RNAi of apple *AGAMOUS* genes leads to increased floral attractiveness and decreased fertility

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Abstract

Sterile hardwood trees have the potential to increase acceptance of genetically engineered (GE) trees by mitigating the risk of gene flow between plantations and wild stands. *AGAMOUS (AG)* is a highly-suitable candidate gene target for obtaining sterile trees, as loss of *AG* function leads to conversion of the reproductive floral whorls into petals. In our proof-of-concept study we used RNAi to suppress expression of the predicted *AG* homolog from domestic apple. Of the 8 events produced, 4 had flowers with a highly showy, polypetalous phenotype and significantly reduced expression of *AG*. These so-called double flowers are highly desirable in cultivated species, and our findings add insight into the genetic basis of this floral trait. Examination of the double flowers revealed that the extra petals were derived from stamens, and retained some stamen-like characteristics, such as the formation of a small number of pollen grains, few of which were viable, and which remained contained within the organ interior. This decrease in pollen formation and loss of pollen shed represent significant impairments to male fertility. Double flowers also had decreased female fertility, with a reduced number of stigmas, partial conversion of styles to petals, and few viable seeds. Despite these floral alterations, trees still set full sized fruit. Such fruit could be decorative, and may be a potential source of food for wildlife. Overall, these data demonstrate the potential of targeting *AG* for the purpose of generating sterile hardwood trees. It is likely that examination of additional RNAi events or the use of a gene-editing mutagenesis method (such as CRISPRs) would lead to the identification of fully-sterile apple trees.

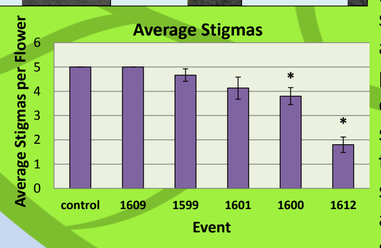
RNAi of apple *AG* led to showy double flowers

Control apple flowers had sepals, 5 petals, 20 stamens and 5 stigmas. Four of our RNAi events looked like control flowers. Four other events had double flowers with numerous extra petals, giving them a showy appearance.

Table 1: Floral phenotypes

Event	Trees	Floral phenotype
1599	**11**	**Double**
1600	**12**	**Double**
1601	**16**	**Double**
1612	**3**	**Double**
1604	1	Single
1607	4	Single
1608	2	Single
1609	6	Single
Control	**9**	**Single**

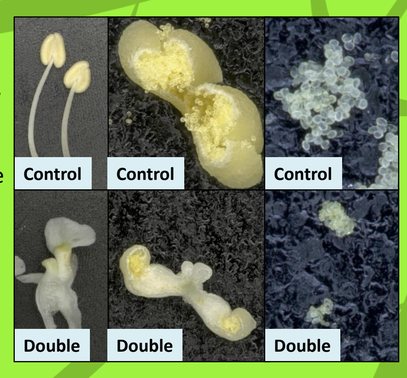
RNAi-*AG* flowers had reduced female fertility



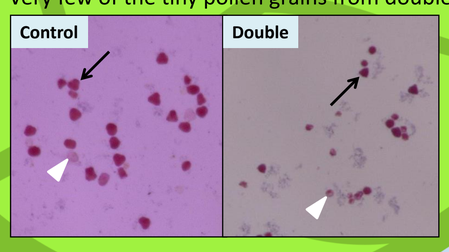
Control flowers had five well-formed styles topped with stigmas. Double flowers had a variable number of styles, often with projections of petal-like tissue, and small stigmas. Quantification of the average numbers of stigmas per flower revealed that events 1600 and 1612 had significantly fewer stigmas than control flowers. Bars show standard error, asterisks indicate significant differences ($P < .05$).

RNAi-*AG* flowers had reduced male fertility

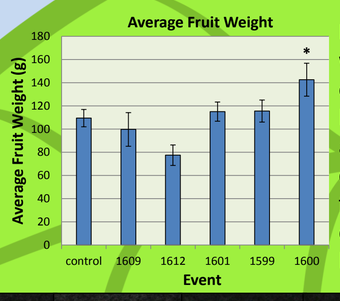
Control flowers had stamens with narrow filaments topped with plump rounded anthers. Cross sectioning of these anthers showed they were full of numerous round pollen grains. By contrast, double flowers had petal-like stamens which contained a small number of tiny pollen grains. These grains did not dehisce and were obtained by dissection.



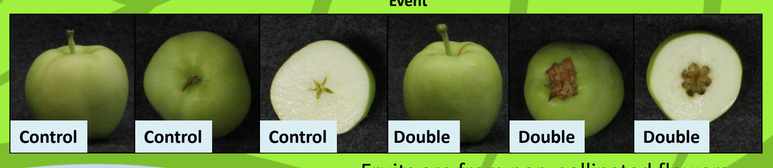
Alexander staining showed that most control pollen grains were viable. Viable grains stain dark pink (black arrow) while non-viable grains remain pale (white arrowhead). Very few of the tiny pollen grains from double flowers were viable.



RNAi-*AG* flowers set full-sized fruit

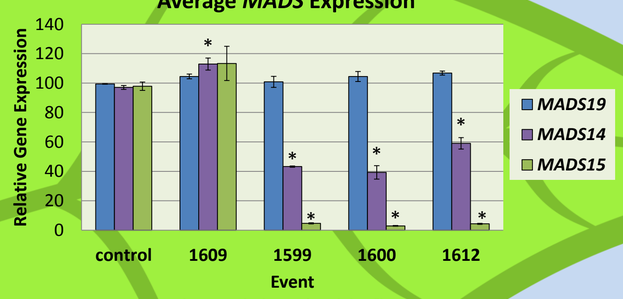


Fruits from double flowers were as large or larger than control fruits. Control fruits had few dry petals on the base and a small five-pointed internal compartment. Fruits of double flowers retained a large cluster of dry petals on their base and had a large central hollow.



Fruits are from non-pollinated flowers

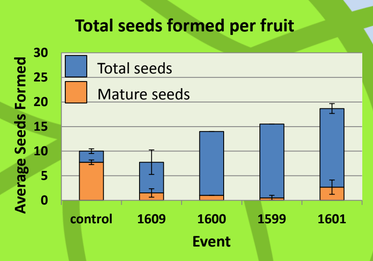
Double flowers had reduced expression of *MADS15* and *MADS14*

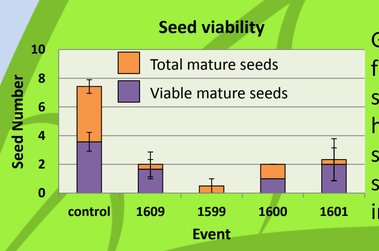


Quantification of *MADS* gene expression showed that double flowers (1599, 1600, 1612) had significantly decreased expression of the *MADS15* target gene and closely-related *MADS14*. The more divergent *MADS19* was unchanged in double flowers. Bars show standard error, asterisks indicate significant differences ($P < .05$).

RNAi-*AG* fruits had few viable seeds

Control fruits formed after cross pollination had an average of 10 seeds, most of which were mature. Fruits of double flowers formed many seeds but most did not develop.





Germination testing of mature seeds from cross-pollinated flowers showed that double flower events had a large reduction in viable seed number. Bars show standard error across individual fruits.

Conclusions

RNAi of apple *AG* leads to showy flowers with additional whorls of petals

RNAi-*AG* flowers had strongly reduced male and female fertility

RNAi-*AG* trees set full-sized fruit

***AG* suppression or mutation is likely to be an effective means for genetic containment in vegetatively propagated apples**

Acknowledgements

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