

***Ranunculus* flowers produce numerous carpels via evolutionarily rewiring of the canonical *AGAMOUS-WUSCHEL* developmental module**

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Abstract

Angiosperm flowers generally have more stamens than carpels in bisexual species, due to the timely repression of *WUSCHEL* (*WUS*) by *AGAMOUS* (*AG*) genes in floral meristems (FMs). In some taxa, however, their flowers have many more carpels than stamens as a morphological innovation, yet the underlying developmental and molecular bases remain unclear. Here, we unveil that within the Ranunculaceae, *Ranunculus sceleratus* with numerous carpels exhibits stronger cellular dynamics at FM apexes than *Nigella damascena* with a few carpels. We also find that compared with the *N. damascena* *NdWUS* gene, the two *RsWUS1* and *RsWUS2* duplicates of *R. sceleratus* respectively display expression repatterning in time and space in developing FMs, and contribute more to carpel than stamen numbers. More unexpectedly, *RsAG1* and *RsAG2* that specify floral organ identities also function together with *RsWUS1/2* to promote carpel numbers. One reason is that the *Ranunculus WUS2*-lineage copies gained two repeated transcription factor binding sites in their promoters, which is key to being activated, rather than repressed, by *RsAG* genes. These findings reveal how gene duplication, expression repatterning and *cis*-regulatory evolution have rewired a canonical *AG-WUS* module to shape the floral organ number diversification.

Introduction

One of the fundamental questions in biology is how the organ number is precisely determined in organism bodies. In angiosperm flowers, for example, their floral organ numbers change dramatically among different taxa, which ultimately impact the floral basic structures and diversities^{1, 2, 3, 4}. Earlier studies have systematically explored the developmental patterns of floral organ number determination. Once the floral meristem (FM) of a typical flower emerges from the inflorescence meristem, four types of floral organ primordia then successively initiate from the peripheral region of the meristem, following the manners of outer to inner, sepal to petal, stamen, and finally carpel primordia^{5, 6}. During the early phase of floral organ initiation, the central stem cells of FM stay their activities till a proper time point, after that the FM activity will cease and all cells will be incorporated into the innermost carpel primordia^{7, 8}. Consequently, the position and number of each type of floral organs are largely determined⁹. These suggest that controlling of the FM activity is the key to appreciating the floral organ number determination.

So far, we have relatively good knowledge regarding this issue in *Arabidopsis thaliana* (hereafter *Arabidopsis*), which is mainly regulated by two well-characterized negative feedback loops. The first one is the *WUSCHEL* (*WUS*)-*CLAVATA* (*CLV*) loop, in which *WUS* determines the stem cell activity and induces the expression of the *CLV3*, while the *CLV3* signal in turn represses the expression of *WUS*^{10, 11, 12, 13}. This feedback loop maintains the stem cell population and then impacts the FM size, through which a certain number of floral organ primordia could be allocated on the FM⁴. For instance, the *clv3-2* mutant produces more floral organs, especially stamens and carpels, due to the significantly enlarged FM size¹¹, while the *wus-1* mutant flower bears reduced floral organ numbers, as the result of disrupted stem

cell activity and smaller sized FM¹⁰. The second one is the *WUS-AGAMOUS* (AG) loop. The AG gene is initially induced by *WUS* together with *LEAFY*^{14, 15, 16}. AG then not only specifies the stamen and carpel identities as the C function of the floral ABC model^{17, 18, 19}, but also represses the *WUS* expression, as evidenced by the *ag* mutant showing the “flower-within-flower” structure^{15, 16, 17}. In addition to *Arabidopsis*, the reduced floral organ numbers were observed in mutants of *WUS* orthologs of tomato (*Solanum lycopersicum*) and snapdragon (*Antirrhinum majus*)^{20, 21}, and the increased carpel numbers were documented in tomato and cucumber (*Cucumis sativa*) whose *WUS* and *CLV* homologous genes show expression changes in FMs²². Moreover, mutations in AG-like genes in many species often generate doubled flowers and “flower-within-flower” structures^{23, 24}. These results demonstrated that the two feedbacks are functionally conserved in controlling the FM activity and the floral organ number across angiosperms.

It is worth to note that, in most angiosperm species, their FM activities would cease immediately along with the emergence of carpel primordia, as marked by the FM shape changing from hemispheric to flattened, from which no more carpels could be produced^{8, 25, 26}. As a result, FMs often produce flowers with fewer carpels than stamens. Indeed, by analyzing floral organ numbers from 824 species covering 63 orders and 350 families of angiosperms²⁷, we confirmed that 368 (45%) and 138 (17%) species have 4–6 and 7–10 stamens, while 480 (58.3%) and 151 (18.3%) have 1–3 and 4–6 carpels, respectively (Supplementary Data 1). Meanwhile, of the 672 species with bisexual flowers, 623 (92.7%) have more stamens than carpels, in which 529 (78.7%) have stamen more than 1.5 times than carpels (Fig. 1a). Despite this, there are also exceptions. For example, 49 (7.3%) species have more carpels than stamens, in which 33 have carpels more than 1.5 times than stamens, even some of them have more than 100 carpels (Fig. 1a; Supplementary Data 1). Notably, these 33 species belong to distinct lineages, such as *Fragaria vesca* and *Duchesnea indica* (Rosaceae) of core eudicots, *Ranunculus sceleratus* (Ranunculaceae) of basal eudicots, *Ranalisma rostratum* (Alismataceae) of monocots, and *Liriodendron chinense* (Magnoliaceae) of basal angiosperms (Fig. 1b; Supplementary Fig. 1). Meanwhile, this specialized character has been considered a morphological innovation and used as a key to identifying the five species and their corresponding genera. The above observations hint that in these flowers, their FM activities might not terminate in time as the initial emergence of carpel primordia, yet the underlying developmental and molecular bases are far from understood, due to lack of a suitable system.

The Ranunculaceae is an excellent system to address this question, since their members show huge diversities and strong flexibilities in floral organ numbers. More importantly, the differences in carpel and stamen numbers are exceptionally eye-catching (Fig. 1e). For example, *N. damascena* generally contains about 60 total floral organs, with 5 carpels vs. 40 stamens; *Delphinium grandiflorum*, about 40 organs, with 3 carpels vs. 30 stamens (Fig. 1h-k), while *Clematis tangutica* has about 140 organs, with 100 carpels vs. 40 stamens; *R. sceleratus*, more than 170 organs, with 150 carpels vs. 17 stamens (Fig. 1f-g). Strikingly, the species of *Laccopetalum giganteum* located in the Andes Mountain has more than 10,000 carpels in a flower. Based on previous studies of floral ontogenesis of this family, such as *R. sceleratus* and *N. damascena*, it has been shown that their FM morphologies are largely similar with each other till

the initiation of stamen primordia, but then become different when the carpel primordia emerge^{24, 28, 29}. Specifically, the *N. damascena* FM quickly changes its shape from hemispheric to flat, from which only about five carpel primordia could be produced²⁴, indicative of immediate termination of the FM activity. In contrast, the *R. sceleratus* FM keeps the hemispheric shape, and there is still enough space for the differentiation of more carpels until they fill up the FM^{28, 29}, suggestive of the extended FM activity. Furthermore, functional study of the C function genes in *N. damascena* revealed that co-silencing *NdAG1* and *NdAG2* produced the “flower-within-flower” structure²⁴, implying that the two genes can repress the expression of *WUS*-like genes as the model species.

The above backgrounds, therefore, beg the following questions concerning the carpel and stamen numbers in the Ranunculaceae: what are the differences of the cellular behavior, as well as the molecular and regulatory mechanisms in FMs between flowers with more and fewer carpels relative to stamens? Here, we primarily focused on the potential *WUS*-AG feedback loop and proposed the following hypothesis. In FMs producing fewer carpels than stamens, their activities terminate upon carpel initiation, due to the repressed *WUS*- by AG-like genes. However, in FMs producing more carpels than stamens, their activities could maintain for a longer time, possibly due to the decreased or even no repression of *WUS*- by AG-like genes. In this study, we selected *R. sceleratus* with much more carpels and *N. damascena* with a few carpels as research systems. Through comprehensive comparisons of cellular dynamics, as well as expression, functional, regulatory, and evolutionary studies for *WUS*- and AG-like genes between the two species, we attempt to explore the developmental and the molecular mechanisms underlying the production of numerous carpels in *R. sceleratus* flowers.

Results

R. sceleratus FM apexes exhibit stronger cellular dynamics

To figure out the differences of FM activities during development between *R. sceleratus* and *N. damascena*, their cellular dynamics of FMs were compared by using the time-lapse live imaging technique (Fig. 2). We defined four time points for each species to conduct the experiment (Supplementary Data 2). For *N. damascena*, the first time point (NTP1) is distinguished by the preliminary initiation of a few stamen primordia at the periphery region of FM, NTP2 by the occupancy of about 1/2 stamens on the FM; NTP3 by the complete of stamen production; NTP4 by the initiation of carpel primordia, at which the FM is almost occupied by floral organs (Fig. 2a-d; Supplementary Fig. 2). For *R. sceleratus*, the first time point (RTP1) is defined by the preliminary initiation of a few stamen primordia, RTP2 by the initial emergence of carpel primordia; RTP3 by the occupancy of about 1/2 carpels on the FM; RTP4 by the FM stuffed carpels with (Fig. 2e-h; Supplementary Fig. 3). At each time point, we conducted cells lineage tracing by imaging the FM apex at a 24-h interval, and then quantified the distribution and rate of cell division, as well as the degree of cell expansion, over the four developmental time points.

We first compared the cell division activities between the two species. In *N. damascena*, relative higher numbers of divided cells on the FM apex were observed at NTP1, while those of *R. sceleratus* were found at RTP2-4 (Fig. 2a-h; Supplementary Figs. 2, 3). To eliminate the differences between species as well as individual FM, and make the results more comparable, we calculated the cell division ratio at each time point, which refers to the number of cells that divided during the 24-h interval divided by the total number of sampled cells at 0 h. The higher this ratio is, the greater proportion of divided cells on the FM apex. As the results shown, the cell division ratio on *N. damascena* FM apex from NTP1 to NTP4 gradually declined, while those in *R. sceleratus* gradually elevated, although their discrepancies were not obvious (Fig. 2i; Supplementary Figs. 2e, 3e). By the same strategy, when the cell expansion ratio on the FM apex were calculated, we found that the ratio values of the two species all higher than 1.0 at each time point, suggestive of increased cell areas (Fig. 2j; Supplementary Figs. 2f, 3f). However, when the four time points were collectively considered, we found that the cell expansion ratio values were higher in *R. sceleratus* than *N. damascena* (Fig. 2j). More importantly, the values of *N. damascena* and *R. sceleratus* FMs exhibited declined and elevated tendencies, respectively, which led to their discrepancies becoming increasingly significant, especially during the last two time points (Fig. 2j). These results imply that the *R. sceleratus* FM could produce bigger receptacle that could bear more floral organs than the *N. damascena* FM. Indeed, the receptacle shapes of mature flowers between the two species are obviously different (Fig. 2k-n; Supplementary Data 3). In particular, the receptacle height and the resultant surface area are significantly higher in *R. sceleratus* than *N. damascena* flowers (Fig. 2k-n), suggesting that more floral organs could be loaded on *R. sceleratus* receptacles, possibly due to the extended FM activity. Taken together, these results showed that the cellular dynamics of FMs in *R. sceleratus* are stronger than *N. damascena*, especially the index of cell expansion. This also implies that once the early carpel primordia emerged from the *R. sceleratus* FM, its activities could still maintain for a longer time relative to that of *N. damascena*, which, therefore, would result in the differences of carpel numbers between the two species.

R. sceleratus RsWUS duplicates show expression repatterning in developing FMs

To explore the molecular mechanisms explaining the differences in carpel numbers between *R. sceleratus* and *N. damascena*, we first focused on the *WUS* homologs that have been well-characterized to specify the FM identity. From the genomes and transcriptomes of different Ranunculaceae species, one putative *WUS*-like copy was respectively identified from *N. damascena*, *D. anthriscifolium*, and *Aquilegia coerulea* that have a few carpels (Supplementary Fig. 4), consistent with the single-copied *WUS* homologous genes in most diploid angiosperm species³¹. However, two *WUS*-like copies were identified from the species of *Clematis* and *Ranunculus* that have much more carpels, respectively (Supplementary Fig. 4). Amino acid residues encoded by these homologs all have the typical homeodomain and *WUS*-boxes, as well as the EAR-like motif (Supplementary Fig. 5). Phylogenetic analysis revealed that these candidates from the Ranunculaceae and other characterized *WUS*-like genes formed a well-supported lineage, confirming their orthology (Supplementary Fig. 4). Moreover, the *WUS*-like genes from *Ranunculus* fell into two strongly supported clades (Supplementary Fig. 4), suggesting that a duplication

event occurred before the diversification of this genus, which were then named as *WUS1*- and *WUS2*-clade, respectively.

To understand the roles of the *WUS* homologous genes in FM activities and floral organ number determination in *N. damascena* and *R. sceleratus*, their expression patterns in FMs were investigated (Supplementary Fig. 6). In *N. damascena*, *in situ* hybridizations for *NdWUS* gene demonstrated that it was specifically expressed in the inner layers of the S3-4 FMs, corresponding to the organizing center (OC) that specify the meristem activity (Supplementary Fig. 3a). At S6 before the initiation of carpel primordia, no signal of this gene could be detected (Fig. 3a), suggestive of timely termination of the FM activities. In *R. sceleratus*, RT-qPCR results for *RsWUS1* and *RsWUS2* genes showed that they were expressed not only in the pooled floral buds at very early developmental stages (S0-S5), but also in dissected stamens and carpels in flowers at later stages (S6-S12) (Supplementary Fig. 7a-b). *In situ* hybridization for *RsWUS1* and *RsWUS2* revealed that at S1-4, both of them were specifically expressed in the OC region of FMs (Fig. 3b-c). From S6 on, their expression patterns became obviously diverged. In details, the *RsWUS1* signal could still be detected in the OC of S6 FMs (Fig. 3b). More interestingly, during S6-9, the signal location of *RsWUS2* transferred from the OC to the stamen primordia (Fig. 3c). Taken together, these expression changes in time (heterochrony) of *RsWUS1* and in space (heterotopy) of *RsWUS2* in *R. sceleratus* suggest that the FM activities could be maintained for a longer time, which provide critical clues for the production of numerous carpels.

R. sceleratus RsWUS duplicates contribute more to carpel than stamen numbers

Next, we attempted to investigate the functions of *WUS*-like genes in determination of floral organ (carpel in particular) numbers in *N. damascena* and *R. sceleratus*, by using the virus-induced gene silencing (VIGS) technique (Supplementary Fig. 6). Meanwhile, Phytoene desaturase (*PDS*) homologous genes were used as marker of gene silencing. In *N. damascena*, the TRV2-*NdPDS*-*NdWUS* treated plants with strong phenotypical changes generally have smaller flowers, along with the significantly decreased total floral organ numbers, relative to the mock (TRV2-*NdPDS*) (Fig. 4a-b, g; Supplementary Fig. 8a-b). Specifically, the median numbers of sepals remained at five, whereas petals decreased from 7–10 to 5–7 (by 2–3), stamens from 21–44 to 10–20 (by 11–24), carpels from five to 3–4 (by 1–2) across the 1st to 3rd branches (Fig. 4g; Supplementary Fig. 8a-b, 9). Meanwhile, the value of the carpel/stamen number ratio was significantly elevated (Fig. 4i), resulting from reduction of more stamens. In accordance with this, very low expression levels of *NdWUS* were detected in floral buds with strong phenotypic changes (Supplementary Fig. 10a), indicating that the silenced phenotypes were indeed the result of downregulation of *NdWUS*.

In *R. sceleratus*, the TRV2-*RsPDS*-*RsWUS1* treated plants also produced smaller sized flowers and significantly decreased floral organs, compared with the mock (TRV2-*RsPDS*) (Fig. 4c-d, g; Supplementary Fig. 8c-d). In strongly silenced flowers, the median numbers of sepals and petals did not change as five, while stamens significantly decreased from 16–17 to 10–12 (by 4–6), and carpels from 106–161 to about 73–79 (by 27–85) across the 1st to 3rd branches (Fig. 4c-d; Supplementary Fig. 11).

The carpel/stamen number ratio of this treatment was decreased but with no significant difference (Supplementary Fig. 11). Silencing of *RsWUS2* alone or both *RsWUS1/2* produced flowers with more severe phenotypes, especially the carpel number (Fig. 4e-f; Supplementary Fig. 8e-f). For example, the median stamen numbers decreased to 7–9 (by 8–9) for *RsWUS1/2*- and to 9–11 (by 6–7) for *RsWUS2*-silenced flowers, while the median carpel numbers decreased to about 48–53 (by 58–108) for *RsWUS1/2*-silenced flowers, and even to about 29–45 (by 61–132, especially 82.0% at the 1st branch) for *RsWUS2*-silenced flowers (Fig. 4h; Supplementary Fig. 8e-f). Unlike the treatment in *N. damascena*, carpel/stamen number ratios of the two treatments in *R. sceleratus* were significantly decreased (Fig. 4j; Supplementary Fig. 11), suggesting the reduction of more carpels. In addition, the two types of silenced flowers also accompanied with the underdeveloped stamens (Fig. 4e-f). In line with the dramatically decreased floral organ numbers of the above treatments, the receptacle lengths and widths of mature flowers were significantly shorter than those of mock (Supplementary Fig. 12; Supplementary Data 3), meanwhile very low expression levels of corresponding gene(s) were detected in floral buds with strong phenotypic changes (Supplementary Fig. 10b). Taken together, these functional studies in the two species all highlight the essential and conserved roles of *WUS*-like genes in maintaining the FM activities and the resultant floral organ numbers. More critically, *RsWUS1* and *RsWUS2* genes of *R. sceleratus* play more important roles in the determination of carpel numbers, possibly due to their effects on the extended FM activity. One possible reason for this is that the traditional repressors of *WUS*-like genes (e.g., *AG*-like genes) might change their functions in *R. sceleratus*.

RsAGs and RsWUSs function together to promote carpel numbers

We then ask whether the above issue relates to *AG*-like genes in *R. sceleratus*. Like many other species of the Ranunculaceae, two *AG* homologs were identified from *R. sceleratus*, which were named *RsAG1* and *RsAG2*, according to their phylogenetic relationships (Supplementary Fig. 13). Gene expression analyses by RT-qPCR and *in situ* hybridization revealed that *RsAG1* was mainly expressed in stamens and carpels, while *RsAG2* specifically in carpels (Fig. 3d-e; Supplementary Figs. 6, 7c-d), consistent with the expression patterns of the counterparts of other studied Ranunculaceous species^{24, 32, 33}. When the *RsAG1* gene was silenced alone (Supplementary Fig. 6), flowers with strong or moderate phenotypic changes showed transformation of partial outer stamens into petals or petal-stamen chimeras, without affecting the carpel identity, as well as the total and respect floral organ numbers (Supplementary Fig. 14, 15b). Simultaneously silencing of both *RsAG1* and *RsAG2* resulted in flowers with two types of strong phenotypes. Type I flowers only showed the organ identity transformations, i.e., all the stamens transformed into petals and carpels into sepals (Fig. 5a-b; Supplementary Fig. 15c). Type II flowers displayed not only the phenotypes as Type I, but also the “flower-within-flower” structure at the tip of receptacle (Fig. 5c; Supplementary Fig. 15c), indicative of disruption of the floral determinacy. Nevertheless, it was unexpectedly to find that, unlike the *N. damascena* *NdAG1/2*-silenced flowers showing no effect on floral organ numbers²⁴, the two types of *RsAG1/2*-silenced flowers in *R. sceleratus* exhibited obvious reduction in their size and the decrease in floral organ numbers (without considering the numbers of the “flower-within-flower” structure) (Fig. 5b-c). Compared with the mock, the median

numbers of sepals and petals kept largely unchanged, and those of stamens or stamen-petal chimeras at the 1st and 2nd branches decreased to 14 – 13 (by 3) but with no significant difference (Fig. 5f). More severely, however, the median number of carpel or carpel-sepal chimeras significantly decreased to about 68 (by 93, 57.8%) at the 1st branch, which accordingly resulted in the significant decrease in the carpel/stamen ratio (Fig. 5f). Accordingly, the receptacle lengths and widths of *RsAG1/2*-silenced flowers were significantly shorter than those of mock (Supplementary Fig. 12), meanwhile very low expression levels of corresponding gene(s) were detected in floral buds with strong phenotypic changes (Supplementary Fig. 16).

Given that these phenotypic changes in floral organs were very similar to the *RsWUS2*- or *RsWUS1/2*-silenced flowers, we, therefore, inferred that the two *RsAG* genes might not have negative roles to *RsWUS* genes. To this aim, we simultaneously silenced *RsWUS1/2-RsAG1* and *RsWUS1/2-RsAG1/2*, respectively. In the *RsWUS1/2-RsAG1*-silenced flowers with strong phenotypic changes, a few outer stamens and outer carpels respectively transformed into petal-stamen and sepal-carpel chimeras, and the other stamens were underdeveloped (Fig. 5d; Supplementary Fig. 15d). In the *RsWUS1/2-RsAG1/2*-silenced flowers with strong phenotypic changes, all stamens transformed into petal-stamen chimeras and carpels into sepal-like organs (Fig. 5e; Supplementary Fig. 15e). Importantly, the median carpel numbers of the above two treatments showed similar decreased patterns like the *RsAG1/2*-silenced flowers (Fig. 5f). In addition, their median stamen numbers significantly decreased to 8–9 (by 8–9) at the 1st and 2nd branches (Fig. 5f), suggestive of reduction of more total floral organ numbers than those of the *RsAG1/2*-silenced flowers. Consistently, the receptacle lengths and widths of the two treatments were significantly shorter than those of mock (Supplementary Fig. 12; Supplementary Data 3), and very low expression levels of corresponding gene(s) were detected in floral buds with strong phenotypic changes (Supplementary Fig. 16). These results collectively suggest that, unlike the traditional functions of AG-like genes in terminating FM activities so as to produce a few carpels, *R. sceleratus* *RsAG1/2* and *RsWUS1/2* genes work together to promote the production of more carpels.

RsAG genes positively regulate RsWUS2 in *R. sceleratus*

To further explore the regulatory relationships between *RsAG1/2* and *RsWUS1/2* genes in *R. sceleratus*, we first examined the gene expression levels by RT-qPCR in the above VIGS silenced flowers. We found that in the *RsWUS2*- and *RsWUS1/2*-silenced flowers, the expression levels of *RsAG1* and *RsAG2* were significantly downregulated, as expected (Fig. 6a; Supplementary Fig. 10b), while those of *RsWUS1* and *RsWUS2* were also significantly downregulated in the *RsAG1*- and *RsAG1/2*-silenced flowers (Fig. 6a; Supplementary Fig. 16). These results are in contrary with the studies in model species, hinting that *RsWUS1/2* and *RsAG1/2* have reciprocal positive regulatory relationships. Here, we focused on the regulatory roles of *RsAG1/2* to *RsWUS1/2*. The promoters about 2-kb regions upstream of *RsWUS1* and *RsWUS2* were cloned, which were used as reporter constructs for dual-luciferase (LUC) assay in tobacco with *RsAG1* or *RsAG2* proteins. Meanwhile, the 2-kb promoter of *NdWUS* and *NdAG1* protein of *N. damascena* were incorporated as a control. The results showed that *NdAG1* repressed the expression of *NdWUS* promoter (Fig. 6b). In contrast, either *RsAG1* or *RsAG2* could activate the expression of *RsWUS2*

(rather than *RsWUS1*) promoter (Fig. 6c-d; Supplementary Fig. 17c-d, g-j). Moreover, when the *N. damascena* NdAG1 and *R. sceleratus* *RsWUS2* promoter were co-infiltrated, positive activation result was also obtained (Fig. 6e; Supplementary Fig. 17e). These results preliminarily suggest that *RsWUS2* is under the direct positive regulation of AG-like genes, and therefore the *RsWUS2* promoter possibly contains key regulatory elements.

To this aim, the 2-kb promoter of *RsWUS2* was subjected to the JASPAR database, through which four putative AG-like transcription factors binding sites (TFBSs) with the featured sequence of CC(A/T)₆GG (CArG-box) were predicted (Fig. 6f). To determine which site(s) are functionally relevant with *RsAG1/2*, the *RsWUS2* promoter was dissected into three fragments *RsWUS2_F1*, *F2* and *F3*, with *RsWUS2_F1* containing two repeated CArG boxes, and the other two fragments each having one (Fig. 6f). They were then subjected to yeast one-hybrid (Y1H) assays with *RsAG1* and *RsAG2* proteins, respectively. The results showed that either *RsAG1* or *RsAG2* can directly bind to *RsWUS2_F1*, rather than *F2* and *F3* (Fig. 6g; Supplementary Fig. 18a-c). With the same strategy, six and four putative CArG boxes were predicated from the *RsWUS1* and *NdWUS* promoters, respectively (Fig. 6f). Then, *RsWUS1* and *NdWUS* promoters were respectively dissected into four fragments to perform the Y1H assays with their corresponding *RsAG1/2* or *NdAG1/2* proteins, which all led to negative interaction results (Supplementary Fig. 18e-h, 19). To further confirm whether this critical *RsWUS2_F1* fragment could be activated by the AG-like proteins from other species, it was inserted into the 2.1-kb promoter of Arabidopsis *WUS* gene to construct a *AthWUSpro+RsWUS2_F1* reporter (Fig. 6h), which was then employed in LUC assays with the Arabidopsis AG protein. Accordingly, we found that this reporter could be significantly activated by AG (Fig. 6i; Supplementary Fig. 17f). These results altogether suggest that the *RsWUS2_F1* is the only key fragment that can be activated by AG-like TFBSs.

Two newly gained TFBSs are required for the activation of *RsWUS2* by *RsAGs*

This finding further intrigued us to ask why and how such a *RsWUS2_F1* fragment is critical for *RsWUS2* to be activated by AG-like genes. To this end, we cloned the promoters of *WUS* homologs from another two *Ranunculus* species, *R. sardous* and *R. cassubicifolius*, as well as the counterparts of *A. coerulea* and *D. grandiflorum*. Sequence analysis revealed that a conserved non-coding sequence (CNS) motif is shared by the promoters of sampled *WUS*-like genes of the Ranunculaceae, which is coincidentally located within the *RsWUS2_F1* fragment (Fig. 6f). Further inspection within this CNS revealed that, the sequences of *WUS1*-lineage promoters from three *Ranunculus* species exhibit higher identity with those of *N. damascena* *NdWUS*, *A. coerulea* *AcWUS* and *D. grandiflorum* *DgWUS*, relative to those of *Ranunculus* *WUS2*-lineage (Fig. 6f), suggesting that the CNS sequences of *WUS1*-lineage promoters retain more ancestral features, and those of *WUS2*-lineage promoters have experienced apparent divergence (Fig. 6f). More interestingly, the two repeated AG-like TFBSs within *RsWUS2_F1* are coincidentally nested within the CNS, and are totally share by the other two *WUS2*-lineage promoters (Fig. 6f). However, no such TFBSs were recognized in the CNS of any other *WUS*-like promoters (Fig. 6f). Furthermore, we found that the two *WUS2*-lineage-specific TFBSs derived from the modification of preexisting ancestral sequence without CArG-box, before the divergence of *WUS2*-lineage genes (Fig. 6f). We, therefore,

speculated that it was the two newly gained TFBSs that reshaped the ability of *Ranunculus WUS2*-lineage genes to be activated by AG-like proteins.

To test this hypothesis, we designed a shorter probe containing the two repeated AG-like TFBSs and their flanking sequences, named *RsWUS2_F1_2CArGs*, to conduct Y1H assays. As expected, this probe could be bound by RsAG1 and RsAG2 proteins (Fig. 6j; Supplementary Fig. 18d). In contrast, the corresponding probe without the two CArG-boxes from the *RsWUS1* promoter named *pRsWUS1_nonCArG*, however could not be bound by RsAG1 and RsAG2 (Fig. 6k; Supplementary Fig. 18i). In addition, the *pRsWUS2_F1*-EMSA probe and its mutagenized probe were also subjected to EMSA assays (Fig. 6l). As expected, this probe could be bound by RsAG1 or RsAG2 proteins, though the binding efficiency of RsAG2 to the probe was very weak, while the mutagenized probe displayed no interaction with RsAG1 or RsAG2 proteins (Fig. 6m; Supplementary Fig. 20a). Furthermore, we wonder if each of the two TFBSs could work with RsAG1 or RsAG2 proteins. To do this, we dissected the *pRsWUS2_F1*-EMSA probe into two shorter probes *pRsWUS2_F1*-EMSA1 and *pRsWUS2_F1*-EMSA2, with each containing only one TFBS, to perform EMSA assays. Unfortunately, no interaction signal was ever detected for any of them (Supplementary Fig. 20b-c). Taken together, these sequence analyses and gene interaction assays all point to the conclusion that the two newly gained AG-like TFBSs are indeed required for the activation of *WUS2*-like genes in *Ranunculus*.

Discussion

It has been well-known that the FM activity is critical for the FM size and the consequential floral organ numbers, which ultimately lay the foundation for floral basic structure and diversity^{34, 35, 36}. These knowledges are largely gained from mutants of model species or artificially selected crops, which all emphasize the effects of cessation or over-proliferation of FM stem cells on the total floral organ numbers^{4, 10}. In consistent with this, the flower of *Pulsatilla chinensis* (Ranunculaceae) can bear more than 250 stamens and 250 carpels, which could be attributed to the prominently enlarged FM just likes an inflated balloon³⁷. It is also worth to caution that the information about the cellular dynamics during FM development exclusively comes from *Arabidopsis*^{38, 39}. Moreover, most of the above model species and their close relatives invariably have only four whorls and fixed numbers of floral organs. In contrast, a wide range of floral organ number variations have been recorded in diverse angiosperm species, while their underlying cellular dynamics of FMs are far from understanding, especially the taxa having much more carpels than stamens. In this study, by selecting *R. sceleratus* and *N. damascena* as representatives from the Ranunculaceae, together with the established time-lapse live imaging technique, we systematically investigated the cellular dynamic properties during the process of FM development. The results revealed that compared to *N. damascena*, stronger cellular dynamics after the initiation of carpel primordia, especially the index of cell expansion, were observed on the FM apex of *R. sceleratus*. This confirms that the FMs activity of *R. sceleratus* could maintain or prolong for a longer time, consistent with the heterochronic and heterotopic expression of *RsWUS1* and *RsWUS2* genes, respectively.

Consequently, more space would be produced on the FMs, and thus available for more additional carpel primordia to arise.

It is also interesting to note that for the species or taxa with much more carpels than stamens, their flowers almost have apocarpous, rather than syncarpous, gynoecia, such as *Fragaria* of the Rosaceae, *Ranunculus* and *Clematis* of the Ranunculaceae, *Ranalisma* of the Alismataceae, and *Liriodendron* of the Magnoliaceae, all of which display a dramatic increase in carpel numbers than their syncarpous relatives. This strong correlation between numerous carpels and apocarpous gynoecium has been noted and their evolutionary significances have been considered in previous studies^{2, 3, 8}. From the developmental point of view, the differences between the two types of gynoecia reside in that syncarpous gynoecium consists of a few carpels fused into a single functional unit that consumes the entire FM very quickly⁸, as exemplified by those of *Arabidopsis*, tomato, and *N. damascena*. Apocarpous gynoecium, however, is comprised of free or separate carpels so that each one initiates and functions independently, and therefore, the FM will not be immediately consumed as the first carpels arise⁸. One reason for this can be attributed to the stronger and prolonged FM activities, as shown in *R. sceleratus* of this study. Moving out of this species or the genus *Ranunculus*, whether the flowers with much more carpels from other taxa have the similar cellular dynamics is also deserved to be explored.

Great amount of genetic and molecular studies in model species and crops have demonstrated that the maintenance time of FM is essential for the determination of floral organ numbers, which concerns very complex regulatory networks. In addition to the well characteristic *WUS-CLV* and *WUS-AG* negative feedback loops, many others are also involved in this process, such as the FM maintenance by cytokinin signal and *Knotted 1-like homeobox (KNOX)* genes, and FM termination by *KNUCKLES (KNU)*^{39, 40}, *AUXIN RESPONSE FACTOR 3 (ARF3)*⁴¹ and *CRABS CLAW (CRC)*⁴². In this study, we provide new evidence that the duplication and subsequent expression repatterning of *WUS* homologs themselves are also critical for the determination of floral organ numbers, especially the increase in carpel numbers. It is worth to note that the *WUS* homologs of most diploid angiosperm species are single copied³¹, which might be essential of the stem cell homeostasis and the production of certain numbers of floral organs. Nevertheless, in the Ranunculaceae, two *WUS* homologous copies were respectively identified in *Ranunculus* and its closely related genera that have much more carpels, while only one copy was respectively identified in the other species with a few carpels (Supplementary Fig. 4). Furthermore, the two copies of *R. sceleratus* displayed heterochronic (*RsWUS1*) and heterotopic (*RsWUS2*) expression changes in developing FMs, which collectively prolonged the FM activities and promote the dramatic increase in carpel numbers (Fig. 7). Moving out of this family, in the Rosaceae of core eudicots, many closely related genera (such as *Fragaria* and *Argentina*) also show much more carpels than stamens, and similarly, two or three duplicated copies were also identified in each species (Supplementary Fig. 4), and more importantly one copy *FvWUS1* from *F. vesca* is also heterotopically expressed in reproductive organs⁴³. These similar duplication and expression changes of *WUS* homologs in distinctly related taxa agree with the notion that duplications provide the raw materials for convergent evolution of genes and phenotypes, possible due to expression and functional divergence⁴⁴. In particular, given the essential

and pleiotropic roles of *WUS* homologs during the process of plant development, expression changes in time, space and level of *WUS*-like duplicates (and some species with single copied *WUS* homologs) in diverse lineages or mutants would not impact their basic functions, which in turn may have more contributions to the stem cell activities and the consequent floral organ numbers. For example, the upregulation of *TaWUS-D1* induces the formation of multiovary florets such that enhances the grain number in bread wheat (*Triticum aestivum*)^{45, 46}. Despite this, this hypothesis needs to be verified in other taxa with numerous carpels.

In this study, we also unexpectedly found that unlike many model species and *N. damascena* that have fewer carpels than stamens, their AG-like genes could terminate the expression of *WUS*-like genes at the appropriate time points, the *RsAG1* and *RsAG2* *R. sceleratus* instead have a direct positive role for regulating *RsWUS2*, which might induce the heterotopic expression change of *RsWUS2* from FMs to stamens (Fig. 7). Coincidentally, a previous study showed that when the coding sequence of *WUS* driven by the AP3 promoter or AG second intron was transformed into Arabidopsis, the heterotopic expression of *WUS* in reproductive organs can indeed produce the massive over-proliferation of gynoecial structures¹⁵. The two evidence suggests that the expression of *WUS* homologs out of FMs can also maintain the FM activity. More critically, the reason for activation of *RsWUS2* by *RsAG1* is that the ancestral promoter of *Ranunculus* *WUS2* lineage gained two AG-like TFBSs (CArG boxes), through modification of preexisting non-CArG box sequence, which can be bound and activated by AG-like transcription factors. As such, a conserved regulatory module between AG- and *WUS*-like genes was rewired. This result highlights the fundamental contributions of *cis*-regulatory evolution through modification to the biological diversification^{47, 48, 49}, especially the origin of novel characters, as exemplified by the wing and abdominal pigmentations in insects⁵⁰. Taken together, this study uncovered the cellular, molecular and regulatory mechanisms underlying an interesting evolutionary innovation of numerous carpels in *Ranunculus*, which concerns several fundamental theories of evolutionary biology, such as morphological parallelism, gene duplication and expression repatterning, *cis*-regulatory evolution, and rewiring of regulatory relationships.

In this study, we have demonstrated that in *R. sceleratus*, the much more carpels in a flower can be attributed to the *RsWUS*-mediated stronger FM activities during the process of floral development. In addition to this, there is another possible reason for the characterized phenotype, the homeotic conversion of floral organs controlled by floral organ identity genes of the ABC model³⁵. Here, we propose that these genes could possibly achieve this through two ways. The first is the interactions between B and C function genes themselves. In *N. damascena*, for example, it has been shown that the ABC function genes can not only specify different types of floral organs but also affect the boundaries between them²⁴. In particular, the stamen identity genes *NdAP3-1* and 3 – 2 repress the carpel identity gene *NdAG2*; silence of *NdAP3-1/2* resulted in the transformation of inner stamens into carpels, so that the stamen and carpel numbers decreased and increased, respectively²⁴. In *R. sceleratus*, it is reasonably to speculate that the B function genes do not repress, but rather promote the C function genes, while the C in turn represses B, through which the carpel identity would occupy a relatively large proportion of

space on the FM surface. The second is the interactions between other floral organ identity genes and the *WUS* homologs. Given that the activation of *RsWUS2* by *RsAG1* and the resultant heterotopic expression of this gene in stamens was largely due to the gain of two repeated AG-like TFBSs that can be recognized and bound by floral MADS-box transcription factors. Therefore, it is very possible that in addition to the C function genes, other floral organ identity (e.g., B and A function) genes might also interact with the two TFBSs to regulate the expression rearrangement of *RsWUS2* (and also *RsWUS1*), which then in turn affect the spatiotemporal expression domains of B and C function genes.

The strong FM activities of *R. sceleratus* trigger another two questions. The first is the reason for the heterochronic expression of *RsWUS1* gene, although the mechanisms for heterotopic expression of *RsWUS2* was addressed in this study. Multiple elements have been demonstrated to be involved in the regulation of *WUS* gene expression in Arabidopsis. For example, WT Plants treated with phytohormone cytokinin induce the temporal expression change of *WUS* gene, which alters the floral development⁵¹; products of class I *KNOTTED1-LIKE HOMEODOMAIN* (*KNOXI*) family gene *SHOOT MERISTEMLESS* (*STM*) can directly interact with the *WUS* protein, which strengthens the binding ability of *WUS* to *CLV3*⁵²; *HAIRY MERISTEM* (*HAMs*) of the GRAS family^{53, 54}, as well as *PHABULOSA* (*PHB*) and *PHAVOLILA* (*PHV*)⁵⁵ are also involved into the maintenance of the *WUS* expression. These studies, therefore, provide with important clues for understanding the heterochronic expression of *RsWUS1* gene in *R. sceleratus*, which is deserved to be explored in the future. The second is how the FM timely was terminated. We found that its C function genes have no conventional roles in repressing *RsWUS* like genes, while simultaneously silencing *RsWUS1/2* could also produce the “flower-within-flower” phenotype in some flowers. This implies that the termination of *RsWUS* duplicates might be achieved through other ways. For example, it has been shown that as the direct downstream genes of C function genes, *KNUCKLES* (*KNU*) and *CRC* are critically for the direct and indirect termination of *WUS* homologs, in both Arabidopsis and tomato, which have been characterized as the “timer” of FM termination^{39, 56, 57, 58}. Therefore, it is worth to compare the roles of *KNU* and *CRC*, as well as other candidates in the termination of *WUS* homologs in *R. sceleratus* with much more carpels and the relatives with a few carpels, which would provide new insights into the determination of floral organs and floral diversifications.

Methods

Plant materials and growth conditions

R. sceleratus were collected from Meixian, Shaanxi Province (China) and were cultivated under laboratory conditions. Mature seeds were subsequently collected and used for experiments. *N. damascena* was maintained under laboratory conditions. Seeds of *R. sceleratus* and *N. damascena* were sown in soil (vermiculite:nutrient soil = 1:1). Plants of both species were grown under a 16-h light/8-h dark photoperiod at 24°C and 60% relative humidity.

Time-lapse live imaging of FM dynamics

Fresh floral buds of *R. sceleratus* and *N. damascena* at four developmental time points (TP1-4) were collected for live confocal imaging. Floral buds were carefully dissected under a stereomicroscope to remove surrounding bracts and sepals, exposing the FM and floral organ primordia. The dissected floral primordia were cultured on solid MS medium (pH 5.8) supplemented with 15 g/L sucrose and 3% agar prior to staining.

Before the live imaging, for nuclear visualization, floral buds of *R. sceleratus* and *N. damascena* were stained with DAPI working solutions at concentrations of 1.00 mg/mL for 10 min and 0.75 mg/mL for 8 min, respectively. Samples were then washed five times with sterile water to remove excess dye. The first live imaging was performed using an AX confocal laser scanning microscope system (Nikon, Japan) equipped with a 40× water-immersion objective. DAPI fluorescence was excited using a 405-nm laser, and emission signals were collected between 429 and 474 nm. Z-stack images were acquired with a step size of 2 μm to generate three-dimensional (3D) representations of FMs. After imaging, samples were returned to growth conditions to allow continued development. The second live imaging was performed at a 24-h interval, with the DAPI staining being repeated before each imaging session. Following this routine, independent time-lapse imaging series were established for the four developmental windows from TP1 to TP4.

The confocal image files were transformed into .TIFF files using ImageJ/Fiji and further processed in MorphoGraphX. Briefly, all image stacks were Gaussian blurred with a radius of 0.8 to reduce imaging noise and improve signal continuity. 3D surface meshes of FMs were generated using the Marching Cubes algorithm with a voxel size of 5.0 μm. The reconstructed meshes were subsequently subdivided and smoothed to improve surface quality while preserving the original morphology of FMs.

Fluorescence signals were projected onto the reconstructed mesh surfaces using the Signal Projection function in MorphoGraphX. Projection distances were adjusted according to sample morphology, with minimum and maximum projection distances of 6 μm and 10 μm, respectively. The projected signals were further Gaussian blurred with a radius of approximately 1.0 to enhance cellular features.

Cells on the reconstructed FM surfaces were segmented using the Watershed segmentation algorithm in MorphoGraphX, and segmentation errors in regions with unclear boundaries were manually corrected. This procedure generated labeled cell meshes and cell identity maps for subsequent quantitative analysis.

For cell lineage tracking, cell label maps from the same FM at two consecutive time points (0 h and 24 h) were imported into MorphoGraphX. The meshes from the two time points were manually aligned through translation, rotation, and scaling, and cell labels from the earlier time point were transferred onto corresponding positions at the later time point to establish parental labeling.

Based on lineage-tracking results, visualized heat maps of cell division and cell expansion were generated in MorphoGraphX (Fig. 2). Cells that did not undergo division were assigned a value of “1”, whereas cells that underwent one division event were assigned a value of “2”. Cell expansion was

quantified based on changes in cell area between consecutive time points, including relative area increase (range: 1-1.8) and absolute area difference (range: 0–80 μm^2) (Fig. 2). Quantitative datasets were exported (Supplementary Data 2) and further analyzed and visualized using R software.

Receptacle height and width measurements

Receptacle height and width shown in Fig. 2k and Supplementary Fig. 12 were measured from images captured using Micro-specimen Imaging System (Xi'an RuiFeng, China). The measurements were performed using ImageJ and Photoshop software. Quantitative data were plotted using Origin 2018 and statistical analyses were performed using the Mann-Whitney U test (Supplementary Data 3).

Gene isolation

To obtain the cDNA sequences of target genes, total RNA from floral buds was reverse-transcribed into cDNA. The target genes were amplified using gene-specific primers with cDNA as the template. PCR products of the expected sizes were purified, cloned into the pMB16K cloning vector (Biomed, China), and sequenced. The promoters of *RsWUS1* and *RsWUS2* were obtained using a PCR-based genome walking method (Takara, Japan). Primers used for gene isolation are listed in the Supplementary Data 4.

Phylogenetic analyses

Putative *WUS* and *AG* homologous genes were retrieved from reference transcriptomes of eleven species of the Ranunculaceae. To obtain reliable gene classification, homologous sequences from additional species were obtained from publicly available databases through BLAST searches (Supplementary Data 5). For each gene lineage, coding sequences of *WUS*- and *AG*-like genes were first aligned using CLUSTAL X 1.83⁵⁹ and manually adjusted using MEGA 12, respectively⁶⁰. Phylogenetic analyses were performed using the aligned nucleic acid sequences in IQ-TREE 2.2.2.7⁶¹ with the maximum-likelihood (ML) method. The best-fit substitution model was automatically selected using ModelFinder, and branch support was assessed with 1000 ultrafast bootstrap replicates. The tree of *WUS*-like genes was rooted with genes from a closely related *WOX* gene family, whereas that of the *AG*-like genes was rooted with the *Amborella trichopoda* *AmtrAG* gene.

Gene expression analyses

The spatial expression patterns of *NdWUS*, *RsWUS1*, *RsWUS2*, *RsAG1*, and *RsAG2* were examined by mRNA in situ hybridization following the procedures described by²⁴. Gene-specific cDNA fragments were used as templates for the synthesis of DIG-labeled antisense and sense RNA probes using the DIG RNA Labeling Kit (Roche, Switzerland). Images were captured using an Olympus BX51 upright microscope (Olympus, Japan). Primers used for probe preparation are listed in the Supplementary Data 4 and the locations of the probe-targeted fragments are shown in Supplementary Fig. 6.

RT-qPCR experiments were performed to analyze the tissue-specific expression patterns of *R. sceleratus* *RsWUS1*, *RsWUS2*, *RsAG1*, and *RsAG2* (Supplementary Fig. 7). Total RNA extracted from different tissues was reverse-transcribed into cDNA, which was used as the template for quantitative analysis using 2 × Q3 SYBR qPCR Master Mix (Universal) (TOLOBIO, China) on a real-time PCR system (Thermo Fisher Scientific, USA). The *R. sceleratus* Actin gene was used as the internal reference for normalization. Gene-specific primers and an internal reference gene were used to quantify transcript levels. Relative expression levels were calculated using the comparative CT ($2^{-\Delta\Delta CT}$) method. Primers and gene fragment used for RT-qPCR are listed in Supplementary Data 4 and Supplementary Fig. 6.

VIGS experiments

Gene-specific fragments were selected for each target gene, and the specificity of these fragments was evaluated using R (Supplementary Data 6). The selected VIGS fragments were subsequently cloned into the TRV2-based pYL156 vector. Single-gene silencing constructs targeting *WUS*-like or *AG*-like genes, as well as constructs for simultaneous silencing of multiple genes, were generated. TRV2-*NdPDS* or TRV2-*RsPDS* constructs were used as mock, as silencing of *PDS* causes visible photobleaching phenotypes. Flowers showing visible phenotypic alterations were photographed, and floral organ numbers were counted and compared (Supplementary Data 6). Because floral organ numbers vary considerably among flowers, only flowers classified according to their positions along the inflorescence were used for phenotypic analyses (Supplementary Fig. 9). Silencing efficiency was assessed by RT-qPCR using cDNAs derived from mock-treated floral buds and floral buds exhibiting strong phenotypic alterations. Primers used for evaluating silencing efficiency are listed in the Supplementary Data 4. The schematic diagram of the VIGS fragment is shown in Supplementary Fig. 6. The unpaired two-tailed Student's t-test was used to assess the significant differences between samples in RT-qPCR (Supplementary Figs. 10, 16). A summary of statistical analyses is provided in Supplementary Data 6.

Protein-DNA interaction (PDI) analyses

Yeast one-hybrid assays were performed using the pGADT7 and pAbAi systems. The CDSs of *NdAG1*, *NdAG2*, *RsAG1*, and *RsAG2* were cloned into pGADT7, while promoter fragments of *NdWUS*, *RsWUS1*, and *RsWUS2* containing predicted CArG-box sites were cloned into pAbAi. The 2-kb upstream promoter regions of *WUS* homologs were analyzed using the JASPAR database to identify putative AG-like transcription factor binding sites. Bait constructs were integrated into the Y1H Gold genome, and the minimal AbA concentration for each bait strain was determined. Prey constructs or empty pGADT7 (negative control) were transformed into bait strains; transformants were selected on SD/-Leu/-Ura and tested on medium containing AbA. Colony growth on AbA-supplemented medium indicated positive interactions. The assays were independently repeated three times with consistent results.

For the dual-Luciferase LUC assay, the coding sequence of *NdAG1*, *RsAG1*, *RsAG2* and *Arabidopsis AG*, as well as the upstream 2000 bp promoters of *NdWUS*, *RsWUS1*, and *RsWUS2* were introduced into effector (pGreenII 62-SK) and reporter (pGreenII 0800-LUC) vectors, respectively. A chimeric promoter *pWUS+pRsWUS2_F1* (2,114 bp), was constructed by fusing *Arabidopsis WUS* promoter fragments (-1 to

-728 and -1,067 to -2,114)⁶² with an allelically replaced fragment from the *R. sceleratus* *RsWUS2* promoter fragment (-729 to -1,065, corresponding to the -474 to -810 region of *RsWUS2*). This chimeric promoter was cloned into the pGreenII 0800-LUC vector to drive LUC reporter expression. The recombinant vectors were then transformed into *Agrobacterium tumefaciens* strain GV3101 and the bacterial mixture carrying different vectors was co-infiltrated into tobacco (*Nicotiana benthamiana*) leaves. For luminescence imaging, infiltrated leaves were sprayed with potassium luciferin substrate and visualized using an In Vivo Plant Imaging System (Lumazone Pylon 2048B; Princeton, USA). For quantitative analysis, firefly and Renilla luciferase activities were measured using the Dual Luciferase Reporter Gene Assay Kit (Beyotime, China), and the relative luciferase activity was calculated as the ratio of firefly luciferase activity to Renilla luciferase activity. Five biological replicates were performed, with each replicate consisting of an independently infiltrated tobacco leaf.

For Electrophoretic mobility-shift assays (EMSAs), the full-length coding sequences of *RsAG1* and *RsAG2* were cloned into the pMAL-c5X vector and introduced into the *Escherichia coli* strain Rosetta (DE3) (Coolaber, China). Recombinant proteins MBP-*RsAG1* and MBP-*RsAG2*, as well as the MBP tag alone, were induced in 200 mL cultures with 0.2 mM isopropyl- β -D-thiogalactopyranoside (IPTG) at 16°C for 16 h. Cells were harvested by centrifugation, resuspended in PBS buffer (pH 7.4), and lysed by sonication. The soluble protein fractions were isolated by centrifugation and purified using MBP affinity chromatography with Dextrin Beads (Smart-Lifesciences, China). Biotin-labeled double-stranded DNA probes containing the two newly gained CARG-boxes in the *RsWUS2* promoter region were synthesized. Wild-type (WT) and mutant probes were generated to examine the specific binding of *RsAG* proteins to these CARG-box-containing sequences. Unlabeled probes with identical sequences were used as competitors at a 10-fold molar excess. Protein-DNA binding reactions were performed using the Chemiluminescent EMSA Kit (Beyotime, China) according to the manufacturer's instructions. The resulting DNA-protein complexes were separated by native polyacrylamide gel electrophoresis, and chemiluminescent signals were detected using an Amersham ImageQuant 800 UV imaging system (Cytiva, USA). The primers used are listed in Supplementary Data 4.

Statistics and reproducibility

In general, the two-sided Mann–Whitney U test was used to assess significant differences between two groups, while unpaired two-tailed Student's *t*-tests were used for RT–qPCR and dual-luciferase reporter assays. Tukey's multiple-comparison test was used where appropriate. Numbers of samples and experimental repetitions for each experiment are indicated in the figure legends and Methods.

Declarations

Data availability

Sequence information and accession numbers for genes analyzed in this study are provided in Supplementary Data 5. The genomic loci corresponding to *RsWUS1*, *RsWUS2*, *RsAG1*, and *RsAG2* are located on NCBI accessions OZ205674.1, OZ205669.1, OZ205668.1, and OZ205667.1, respectively. The

data supporting the findings of this study are available within the Article, Supplementary Information and Source Data files. Additional plant materials and datasets generated and analyzed during this study are available from the corresponding author upon reasonable request.

Competing Interests

The authors declare no competing interests.

Author Contributions

R. Zhang, X. D., X. M., and Y. M. conceived and designed the research. X. D., B.S. and G. Z. collected some species used for floral organ counting. R. Zhao, N. H., and K. L. performed the time-lapse live imaging and cellular dynamics analyses, with the assistance of L. G. and J. Q.. X. M., Z. X., and X. D. carried out the phylogenetic analyses and statistics of floral organ numbers. X. M. carried out the RT-qPCR, mRNA *in situ* hybridization, VIGS, and PDI experiments with the assistance of Z. X., K. L., Q. L., J. L. and X. D.. The manuscript was drafted by R. Zhang, X. M., X. D., J. L. and Y. M., with all authors contributing to its revision and final approval.

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Figures

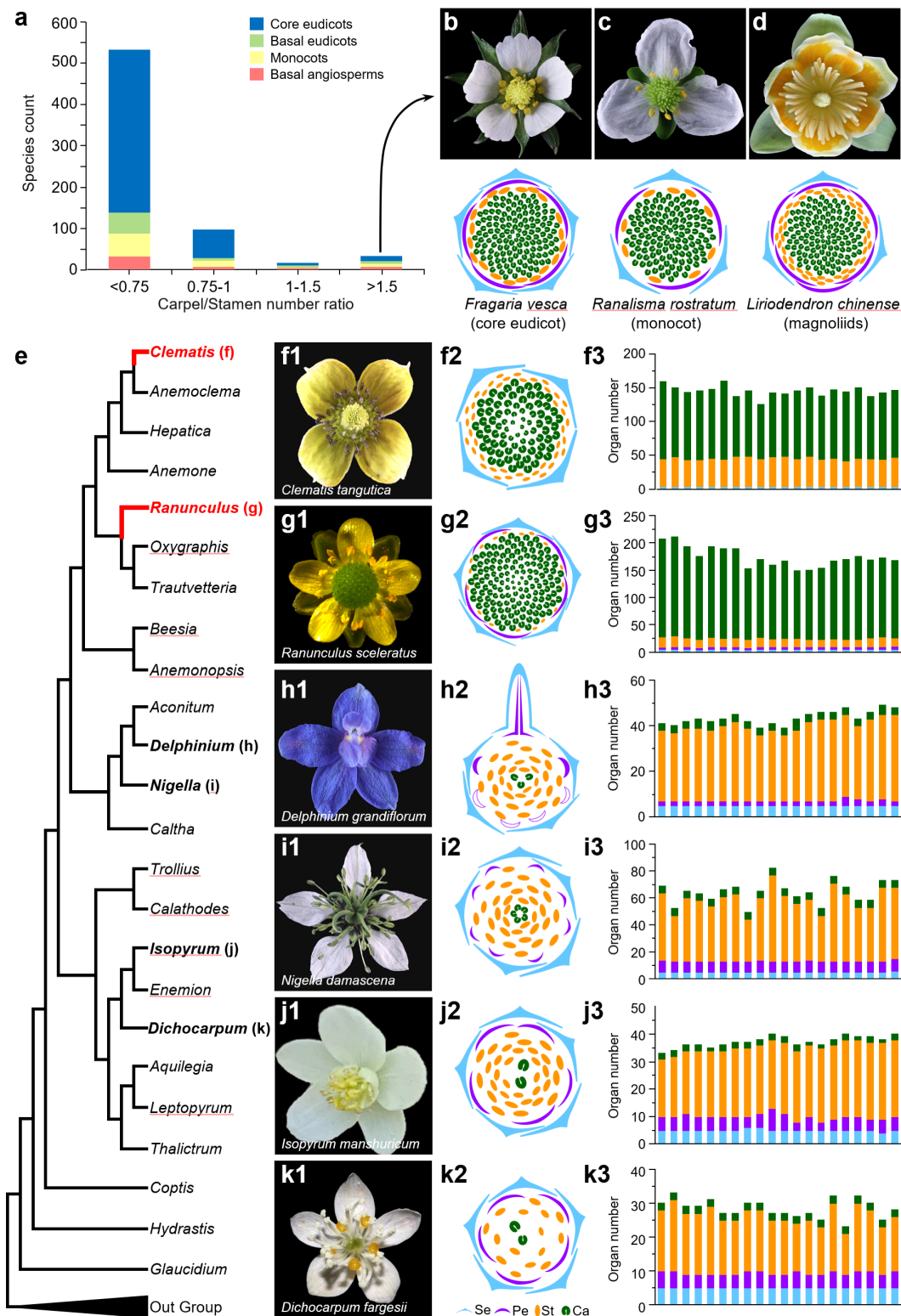


Figure 1

Variation in carpel and stamen numbers in flowers of angiosperms and the Ranunculaceae. (a) Frequency distribution of the carpel/stamen number ratio for species with bisexual flowers. The proportion of major groups is indicated by different colors: core eudicots (blue), basal eudicots (green), monocots (yellow), and basal angiosperms (red). (b-d) Flowers and floral diagrams of representative species from *Fragaria vesca* of core eudicots (b), *Ranalisma rostratum* of monocots (c) and *Liriodendron*

chinense of magnoliids (d). (e) Phylogenetic framework of the Ranunculaceae³⁰ and the diversification of floral organ numbers. The genera selected for floral organ counts are shown in thick, in which those have more carpels than stamens in a flower are highlighted in red. (f-k) Flowers (f1-k1), floral diagrams (f2-k2) and floral organ number counts (f3-k3) from representative Ranunculaceous species of the Ranunculaceae.

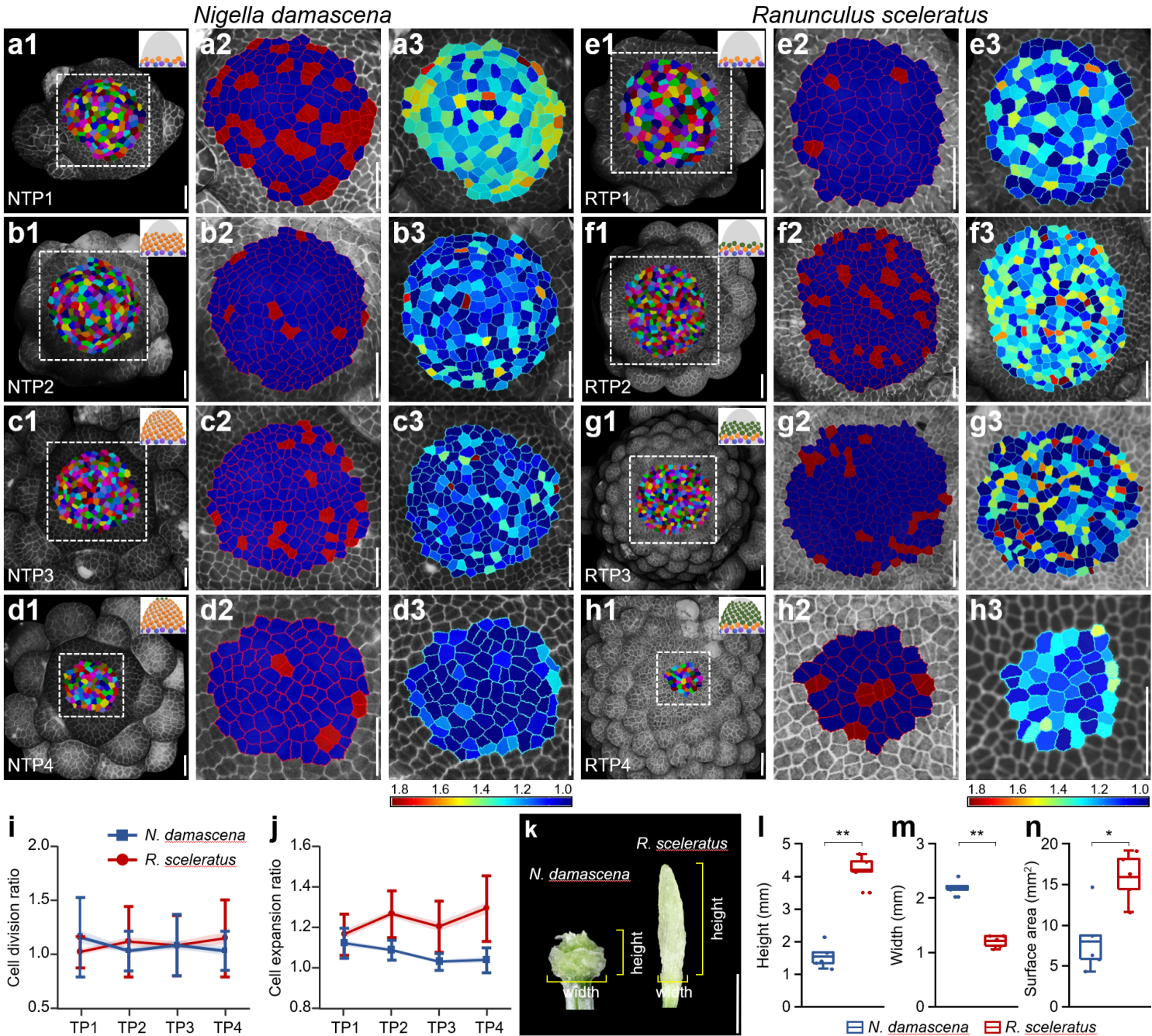


Figure 2

Comparisons of cellular dynamics at floral meristem (FM) apices between *N. damascena* and *R. sceleratus*. (a-d) Time-lapse live imaging and quantitative cellular heatmaps of *N. damascena* FMs at four representative time points (TPs): NTP1 (a), NTP2 (b), NTP3 (c) and NTP4 (d). (e-h) Time-lapse live

imaging and quantitative cellular heatmaps of *R. sceleratus* FM s at four TPs: RTP1 (e), RTP2 (f), RTP3 (g) and RTP4 (h). The FM diagrams representing the coverage status of floral organs are shown at the top right corner of the first column. For each panel group, images arranged from left to right are as follows: top view of cell lineage tracing map (left), where distinct color labels mark individual cells; cell division heatmap (middle), with the divided daughter and undivided cells being labeled with the red and blue, respectively; cell area expansion heatmap (right), with the color scale values indicating the fold change of cell expansion. Scale bar: 50 μ m. (i-j) Comparisons of dynamic trends of cell division ratio (i) and cell area expansion ratio (j) across the four TPs between the two species. The shaded areas around the trend lines represent the 95% confidence intervals. (k) Stereomicrographs of mature receptacles from the two species, with labeled axes for receptacle height and width measurements. (l-n) Box plots showing statistical quantification of mature receptacle height (l), width (m), and surface area (n) in *N. damascena* and *R. sceleratus*. Statistical significance was determined using the Mann-Whitney U test. Asterisks indicate significant differences compared with *N. damascena* and *R. sceleratus*: * P < 0.05, ** P < 0.01, and *** P < 0.001. Scale bar: 2 mm.

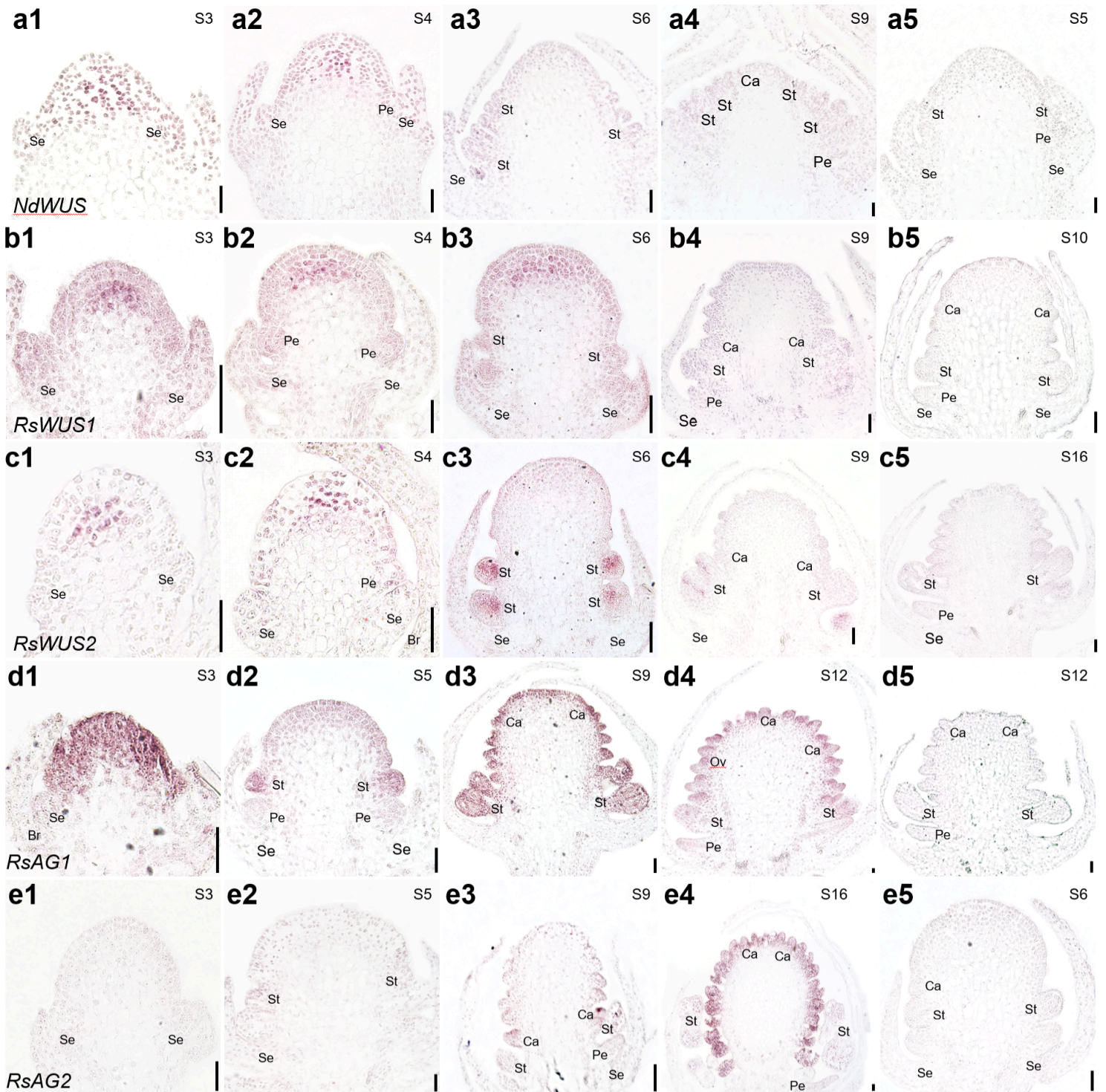


Figure 3

mRNA *in situ* hybridization results of *WUS*- and *AG*-like genes. (a-e) Expression patterns of *NdWUS* in *N. damascena* (a), *RsWUS1* (b), *RsWUS2* (c), *RsAG1* (d), and *RsAG2* (e) in *R. sceleratus* FMs. Sense probes are shown in the last column. The stages of the flowers are shown in the top right corners of each image. All results are representative of at least two independently repeated experiments. Br, bract; Se, sepal; Pe, petal; St, stamen; Ca, carpel. Scale bars: 50 μm.

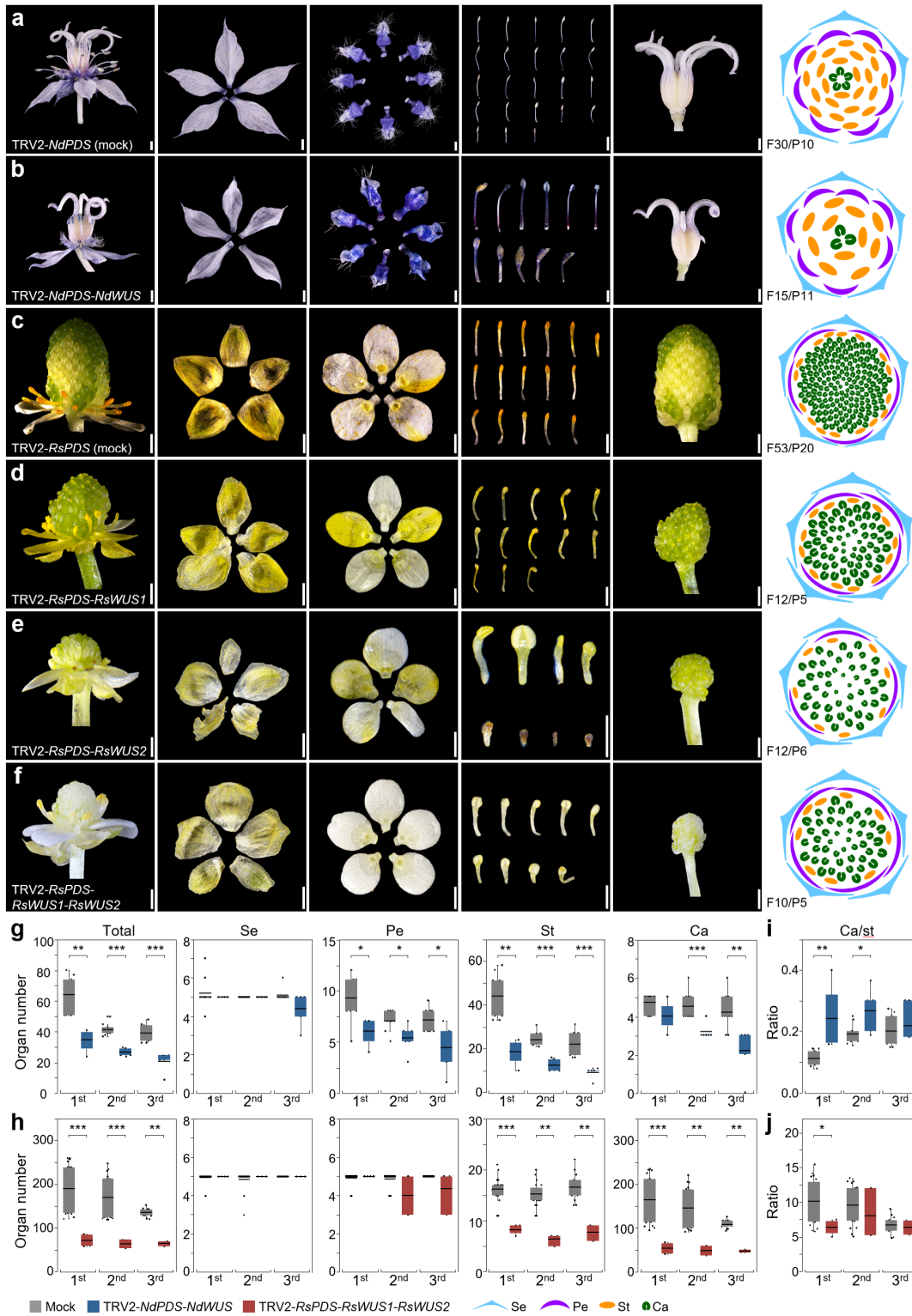


Figure 4

Functions of *WUS*-like genes as revealed by VIGS. (a-b) Flowers with strong phenotypic changes of TRV2-NdPDS- (a) and TRV2-NdPDS-NdWUS-treated (b) plants showing side view of flowers, as well as their floral organs and diagrams in *N. damascena*. (c-f) Flowers with strong phenotypic changes of TRV2-RsPDS- (c), TRV2-RsPDS-RsWUS1- (d), TRV2-RsPDS-RsWUS2- (e), and TRV2-RsPDS-RsWUS1/2- (f) treated plants showing side view of flowers, as well as their floral organs and diagrams in *R. sceleratus*.

Numbers of examined flowers/plants (F/P) are indicated at the bottom left corners of floral diagrams. (g-j) Box plots showing the floral organ number variations in flowers with strong phenotypic changes from TRV2-*NdPDS*-*NdWUS*-treated (g) and TRV2-*RsPDS*-*RsWUS1/2*-treated (h) plants, as well as their resultant carpel/stamen number ratios (i-j). The 1st, 2nd, and 3rd bars represent flowers on the primary, secondary, and tertiary branches, respectively. Significance was determined by Mann-Whitney U test. Asterisks indicate significant differences compared with the mock: **P* < 0.05, ***P* < 0.01, ****P* < 0.001. Se, sepal (cyan); Pe, petal (purple); St, stamen (orange); Ca, carpel (dark green). Scale bars: 2 mm.

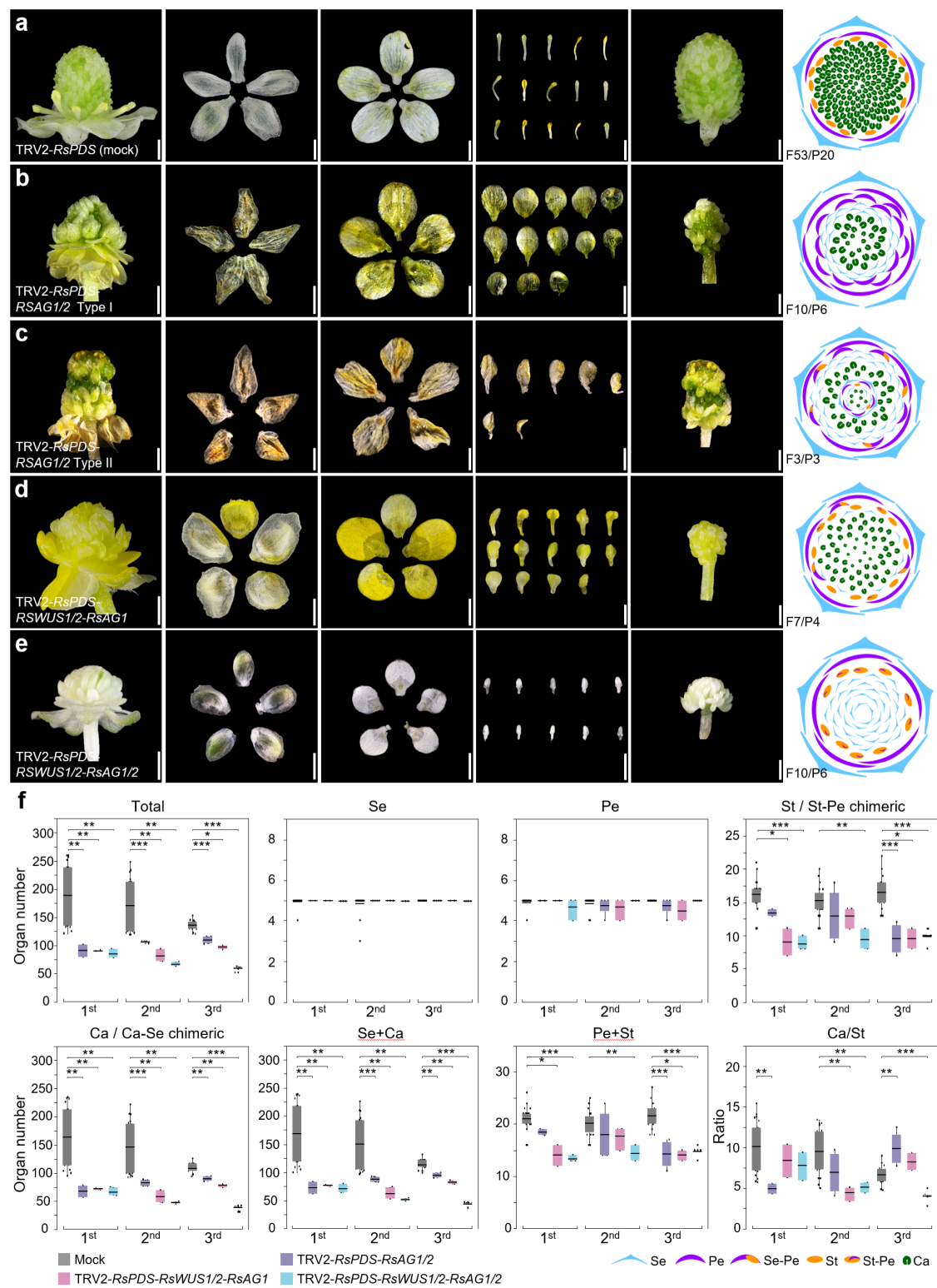


Figure 5

Functions of *RsAG1/2* and *RsWUS1/2* genes as revealed by VIGS in *R. sceleratus*. (a-e) Flowers with strong phenotypic changes of TRV2-*RsPDS*- (a), TRV2-*RsPDS*-*RsAG1/2*- (b-c), TRV2-*RsPDS*-*RsWUS1/2*-*RsAG1*- (d), and TRV2-*RsPDS*-*RsWUS1/2*-*RsAG1/2*- (e) treated plants showing side of flowers, as well as their floral organs and diagrams. Numbers of examined flowers/plants (F/P) are indicated at the bottom left corners of floral diagrams. (f) Box plots showing the floral organ number variations in flowers with strong phenotypic changes from the above VIGS-treated plants, as well as their resultant carpel/stamen number ratios. The 1st, 2nd, and 3rd bars represent flowers on the primary, secondary, and tertiary branches, respectively. Significance was determined by Mann-Whitney U test. Asterisks indicate significant differences compared with the mock group: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Se, sepal (cyan); Pe, petal (purple); St, stamen (orange); Ca, carpel (dark green). Scale bars: 2 mm.

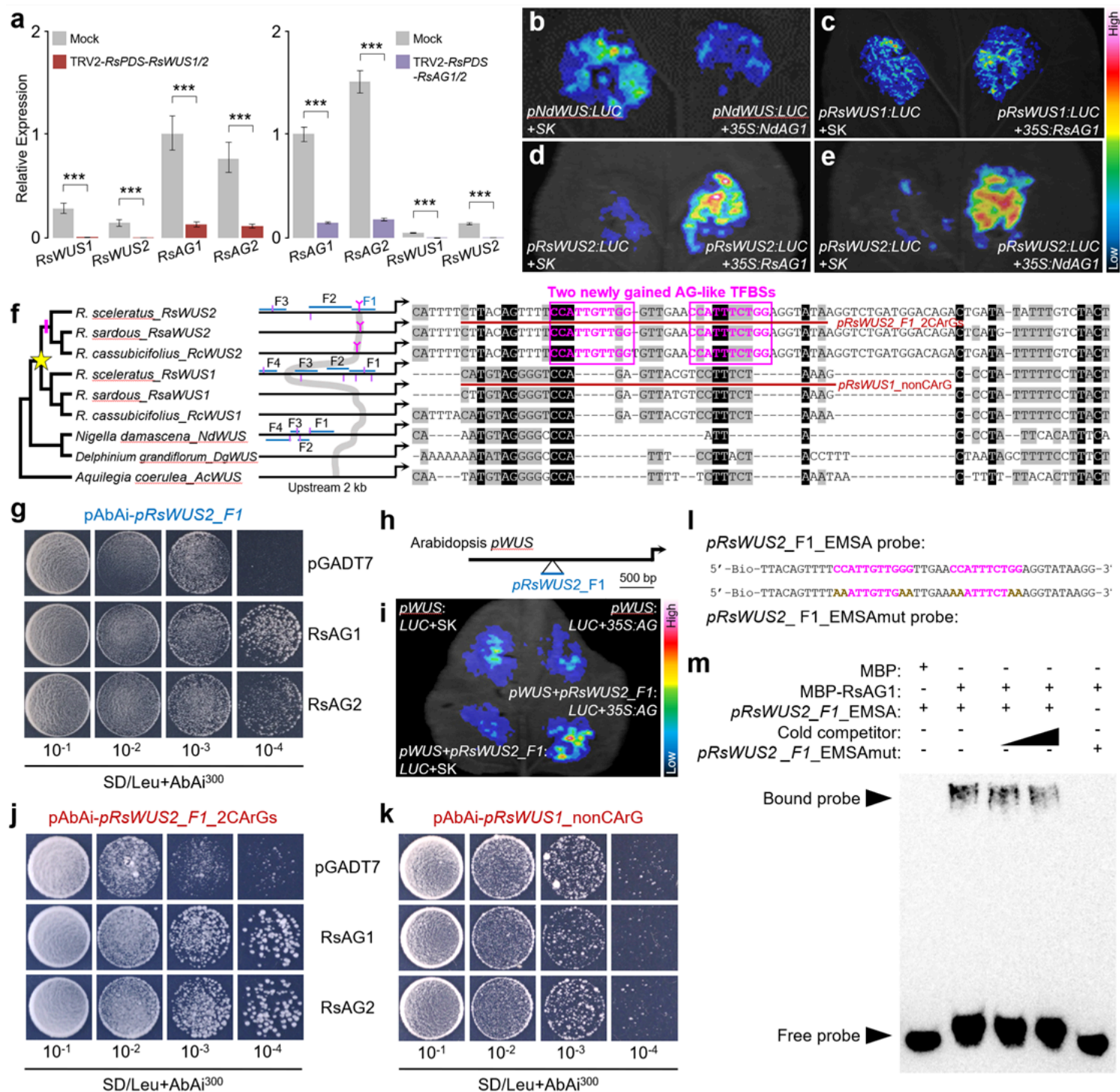


Figure 6

Regulatory links between AG- and WUS-like genes in *R. sceleratus*. (a) RT-qPCR analysis of *RsWUS1*, *RsWUS2*, *RsAG1*, and *RsAG2* in mock-, TRV2-RsPDS-RsWUS1/2-, and TRV2-RsPDS-RsAG1/2-treated plants. Error bars indicate the SD of three technical replicates. (b-e) LUC reporter assays showing the effects of AG1-like proteins on WUS-like promoters in *N. damascena* and *R. sceleratus*. Reporter and effector constructs and LUC quantification are shown in Supplementary Fig. 17. Error bars represent means $\pm$ SD from five biological replicates; significance was determined using Student's *t*-test. (f) Evolution of WUS-like genes and their AG-like transcription factor binding sites (TFBSs) in the

Ranunculaceae. Left, phylogeny of *WUS*-like genes; the yellow star indicates the duplication event before the divergence of *Ranunculus*, and the magenta bar marks the inferred node where the *Ranunculus WUS2* lineage gained two repeated AG-like TFBSs. Middle, predicted AG-like TFBSs and conserved non-coding sequences (CNSs) in the promoters. TFBSs outside the CNSs are shown in light purple, and the two repeated TFBSs within the CNS are shown in magenta. Gray lines indicate CNSs and promoter fragments used for Y1H and LUC assays. Right, alignment of CNS sequences; the repeated TFBSs are highlighted in magenta and fragments used for Y1H and EMSA are underlined in red. (g, j-k) Y1H assays showing binding of RsAG1 and RsAG2 to *pRsWUS2_F1* (g), *pRsWUS2_F1_2CARGs* (j), and *pRsWUS1_nonCARG* (k). Additional Y1H results are shown in Supplementary Figs. 18 and 19. Yeast transformants were grown on selective medium (SD-L) containing 300 ng/mL AbA. L, leucine; AbA, Aureobasidin A. (h) Schematic of the *Arabidopsis WUS* promoter containing the *RsWUS2_F1* fragment (*pWUS+pRsWUS2_F1*). (i) LUC assay showing activation of this reporter by AG; quantification is shown in Supplementary Fig. 17f. (l) *RsWUS2* probes used for EMSA, with mutated nucleotides shown in brown. (m) EMSA showing specific binding of RsAG1 and RsAG2 to the *pRsWUS2_F1_EMSP* probe; unlabeled probe was used as a 10× competitor. See also Supplementary Fig. 20.

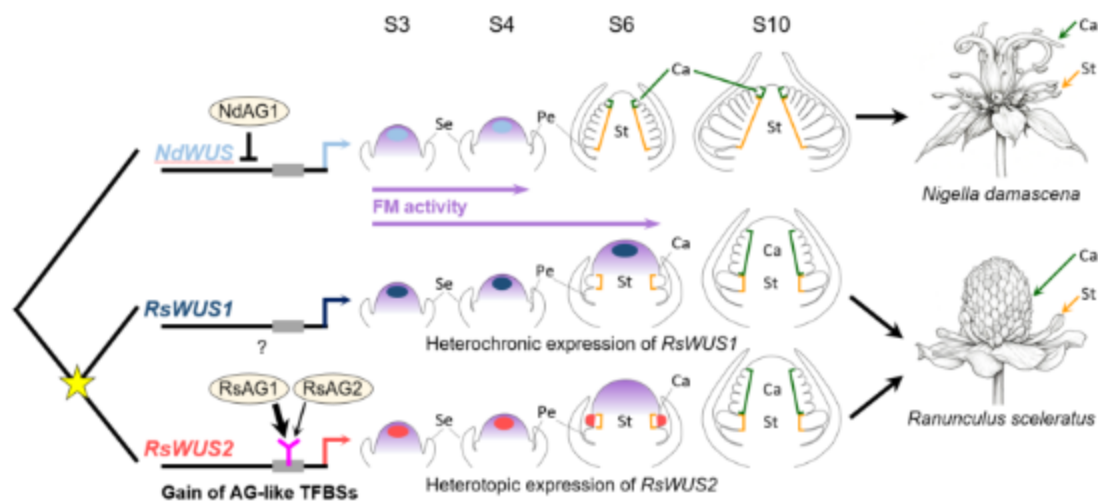


Figure 7

Proposed evolutionary model explaining the production of numerous carpels in *R. sceleratus* flowers.

The yellow star indicates the duplication event of *WUS* orthologs before the divergence of *Ranunculus*. Black horizontal lines, gray boxes and magenta folks denote the promoters, CNS and two repeated AG-like TFBSs. The spatiotemporal expression patterns in FMs of corresponding *WUS*-like genes and corresponding FM activities are shown in the middle part. These combined factors finally result in the distinct floral structures in terms of stamen and carpel numbers between *N. damascena* and *R. sceleratus*. Se, sepal; Pe, petal; St, stamen; Ca, carpel.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [RascWUSSupplementaryData1Informationaboutthefloralorgannumberanalysis.xlsx](#)
- [RascWUSSupplementaryData3Receptaclesizedata.xlsx](#)
- [RascWUSSupplementaryData2ComparisonsofcellulardynamicsatthefourTPsonFMapex.xlsx](#)
- [SourceData.xlsx](#)
- [RascWUSSupplementaryData4Primersusedinthisstudy.xlsx](#)
- [RascWUSSupplementaryData5Geneinformationusedinthisstudy.xlsx](#)
- [RascWUSSupplementaryData6VIGSdataandanalyses.xlsx](#)