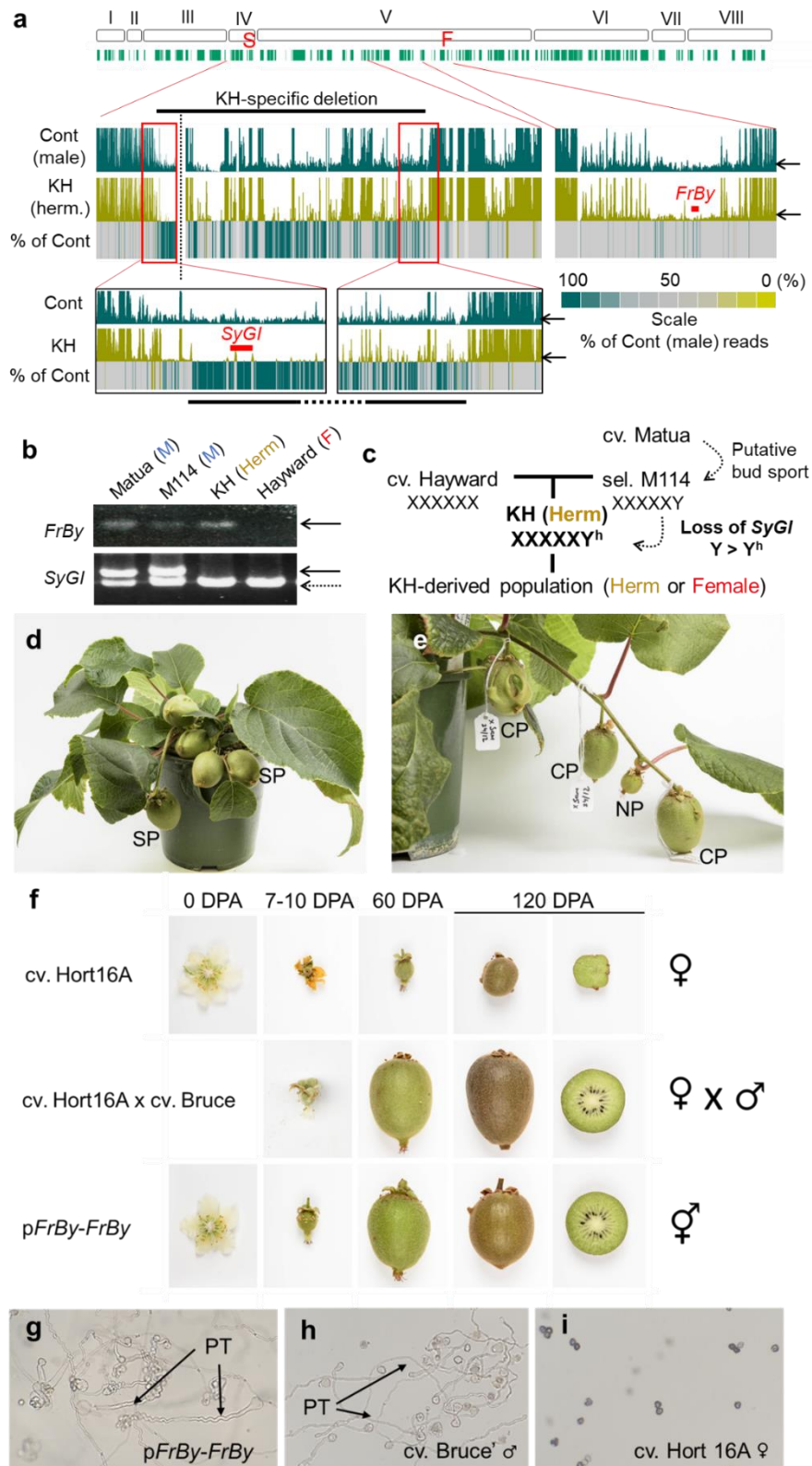
Figure 4. Lost of *SyGI*, or gain of *FrBy* resulted in a natural and synthetic hermaphrodite kiwifruit, respectively

a, Genomic context in the Y-chromosomal contigs, in male cv. Matua (control), and a natural hermaphrodite line, KH. The KH line carries long deletions across contigs IV-V, including *SyGI*.

This was confirmed by PCR analyses (Supplemental Figure S13). The right arm of contig V was conserved in the KH line. Arrows indicated estimated coverage of simplex Y allele. **b**, conservation of *FrBy* and *SyGI* in KH and its parental cultivars/lines, based on the pedigree of the KH lines (as shown in **c**). Solid arrows indicated *FrBy* and *SyGI*. Dotted arrow showed a paralog of *SyGI* with high sequence homology located on an autosome (Akagi et al. 2018). M: male, F: female. *SyGI* is absent specifically in the KH line, while *FrBy* was conserved in both parental males and the KH lines. **c**, pedigree of the KH line and model for the establishment of the *SyGI* null Y^h chromosome. **d-i**, development of hermaphrodite kiwifruit by transformation with *FrBy* under the native promoter (p*FrBy-FrBy*). **d**, The p*FrBy-FrBy* lines showed fertilized fruits with self-pollination (SP) in 120 days post anthesis (DPA). **e**, Control female cv. Hort16A showed unfertilized fruit in non-pollination (NP), while cross-pollination with p*FrBy-FrBy* pollen (CP) resulted in fertilized fruit. **f**, In control female cv. Hort16A (top panels), flowers aborted or developed small parthenocarpic fruit without seeds. In cross pollination of control female and a male cv. Bruce (middle), normal fruits bearing fertile seeds were developed on pollinated cv. Hort16A. In self-pollination of transgenic cv. Hort 16A with p*FrBy-FrBy* (bottom), normal fruits with fertile seeds were developed, comparable to normal female x male. Pollen grains from p*FrBy-FrBy*-induced female cv. Hort16A (**g**) had the ability to grow pollen tubes (PT), as well as from male cv. Bruce (**h**), while pollen grains from control female cv. Hort16A were sterile (**i**).
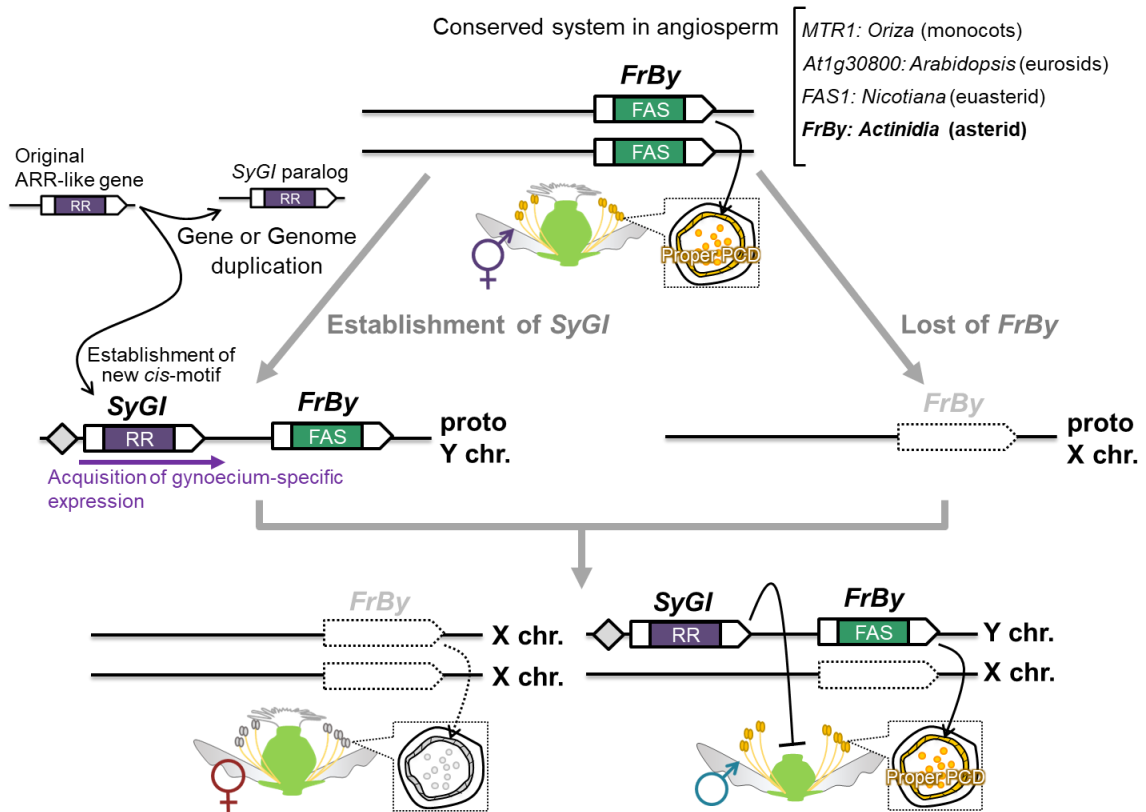
Figure 5: Evolutionary model for the establishment dioecy in *Actinidia*
The putative hermaphrodite ancestor bears an intact *FrBy* ortholog, which carries a function that is conserved across angiosperm. The loss of function in *FrBy* generated a proto X-chromosome, while the gain of function in *SyGI* (15), which occurred next to the intact copy of *FrBy*, resulted in a proto Y-chromosome. Together, these proto X and Y chromosomes evolved to derive the current XY system in the *Actinidia* genus.