VviPLATZ1 is a major factor that controls female flower morphology determination in grapevine

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Plant genetic sex determinants that mediate the transition to dioecy are predicted to be diverse, as this type of mating system independently evolved multiple times in angiosperms. Wild Vitis species are dioecious with individuals producing morphologically distinct female or male flowers; whereas, modern domesticated Vitis vinifera cultivars form hermaphrodite flowers capable of self-pollination. Here, we identify the VviPLATZ1 transcription factor as a key candidate female flower morphology factor that localizes to the Vitis SEX-DETERMINING REGION. The expression pattern of this gene correlates with the formation of reflex stamens, a prominent morphological phenotype of female flowers. After generating CRISPR/Cas9 gene-edited alleles in a hermaphrodite genotype, phenotype analysis shows that individual homozygous lines produce flowers with reflex stamens. Taken together, our results demonstrate that loss of VviPLATZ1 function is a major factor that controls female flower morphology in Vitis.
Traits associated with flower development and fertilization are key for increasing yield in agriculture and horticulture crops. Plants evolved multiple reproductive systems to promote outcrossing, including self-incompatibility, dioecy and dichogamy. In a dioecy mating system, individual plants produce either male or female flowers that are morphologically distinct. During plant speciation, the dioecy reproductive system independently evolved multiple times indicating that the underlying mechanisms that control female and male flower development are diverse. While approximately 5–6% angiosperms are dioecious, a number of economically important crops utilize this mating strategy.

In the past decade, one- and two-gene sex determination systems were characterized in a subset of dioecious plants. A one-gene model of sex determination described in Diospyros and Populus is dependent upon the duplication of a single gene, which gives rise to small non-coding RNAs that alter flower sex identity. Actinidia and Asparagus are two species that exhibit a two-gene model of sex determination involving DNA polymorphisms that result in: (1) ectopic expression of a male-promoting factor that suppresses pistil formation and (2) a recessive mutation in a single gene required for tapetum development and pollen fertility.

Grapevine cultivars derived from Vitis vinifera are a major perennial crop of economic significance throughout the world for wine, table and dried grape production. Modern domesticated grapevines produce hermaphrodite flowers with functional stamens and pistils for self-pollination, which is a fundamental trait for productivity acquired during domestication. Interestingly, the wild progenitor of Vitis vinifera grapevine cultivars, as well as other Vitis species, are dioecious. In wild Vitis species, females initiate flowers that produce short reflex stamens that bend away from the receptive pistil, which likely reduces self-fertilization. The reflex stamen phenotype is utilized by grapevine breeders and biologists to identify female vines for cultivar development. While pollen derived from female flowers is viable, fertility is absent due to an impairment in germination. In males, flowers produce erect functional stamens, while pistil formation is aborted early in development, which prevents self-fertilization.

In Vitis species and hybrids, the SEX-DETERMINING REGION (SDR) is located on chromosome 2. Depending on the Vitis species/hybrid accession and cultivar, the size of the locus varies from 111 kb to 837 kb; however, gene identity and content are conserved. One hypothesis predicts that grape sex determination is mediated by the two-gene model. According to this hypothesis, female flower formation is mediated by a recessive mutation that controls stamen development. Therefore, the genotype of female individuals is iff. In contrast, male flower development is determined by a dominant male-promoting factor (M) that suppresses pistil development. Lastly, domesticated hermaphrodite grapevines with an H/H or H/h genotype produce perfect flowers that contain a modified and less dominant M-factor.

Through sequence analysis of M-, H- and f-haplotypes derived from domesticated and wild Vitis species, the structure of the SDR was determined and candidate sex-determining genes were identified. Genetic association analysis of M- and f-specific DNA polymorphisms and phylogenetic studies of sex-linked genes indicate that male and female flower development is specified by genetic determinants located in the 5’ and 3’ region of the SDR, respectively. The male sex-determining region (MSDR) consists of two genes, Vitis vinifera YABBY3 (VviYABBY3) and VviSKUS, while 11 genes from TREHALOSE-6-PHOSPHATE PHOSPHATASE (TPP) to WRKY21 reside in the female sex-determining region (FSDR). The H-haplotype is structurally similar to the f-haplotype from VviYABBY3 to the aldolase gene but structurally related to the M-haplotype from TPP to the WRKY21 transcription factor. Initial studies suggested that VviAPT3 was the candidate M-factor that controls male flower formation through the inactivation of cytokinin, which is required for pistil development.

In support of these studies, transcript levels for VviAPT3 are significantly increased in male flowers compared to hermaphrodite and female flowers, and the mRNA for this gene localizes to cells in the male flower meristem that gives rise to the pistil. Consistent with the increased expression of VviAPT3 in the male flower meristem, applications of cytokinin restore pistil development. However, recent studies indicate that VviAPT3 is not located in the MSDR. Therefore, VviYABBY3 emerged as the primary candidate M-factor as this is one of two genes located in the MSDR and the expression of this gene associates with the formation of male flowers. Further, YABBY transcription factors are critical for the development of lateral organs, including carpels.

Genetic studies indicate that a candidate DNA polymorphism located in Vitis INAPERTURATE POLLEN1 (VviINP1) is the underlying female sterility determinant, as this gene is predicted to play a role in pollen development. While candidate f-haplotype sex genes have been identified from the above studies, functional analysis demonstrating a role for these genes in pistil and pollen development have yet to be determined.

Here, we show through genetic and molecular studies that the VviPLATZ1 (plant AT-rich sequence-and zinc-binding protein) transcription factor is a candidate factor involved in female flower development. To this end, VviPLATZ1 localizes to the FSDR and low expression of this gene correlates with the formation of reflex stamens. To validate the role of VviPLATZ1 in reflex stamen development, the CRISPR/Cas9 system is used to generate multiple alleles of this gene lacking the conserved PLATZ domain in a rapid cycling hermaphrodite genotype. Phenotype evaluation shows that homozygous individuals harboring gene-edited alleles of VviPLATZ1 produce flowers that initiate stamens with a reflex architecture. Taken together, results show that the formation of reflex stamens is mediated by the loss of VviPLATZ1, which is a factor involved in controlling female flower morphology in Vitis.

Results

Genetic and molecular analysis of VviPLATZ1. As shown in Fig. 1a, association genetics indicates that the FSDR is comprised of 11 genes from TPP to WRKY21. To further narrow down the number of genes that reside in the FSDR, 21 SNPs spanning the PN40024 SDR were identified (Supplementary Table 1). Three genetic mapping resources were genotyped with this SDR SNP set: (1) Vitis vinifera cultivars with known flower sex genotypes (f/f, H/H or H/h), (2) an F1 00C001V0008 (f/f) x Ugni Blanc (H/h) mapping population, and (3) self-fertilized hermaphrodite microvigne (H/h) progenies (S1 to S5). However, as the PN40024 SDR contains a mixture of H- and f-sequences, this reference genome was not suitable for fine genetic mapping of the reflex stamen phenotype, as gene content between the H- and f-haplotypes differs in the SDR.

Genomic studies indicate that gene content and order in the SDR of f-haplotypes is conserved. Genotype and sequence data from the F1 00C001V0008 (f/f) x Ugni Blanc (H/h) individuals further supported the location of the 5’ FSDR boundary in TPP (Supplementary Data 1). A single
recombination event at VvMT72 in the S5-microvine, 06C008V0018, defined the 3' FSDR boundary located in FLAVIN-CONTAINING MONOOXYGENASE 3 (FMO3; Fig. 1b; Supplementary Table 1, Supplementary Data 1). Taken together, fine genetic mapping demonstrated that the FSDR contained seven genes: VviINP1, Exostosin, 3-ketoacyl-acyl carrier protein synthase III (KASIII), VviPLATZ1, FMO1, FMO4, and FMO2 (Fig. 1b).

The recessive mutation resulting in the formation of reflex stamens is predicted to reduce the expression and/or function of a candidate gene required for stamen development. Of the seven genes located in the FSDR, transcript abundance for VviPLATZ1 appeared to correlate with the development of reflex
Genes localized within the FSDR and molecular analysis of VviPLATZ1. a Diagram of the SDR structure derived from the Cabernet Sauvignon f-haplotype. Genes localized within the FSDR are displayed in yellow. Gray arrows depict genes located outside of the SDR. Black arrows were used to display genes flanking the SDR. b Fine genetic mapping narrowed the SDR to seven genes, highlighted in yellow. The black vertical arrows indicate the location of the VvMT54 and VvMT72 SNPs in TPP and FMO3, respectively, which mark the boundaries of the FSDR. c Transcript levels determined for VviPLATZ1 in 04C023V0006 (H/H) and 04C023V0003 (f/f) flowers at eight, six and four weeks prior to anthesis (WPA), as well as leaves (L). Note: stamen development and pistil initiation occurred at eight WPA. Filament elongation and ovule development occurs at six WPA. Stamen development is nearly complete by four WPA. Data are presented as mean values ± SD. Significance determined by two-tailed Student’s t test indicated by asterisks (*p = 0.0089; **p = 0.00027; n = 3); not significant, ns. d Diagram of VviPLATZ1 genomic structure, which contains five exons (boxes). Exons 3 and 4 displayed in green encode the PLATZ domain. In the f-haplotype, DNA polymorphisms altered the nucleotides at −13 bp and −5 bp in the 5′ UTR, as well as the first codon (underlined nucleotides). The arrowhead and gray highlight illustrate the adenine insertion. The start codon for the hermaphrodite (H/H) and female (f/f) VviPLATZ1 alleles is highlighted in yellow. Point mutations that alter amino acid content in exons 3, 4, and 5 are displayed by asterisk (*). The GenBank accession number for VviPLATZ1 derived from 04C023V0006 is MW548436. For the VviPLATZ1 allele derived from 04C023V0003, the GenBank accession number is MW548435. Source data underlying Fig. 1c are provided as a Source Data file.

Despite the results from these expression studies, a role for the loss of VviPLATZ1 function in reflex stamen development was not recognized. Therefore, to support the hypothesis that loss of VviPLATZ1 results in the formation of reflex stamens, transcript levels for this gene were examined in 04C023V0006 (H/H) and compared to 04C023V0003 (f/f) flowers at eight, six, and four week prior to anthesis (WPA), as previously defined. Results showed that transcripts for VviPLATZ1 were readily detectable in 04C023V0006 flowers at eight and six WPA (Fig. 1c), which correspond to a developmental time in which stamens differentiate and filaments elongate, respectively. At four WPA, when flower development is nearly completed, the mRNA levels for this gene were significantly decreased. In addition, VviPLATZ1 mRNA levels were readily detected in male flowers of 03C003V0060 (M/f) during stamen development at eight and six WPA (Supplementary Fig. 1). In contrast to 04C023V0006 and 03C003V0060, transcript levels for VviPLATZ1 were significantly reduced in 04C023V0003 during stamen development at eight and six WPA (Fig. 1c and Supplementary Fig. 1). Transcript levels for VviINP1, Exostosin, KASIII, FMO1, FMO2, and FMO3, which are located in the FSDR, did not correlate with the formation of reflex stamens.

DNA sequence comparisons between 04C023V0006 (H/H) and 04C023V0003 (f/f) identified female-specific DNA polymorphisms in VviPLATZ1. First, a thymine to adenine point mutation and adenosine insertion were identified in the first codon, resulting in a frame-shift mutation that altered the start of the codon (Fig. 1d). In addition, two SNPs were identified at −5 bp and −13 bp in the 5′ UTR. Through the alignment of hermaphrodite, male and female VviPLATZ1 alleles from sequenced haplotypes of domesticated and wild Vitis species, results showed that female DNA polymorphisms at −13 bp, −5 bp, and +2 bp were conserved (Supplementary Fig. 2). However, the adenosine insertion at +3 bp in the 04C023V0003 VviPLATZ1 allele was found only in a subset of female-specific VviPLATZ1 alleles. Taken together, the conserved female-specific DNA polymorphisms at −13 bp, −5 bp, and +2 bp may act to reduce transcription and/or alter mRNA decay of the female VviPLATZ1 allele. In addition to these DNA polymorphisms, seven nonsynonymous SNPs were identified in the 04C023V0003 VviPLATZ1 allele, which may reduce protein function, including a glutamic acid to lysine substitution in the PLATZ domain (Fig. 1d). To investigate whether these seven nonsynonymous SNPs were conserved, the 04C023V0003 VviPLATZ1 amino acid sequence was aligned with hermaphrodite, female and male VviPLATZ1 proteins derived from sequenced domesticated and wild Vitis species. Results showed that all seven nonsynonymous SNPs were conserved in the female VviPLATZ1 alleles (Supplementary Fig. 3). It should also be noted that the reduction in the number of transposable elements upstream of the female VviPLATZ1 allele compared to hermaphrodite and male alleles, as well as differences in RNA editing between male and female VviPLATZ1 transcripts, may also be important factors attributed to low VviPLATZ1 transcript accumulation in female flowers. As the reflex stamen phenotype is a recessive inherited trait, the female allele is referred to as Vviplat1.

Tissues-specific expression of VviPLATZ1 during flower development. To gain insight into the proposed function of VviPLATZ1 in stamen development, the expression pattern of this gene was analyzed in 04C023V0006 (H/H) and 04C023V0003 (f/f) flowers during filament elongation at six WPA. At this time point, transcript abundance of VviPLATZ1 was significantly higher in 04C023V0006 hermaphrodite flowers compared to 04C023V0003 female flowers (Fig. 1c). In hermaphrodite flowers, VviPLATZ1 was primarily expressed in elongating filaments, as well as developing anthers and microspores (Fig. 2a–c). A similar pattern of expression was detected in flowers derived from the male microvine 03C003V0060 (M/f; Supplementary Fig. 4). In contrast, VviPLATZ1 expression was relatively low in female filaments and anthers, as well as microspores (Fig. 2d–f). The expression of VviPLATZ1 in ovules of hermaphrodite and female flowers (Fig. 2a, d, respectively), as well as male flowers (Supplementary Fig. 4) suggests that this gene may play a role in ovule development. Based on the molecular and gene expression studies described above, we propose that reduced VviPLATZ1 function is a major factor that results in the development of reflex stamens.

Gene-editing of VviPLATZ1. Efficient validation of candidate genes implicated in flower development via reverse genetics is constrained by the perennial nature of grapevine, which displays a long juvenile phase and annual reproductive cycle. The microvine model system has been utilized to overcome the genetic limitations of grapevine. This model system is highly amendable to rapid reverse genetics assessment, as the microvine can be stably transformed and displays a continuous flowering phenotype with a reduced generation cycle. Therefore, the role of VviPLATZ1 in stamen development was functionally validated in a homozygous hermaphrodite microvine genotype, 04C023V0006 (H/H), using the CRISPR/Cas9 system. Two guide RNAs designated FS1 and FS4 were developed to induce indels at the 3′ end of exon 2 upstream of the coding region for the PLATZ domain (Fig. 3a). Eight edited alleles were identified in T0 plants, two of which contained a single bp insertion, while the remaining six alleles contained single or multiple bp deletions (Fig. 3b). All alleles altered the reading frame resulting in a truncated protein lacking the conserved PLATZ domain, which likely abolished VviPLATZ1 function (Fig. 3c). Interestingly, one of the T0 plants...
identified harbored both Vviplat-z1-1 and Vviplat-z1-2 alleles and was referred to as Vviplat-z1-1/2. The other six T0 individuals were heterozygous for only one of the remaining mutated alleles. All seven T0 plants were self-pollinated and the flowers of homozygous T1 Vviplat-z1-(1/2-8) plants were analyzed and compared to hermaphrodite and female flowers.

**Loss of VviPLATZ1 function produced flowers with reflex stamens.** To determine if reduced VviPLATZ1 function plays a role in female flower formation, stamen morphology was examined in hermaphrodite (H/H; VviPLATZ1/VviPLATZ1), female (f/f; Vviplat-z1/Vviplat-z1) and homozygous T1 Vviplat-z1-(1/2-8) plants. Flowers produced in hermaphrodite individuals initiated upright stamens with an elongated phenotype (Fig. 4a, g). The average length of stamens in hermaphrodite individuals was 3.0 mm (Supplementary Fig. 5). In contrast, female flowers initiated reflexed stamens 98% of the time (Fig. 4b, g). The remaining 2% of female stamens displayed an upright growth pattern but were significantly shorter with an average length of 1.2 mm (Supplementary Fig. 5). Flowers produced in Vviplat-z1-3, -5, -7, and -8 displayed an overall morphology strikingly similar to female flowers (Fig. 4c–f, respectively). Flower morphology displayed in Vviplat-z1-1/2, -4, and -6 was similar to the structure of female flowers (Supplementary Fig. 6). In all seven Vviplat-z1-(1/2-8) plants analyzed, 95 to 99% of stamens displayed a reflex architecture, while the remaining stamens were stunted with an average length of 1.4 mm (Supplementary Fig. 5). Taken together, loss of VviPLATZ1 transforms hermaphrodites into females that initiate flowers with reflex stamens during flower development.

**Discussion**

Diocious mating systems evolved mechanisms to promote outcrossing through the arrested development of male and female reproductive organs and haploid sex cells in female and male individuals, respectively. Floral morphology is a key factor that influences outcrossing, as exemplified by the spatial separation of anthers and stigmas in heterostyly flowers. The formation of reflex stamens that spatially separates anthers from stigmas is a unique dioecious feature displayed by Vitis female flowers, which likely contributes to the outcrossing potential displayed in this dioecious mating system.

The formation of male and female flowers is influenced by hormones, which not only affects ovule and pollen development but also the morphology of floral organs, including petals, stamens, and pistils. Experimental studies indicate that asymmetric distribution of auxin regulates organ bending during root and hypocotyl development. In developing stamens, auxin biosynthesis, signaling and transport is critical for filament elongation. Therefore, the symmetric distribution of auxin during filament elongation is predicted to cause organ bending for the reflex stamen phenotype. It would be interesting to determine whether auxin is involved in the formation of reflex stamens and whether the distribution of this hormone is regulated by VviPLATZ1.

PLATZ genes encode plant-specific transcription factors that regulate a diverse array of reproductive processes including endosperm development, grain length and filling. Experimental studies indicate that the Zea mays floury3 (f3) and Oryza sativa GL6 regulate grain development through interaction with the RNA polymerase III (RNAPIII) complex, which transcribes transfer RNAs (tRNAs) and 5 s ribosomal RNA (5 s rRNA). Of the 17 PLATZ proteins identified in maize, 14 interact with a component of the RNAPIII complex indicating that PLATZ proteins regulate developmental processes via tRNA and 5 s rRNA biogenesis. The role of VviPLATZ1 in stamen development further demonstrates that this class of plant-specific transcription factors regulates a wide range of reproductive processes. Further, it is tempting to speculate that loss of VviPLATZ1 function reduces the pool of tRNAs and 5sRNAs required for proper translation during stamen development. Lastly, phylogenetic analysis showed that VviPLATZ1 is a member of a clade that includes GL6, as well as uncharacterized PLATZ proteins from Zea mays, Oryza sativa and Arabidopsis thaliana (Supplementary Fig. 7).

While female flower morphology is determined by loss of VviPLATZ1 function, the f-haplo-type-specific 8-bp deletion in the Vitis
INAPERTURATE POLLEN1-like gene (VviINP1) is a candidate mutation implicated in pollen fertility. Indeed, inaperturate pollen is produced in female flowers of *Vitis riparia* and wild *Vitis vinifera* individuals. While pollen fertility is not affected in *Arabidopsis* *inpl* plants, studies in maize indicate that ZmINP1 is crucial for germination. As a result, gene-editing of *INAPERTURATE POLLEN1* is necessary to validate a role for the 8-bp deletion in *Vvinifera*.

In this manuscript, results from this manuscript show that VviPLATZ1 is a key regulator of female flower formation in grapevine. This is supported by the fact that VviPLATZ1 is expressed in the filaments and anthers of hermaphrodite flowers prior to and at the time in which stamens elongate. Moreover, using the CRISPR/Cas9 gene-editing system in the rapid cycling hermaphrodite microvine, functional analysis demonstrated that loss of VviPLATZ1 is a key factor that controls reflex stamen development during female flower formation.

**Methods**

**Plant materials.** The Pinot Meunier L1 mutant, referred to as the microvine, contains a *GIBBERELLIC ACID INSENSITIVE1*-like (VviGAI1) gain of function allele. While Vvega1 vines display a dwarf phenotype, this mutation does not alter flower morphology. The 04C023V0006 (H/H) and 04C023V0003 (h/f) microvines, which were derived from a Grenache (H/h) x L1 microvine cross, were used for molecular and gene expression analyses. Gene-editing was performed in 04C023V0006. Gene expression studies was also performed in 03C003V0060 (M/M), which was derived from 00C001V0008 (selfed L1 Pinot Meunier microvine) x Richter 110 (V. berlandieri cv. Boutin B x V. rupestris cv. du Lot). Plant material used for fine genetic mapping was derived from F1 00C001V0008 (f/f) x Ugni Blanc (H/h) individuals and successive self-pollinated microvines (H/h) from first (S1) to fifth generation (S5). These mapping lines were maintained in glasshouses and growth rooms under long-days (16 h light/8 h dark) with 27 °C and 22 °C day and night temperatures, respectively. *Vitis vinifera* cultivars used in this study were derived from the cultivar collection at the CSIRO vineyard in Merbein, VIC, Australia.

**DNA marker development and fine genetic mapping of the FSDR.** Sequences for the SSR markers, UDV027, VVIB23, VMC3B10 and VMC6F1, flanking the previously identified SDR2, SDR23, SDR24, were used to identify the genomic region of this locus in the 8X PN4002459 and Pinot Noir genomes. The genomic sequences of annotated genes regularly spaced between UDV027 and VMC6F1 were used for local alignment search tool (https://blast.ncbi.nlm.nih.gov). Thirty-three candidate SNPs were identified in exons and/or introns of selected genes in the region from VIT_202s0025g04920 to VIT_202s0154g00230 (Supplementary Table 1). The Agena Bioscience MassARRAY platform (Agena Bioscience, San Diego, California, USA) was used to validate the 33 SNPs by genotyping *Vitis vinifera* cultivars (n = 33) and self-pollinated...
Reflexed Stunted

Elongated stamens (blue) with an upright growth pattern and an average 0.4 cm (this procedure, DNA was isolated from immature leaves using the NucleoSpin

Representative images of function. with this SDR SNP set. The MassARRAY genotyping was performed at the Aus-

vines (green). In addition, 1% of the stamens initiated in f/f and homozygous mutants were stunted and grew to an average length of 1.2 and 1.4 mm, respectively. The scale bar is 1.0 mm and is representative for all flower images (a-f). Source data underlying Fig. 4g are provided as a Source Data file.

microvines (n = 37) outlined in Supplementary Data 1. In addition, the F1, 04C023V0006 x Ugni Blanc mapping population (n = 101) was also genotyped with this SDR SNP set. The MassARRAY genotyping was performed at the Aus-

rRNA was extracted from 100 mg of flowers derived from at least three inflorescences from a single 04C023V0006 (H/H), 04C023V0003 (f/f) and 03C003V0006 (M/M) plant at eight, six and four WPA (Supplementary Fig. 8)38, using the Spectrum Plant Total RNA Kit (Sigma-Aldrich

P}-like. Sydn. Nsw, Australia, #5TRN250). After RNA extraction, samples were purified using the RNA Clean & Concentrator" (Zymo Research, Irvine, California, USA, #R1013). Total RNA was also extracted and purified from 100 mg of leaf tissue derived from 4 immature leaves, approximately 25 cm2 in size, from 04C023V0006, 04C023V0003, and 03C003V0060. Three biological replicates were used for qRTPCR. Fifteen FSDR transcript abundances in developing flowers at eight, six and four WPA, as well as leaves, for 04C023V0006 and 04C023V0003. For 03C003V0060, only two biological replicates were used for determining Vvi-

Fig. 4 Reflex stamen development is controlled by loss of VviPLATZ1 function. Representative images of flowers derived from (a), 04C023V0006 (H/H, VviPLATZ1/VviPLATZ1); (b) 04C023V0003 (f/f, VviPLATZ1/VviPLATZ1); (c) homozygous VviPLATZ1-3; (d) VviPLATZ1-5; (e) VviPLATZ1-7; (f) VviPLATZ1-8 plants. g Quantification of stamen architecture in 04C023V0006, 04C023V0003, and homozygous mutated plants. Elongated stamens (blue) with an upright growth pattern and an average length equal to 3.0 mm was initiated in H/H flowers. Reflex stamens that bend away from the pistil were produced in f/f and homozygous mutated flowers (green). In addition, 1-5% of the stamens initiated in f/f and homozygous mutated flowers were stunted and grew to an average length of 1.2 and 1.4 mm, respectively. The scale bar is 1.0 mm and is representative for all flower images (a-f). Source data underlying Fig. 4g are provided as a Source Data file.

Cloning VviPLATZ1 alleles. To clone VviPLATZ1, the B26 primer (GACTC-

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GCGCTTTTTCTTCACGAACT), VviUbi6 and VviActin263 with a final concentration of 0.75 µM. Amplification was performed using the Roche LightCycler

Chadstone, Victoria, Australia, #231222). After sequencing the colonies, the DNA concentration (ng/µl) for each construct was determined using the Quant-iT™ PicoGreen dsDNA Quantification Kit (Promega, Madison, Wisconsin, USA, #E2670) and the GloMax® Discover Microplate Reader (Promega Madison, Wisconsin, USA, #GM3000). DNA copy number was calculated in each standard dilution65. After amplification, a standard curve derived from the absolute DNA copy number values from the dilution series for each gene was used to calculate transcript copy number for VviPLATZ1, VviUbi and VviActin2 in each of the samples using the Roche LightCycler 480 software. Transcript copy number for VviPLATZ1 was normalized against reference genes, VviUbi and VviActin2 for each sample.

mRNA in situ hybridization. In situ hybridization of VviPLATZ1 was performed in sectioned 04C023V0006 (H/H, 04C023V0003 (f/f) and 03C003V0006 (M/M) flowers at stage six WPA using a modified procedure previously described66. Briefly, in situ

exones were fixed in 4% paraformaldehyde (PFA) phosphate buffered solution (pH 7.4) and embedded in 45% Triton X-100 and 5% DMSO for 12 h at 4 °C. Ideal tissues were dehydrated using a graded ethanol series (H2O, 15%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% and 100%) at 4 °C and cleared using a graded Histoclear solution (Sigma-Aldrich, Sydney, New South Wales, Australia, #117279-11; 25%, 50%, 75% and 100%) at 20 °C before embedding tissue in Paraplast Plus (Sigma-Aldrich, Sydney, New South Wales, Australia, #125387-89-S). Microtome sections (8–10 µm thick) were prepared and adhered to ProbeOn Plus™ microscope slides (ThermoFisher Scientific, The-}

barn, South Australia, South Australia, Australia, #15-188-51) followed by deparaffinization with Histoclear. Next, slides were rehydrated via a graded ethanol series (100%, 95%, 85%, 75%, 50%, 30%, 15%, H2O at 20 °C then treated with protease K (Promega Madison, Wisconsin, USA, #F9481; 1.0 µg/ml in 10 µl Tris-HCl buffer, pH 8) for 10 min at 37 °C. After washing with PBS, the slides were dehydrated under vacuum. A digoxigenin (DIG) labeled anti-sense probe from nucleotide position 54 to 870 in the VviPLATZ1 coding sequence was used to probe the T7 polymerase (Promega, Sydney, New South Wales, Australia, #P207). Hybridization was performed in saline-sodium citrate (SSC) solution containing 1% SDS, 50% formamide, 100 µg/ml RNAse A 2 h at 32 °C. After washing with SSC solution, the slides were incubated with the Boehringer Blocking Reagent (Roche Diagnostics, Mannheim, Germany, #1109676001) for 2 h at 4 °C. Next, the slides were incubated with a 1:1000 dilution of anti-DIG-alkaline phosphatase-conjugated Fab fragments (Roche Diagnostics, Mannheim, Germany, #11093274910), in phosphate-buffered solution (pH 7.2) containing 0.4% Nonidet P-40 (NP-40) and 0.1% Triton X-100 at 4 °C for 1 h. After washing with phosphate-buffered solution (pH 7.2), the slides were incubated with the alkaline phosphatase substrate Western Blue® (Promega, Sydney, New South Wales, Australia, #53841) for
12 h at 20 °C in order to visualize of VviPLATZ1 transcripts. Colorization was terminated by incubating slides in TE buffer (10 mM Tris-HCl/1 mM EDTA, pH 7.5). The sections were hybridized with a digoxigenin-labeled oligonucleotide probe (Zea Axioskop2, Oberkorn, Germany). Three biological replicates were derived from 04C023V0006, 04C023V0003 and 03C003V0060 plants. Each biological replicate was derived from a single inflorescence.

CRISPR/Cas9 vector construction. The VviPLATZ1 DNA sequence was scanned for 20 bp guide sequences followed by the NGG protospacer adjacent motif (PAM) using Benchling (https://benchling.com), which has been calculated and off target scores76,68. The two guide RNA sequences, FS1 and FS4, that targeted exon 2 were selected, as gene-edited mutations would result in the production of truncated proteins lacking the conserved PLATZ domain. These guide RNAs were also selected for their high cleavage efficiency using the Guide-IT software in Vitro Transcription and Screening Kit (Takara Bio USA, Inc). The CRISPR-associated protein-9 nuclease gene (Cas9)- single guide RNA (sgRNA) cassette was synthesized at GenScript Biotech (Piscataway, NJ, USA) then subcloned into the pCLBI301 binary vector (Supplementary Fig. 9)76. The Streptococcus pyogenes Cas9 sequence containing nuclear localization sequences and the potato IV2 intron69 was codon optimized for Vitis. Cas9 transcribed was controlled by the Arabidopsis UBQUITIN1 promoter and terminator70, which drives expression in vegetative and reproductive tissues71. The UBQUITIN6 promoter was used to drive the expression of FSI and FS4 sgRNAs76. The two binary vectors pVCAS9FS1 and pVCAS9FS4 contained the FSI or FS4 guide RNA, respectively. Lastly, binary vectors containing HygR (HygR), and the endoplasmic reticulum localized green fluorescent protein (GFP-SEr) reporter for selecting transgenic plants73.

Transformation and identification of gene-edited VviPLATZ1 plants. To edit VviPLATZ1, pVCAS9FS1, and pVCAS9FS4 were transformed into the Agrobacterium tumefaciens strain EHA105 and selected using the kanamycin resistance gene, KanR. Next, transgenic 04C023V0006 (UH1) plants were produced by incubating the EHA105 strains with somatic embryogenic callus derived from anthers72. Transgenic somatic embryos were selected using the HygR and GFP-SEr73. Shoot growth was induced in transgenic somatic embryos with 5 µM 6-Benzylaminopurine (Sigma-Aldrich, Sydney, New South Wales, Australia, #B327). After shoots developed, root growth was induced with 0.5 µM 6-Benzylaminopurine (Sigma-Aldrich, Sydney, New South Wales, Australia, #N0640). Finally, rooted transgenic plantlets maintained in tissue culture, were propagated, potted into soil and transferred to the glasshouse and/or growth chamber for further analysis.

To identify gene-edited plants, DNA isolation was performed on immature leaves from GFP-positive plants. The primers PLa_F (ATAAGGCTCAACCCCCACTT) and PLa_R (ACACCCCAATAAAACGCAAA) were used to PCR amplify the target sequence followed by the NGG protospacer adjacent motif (PAM) driving transcription and Screening kit (Takara Bio USA, Inc). The CRISPResso pipeline to identify indels76. Mutations were calculated by counting the number of sequence reads with an indel at the guide RNA target region divided by the total number of sequence reads. The value was corrected by subtracting the data obtained for the untransformed 04C023V0006 (H/H) control. After this analysis was completed, seven first-generation plants (T0) containing gene-edited alleles were identified and self-pollination was performed to evaluate the flower sex phenotype in T1 plants. After DNA isolation from T1 root and cotyledon tissues, the guide RNA target region was amplified by PCR using Pla_F and PLa_R primers. Sanger sequencing performed at AGRF was used to genotype the embryos to identify T1 plants homozygous for the mutated alleles (VviplatZ1-1/2-8).

Flower sex determination. Phenotyping for flower sex was performed by morphological scoring using the OIV descriptor, No 151, of recently opened flowers. The sections were mounted with glycerol and imaged using an optical microscope (Zeiss Axioskop2, Oberkorn, Germany). Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
All relevant data presented in this manuscript are available within the article and its Supplementary Information files. The GenBank sequence identifier for VviPLATZ1 derived from 04C023V0003 is MW548436. For VviPLATZ1 derived from 04C023V0003, the GenBank sequence identifier is MW548435. Source data are provided with this paper.

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Author contributions
M.R.T. and L.T. designed and supervised the genetic mapping; M.R.T. designed and supervised the CRISPR/Cas9 gene-editing work. J.C. identified the SEX LOCUS SNPs, performed the genetic mapping and identified VviPLATZ1 as a candidate gene. P.C. performed the RT-qPCR analysis, cloning, CRISPR/Cas9 gene-editing and phenotyping. D.M. participated in the flower sex phenotyping for mapping, breeding and maintenance of plants in glasshouse and growth cabinets. H.M.S. performed the mRNA in situ hybridization and wrote the manuscript with input from the authors.

Competing interests
The authors declare no competing interests.

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