Coreless apples generated by the suppression of carpel genes and hormone-induced fruit set

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Abstract

Seedless fruits have high consumer appeal and have made seeded varieties obsolete in some crops. In seedless apple varieties, core tissues which normally contain the seed can be unpalatable, reducing the seedless appeal. Apples are accessory fruit with edible flesh derived from hypanthial tissue – a floral tube fused to a compound ovary. Here we show that through suppression of *AGAMOUS*-like carpel identity genes and hormone induced fruit set, it is possible to generate coreless and therefore seedless apples. Suppression of *AGAMOUS*-like genes increased petal whorls and fully eliminated carpel development. Treatments with a combination of gibberellin, cytokinin and auxin, rather than single treatments, were required for fruit initiation in these lines. Transcriptomic analysis of *agamous* RNAi lines suggested conservation of *AGAMOUS*-dependent gene networks between apple and *Arabidopsis*. In the absence of all sexual tissues, the developing fruit continues to grow and follow a ripening process similar to that of a regular apple. The coreless phenotype offers a new concept for pipfruit consumers improving convenience and reducing food waste.

INTRODUCTION

Fleshy fruit texture and whole-fruit eating experience are drivers of consumer acceptability, and removal of inedible elements such as seeds enhances marketability. For many fruits, seedless varieties have greater consumer appeal and often improved fruit quality, which has led to seedless varieties such as grapes, citrus, bananas, and watermelons outcompeting seeded cultivars for market share[1,2]. In these crops, breeding programmes often focus on the seedless character as a primary attribute for new varieties[3]. In pipfruit (apple, pear), seedless varieties are available such as 'Spencer Seedless', 'Rae Ime', and 'Wellington Bloomless', but market domination is not achieved because while seeds are removed, the cartilaginous core remains, which is unpalatable and inedible for most consumers.

Fruit morphology and sub-structures of the fruit are determined by the morphology of the flower. Most fruit derive from carpel tissues, while others, such as apple and strawberry, derive from extra-carpellary tissues such as floral tube/hypanthium and receptacle, respectively. In turn, the four organ types of a perfect flower are determined by floral organ identity genes, as described by the ABCE model[4]. In this model, most genes are MADS-box transcription factors, including A-type *APETALA1* (*AP1*), B-type *PISTILLATA* (*PI*) and *APETALA3* (*AP3*), C-type *AGAMOUS* (*AG*) and E-type *SEPALLATA1-4* (*SEP*). Specific combinations of ABCE type transcription factors interact in quaternary complexes to determine organ identity in each floral whorl[5]. Carpel identity is determined by E- and C-type transcription factors, while B-, C-, E-type combination determines stamen identity. C-type *AG* therefore contributes to both carpel and stamen identity, collectively known as C-function.

The function of E- and C-type transcription factors often varies between species. In dry-fruiting *Arabidopsis*, loss of C-type *AG* function resulted in conversion of stamens to petals, and carpels to indeterminate flowers with sepal-petal-petal patterning and owing to loss of carpel tissues, no siliques were produced[6]. In tomato, loss of C-type *AG* with precision gene editing techniques had weaker effects, generating flowers with altered stamen identity and carpels with loss of determinacy exhibited by seedless fruit and fruit-in-fruit phenotypes[7,8]. In apple, suppression of C-type *AG* increased the number of petal whorls, like *Arabidopsis*, but did not abolish carpel tissues and fruit were produced[9]. In *Arabidopsis*, considerable efforts have sought to identify *AG*-regulated gene networks controlling floral organ identity[10,11]. Partial redundancy in function is supplied by genes of a monophyletic clade with *AG* which are additionally regulated by *AG*, namely *SHATTERPROOF* (*SHP1/2*)[12] and *SEEDSTICK* (*STK*)[13], which were found to control *AG*-independent carpel and ovule identity determination[13]. In contrast to the conserved nature of the *AG* monophyletic clades across species, E-type transcription factors are more functionally diverse owing to extensive clade expansions in this family[14]. *Arabidopsis* has four E-type *SEP* genes, with

SEP3 being an important network hub protein, while *SEP1/2/4* are mostly redundant but with some specialised functions[15,16]. In apple, suppression of E-type *SEP1/2*-like *MADS8/9* modifies floral morphology and significantly reduces hypanthial-derived fruit flesh, but core tissues are mostly unaffected, which suggests flesh and core development are independently regulated[17]. In support of this, partial *AG*-suppressed apples reported full-sized fruit and thus no effect on hypanthial-derived flesh tissue[9].

Carpel tissues are an important site of integration of fruit initiation cues and in all available seedless fruit the carpel necessarily persists. Seedless fruits typically arise when fruits develop in the absence of fertilisation (parthenocarpy) or from post-fertilisation seed abortion (stenospermocarpy). Many lines of evidence demonstrate the inherent properties of carpel tissues to integrate fruiting signals and trigger fruit development, for instance, incomplete pollen tube growth restricted to the female style can set fruit; presence of additional carpelloid organs or loss of stamens correlates with parthenocarpy; and hormone-mediated pistil growth can last longer than ovule viability[18]. Treatment with phytohormones at flowering is a well-established method for generating seedless fruit. Auxin, gibberellin and cytokinin, alone or combined, have all been linked to parthenocarpic induction[19]. In apple, various combinations of all three hormones, singly or in combination, have been trialled with varying results, but predominantly success is achieved with a combination of gibberellins and cytokinins[20,21]. Recently, gibberellins alone were sufficient to induce parthenocarpy in 'Honeycrisp' apples but it was noted that cytokinin was putatively required for proper ovary development[22]. In all mechanisms to induce fruit without fertilisation, carpel tissues persist to enable integration of fruit initiation cues; in apple, this also means the inedible core persists.

In this study, we aimed to investigate the possibility of creating coreless apples through suppression of C-type *AG*-like genes and induction of fruit set with hormone treatment. The purpose of the investigation was two-fold. One, to provide a proof-of-concept for a coreless apple from which the pipfruit industry could harness the economic benefits current seedless varieties cannot provide. Two, to address the biological question of whether fruit tissues can develop in the absence of all sexual tissues.

RESULTS

Identification and suppression of C-type *AG*-like genes in apple

Consistent with the ancient genome duplication in apple[23], six apple genes occur within the *AG* clade. Phylogenetic analysis revealed the two closest apple homologues to *AG* were *MADS15*[24] and *MADS115*, to *SHP1/2* were *MADS14*[24] and *MADS114*, and to *STK* were *MADS10*[25] and *MADS110* (Fig. 1c, Supplemental Table S1). To eliminate carpel tissues, the most similar apple gene to *AG*, *MADS15*, was used as the basis for RNAi construct design (Supplemental Fig. S1). The MADS box region was included for broader targeting of the *AG*-like clade, as specific targeting of *AG* in tomato and apple did not remove carpel tissues[7–9]. Twelve independent

RNAi lines containing the *MADS15* hairpin construct (*ag*) were generated. Five showed a range of severity in loss of carpel tissue and an expected increase in repetitive whorls of petals commonly observed in *ag* mutants (Fig. 1a, b), as well as varying degrees of premature yellow patching on mature leaves (Supplemental Table S2). One line, *ag-05*, showed an increase in the number of petal whorls, and closer examination revealed remnant ovule- and stamen-like structures in central whorls, consistent with a partial knock-out phenotype (Fig. 1b). Despite the existence of these structures and repeated attempts to pollinate, fruit set was not successful. An extreme line, *ag-21*, showed no ovule-like tissues, but inner most petals were slightly stamenoid and contained pollen-like granules (Fig. 1b, d) suggesting residual C-function at the centre of the meristem; similarly to line *ag-05*, repeated pollination attempts were unable to set fruit. The phenotype of both *ag-05* and *ag-21* surpassed the *AG*-knock-down phenotype described previously[9] in achieving near or complete removal of carpel tissues, respectively, and an inability to set fruit with pollination.

mRNA-seq analysis was used to assess the effects of RNAi suppression on the *AG*-like clade and other MADS-box genes. In *Arabidopsis*, *AG* is expressed early in flower development to determine carpel and stamen organ identity[26], but is also
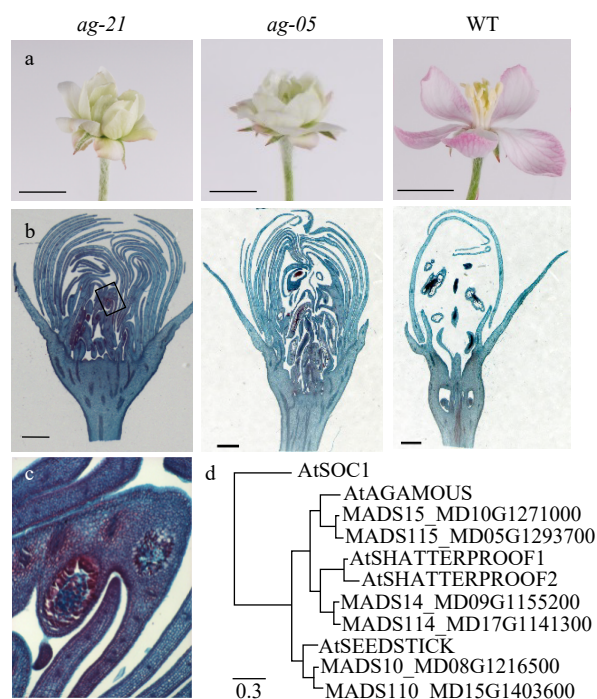

Fig. 1 Removing apple carpel tissues through suppression of *AG*-like genes. (a, b, c) Floral phenotype of wild-type and RNAi lines. (a) Flowers at full bloom (open stage). Scale bar, 1 cm. (b) Longitudinal sections of flowers at balloon stage stained with safranin fast-green. Rectangle on *ag-21* image borders tissues with pollen-like granules and demarcates boundary of magnified image in (c). Scale bar, 1 mm. (c) Magnification (10X) of pollen-like granules in *ag-21*. (d) Phylogenetic tree of *AG*-like genes in apple and Arabidopsis, model and accession numbers listed in Supplementa Table S1, shown with AtSOC1 (AT2G45660.1) as outgroup. Scale indicates 0.3 substitutions per site.

highly expressed in silique tissues at the beginning of fruit development. However, AG function at this development stage is not known[27]. For the latter, the same is true for *AG* orthologues in apple[17]. To determine the effects of *AG*-suppression we chose two late floral stages in which fruit-competent floral tissues are preparing for eventual fruit set: balloon (when the flower is immature and expanding) and open (when the flower is receptive to pollination). It was found that *AG*-like *MADS15/115* were significantly down-regulated in both RNAi lines ($p \leq 0.05$; Fig. 2a). *AG* clade members, *SHP1/2*-like *MADS14/114* and *STK*-like *MADS10/110* were also significantly down-regulated ($p \leq 0.05$; Fig. 2a) and were overall more suppressed in the extreme line *ag-21*, likely accounting for the more extreme phenotype. Transcripts of the *AG* sister-clade *SOC1*-like genes, however, were not down-regulated (Supplemental Fig. S2) thus demonstrating constraint of RNAi specificity to the *AG* clade.

Transcriptomic analysis of *AG*-suppressed lines shows conservation of *AG*-dependent gene networks

The mRNA-seq data were assessed for effects on apple homologues to known direct targets of *AG* in *Arabidopsis*. Analysis of the *AG*-dependent gene regulatory network in

Arabidopsis found 225 direct targets of *AG*[10]. Apple gene models were identified by BLASTp for 177 of the 225 direct *AG* targets (48 returned low or no identity) resulting in 329 apple homologues consistent with whole genome duplication-in apple. Of these, only 10 (3%) were not expressed at all, 47% (156 genes) were significantly ($p \leq 0.05$) differentially expressed with a 2-fold cut-off, and 66% were DE with a 1.5-fold cut-off ($p \leq 0.1$). Apple homologues of *AG* direct targets significantly down-regulated ($p \leq 0.05$, Log2FC ≤ -1) included downstream carpel-sub-structure identity genes *CRABS CLAW* (*CRC*), *AGAMOUS-LIKE 18* (*AGL18*) and *AGAMOUS-LIKE 32/ARABIDOPSIS B-SISTER/TRANSPARENT TESTA 16* (*AGL32/ABS/TT16*), and the organ boundary gene *SUPERMAN* (*SUP*) (Fig. 2b). These effects may be due to the absence of carpel tissues rather than direct regulation, but the trend of down-regulation of these genes mirrors the effects seen in *Arabidopsis* when *AG* is suppressed.

Analysis of all significantly differentially expressed genes (DEGs) ($p \leq 0.05$ and $-1 \geq$ Log2FC ≥ 1) was conducted to identify transcriptomic trends related to removal of the carpels from fruit-competent floral tissues. Removal of carpel tissues was incomplete in the partial line (*ag-05*), and consistent with this, the extreme line (*ag-21*) exhibited more
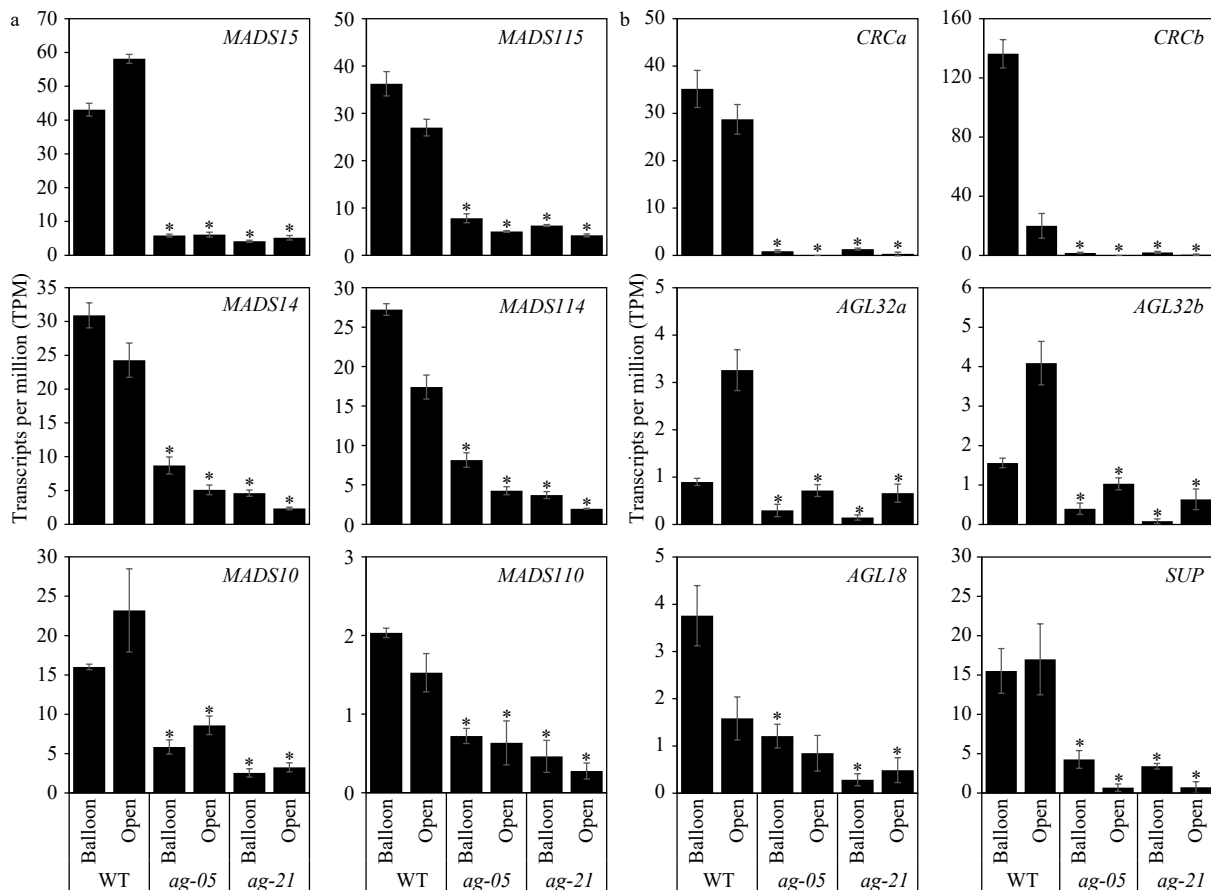

Fig. 2 mRNA-seq analysis of apple AG-like gene models (a) and apple homologues to direct targets of AG (b) in wild-type (WT) and RNAi lines *ag-05* and *ag-21*. Gene expression (transcripts per million, TPM) as found in whole flowers at two development stages (balloon and open flower). Values are mean ± SEM (n = 3). * $p \leq 0.05$, $-1 \geq$ Log2FC ≥ 1 compared with WT stage. Apple AG clade accession and model numbers provided in Supplemental Table S1. HFTH1 gene model IDs for apple homologues are: CRCa, HF07613; CRCb, HF42325; AGL32a, HF11534; AGL32b, HF23066; AGL18, HF20484; SUP, HF14066.

consistent and greater DEGs overall (Fig. 3a), and set analysis showed a greater number of DEGs in the *ag-21*-only DEG set (n = 2,051) compared with the *ag-05*-only DEG set (n = 1,331) (Fig. 3b). Based on homology to TAIR loci, and using keyword search for terms of interest (see Methods), the *ag-21*-only DEG set had proportionally more DEGs with carpel- and stamen-related terms (Supplemental Fig. S3a), consistent with more complete *AG* knock-out, while the *ag-05*-only DEG set had fewer DEGs with gibberellin-related terms, suggesting a more wild-type (WT) state of gibberellin-related transcription (Supplemental Fig. S3b).

Ovary tissues are important sites of integration of fruit initiation signals and many key genetic regulators of fruit initiation have been identified in species with ovary-derived fruits[28]. Apple homologues to these genes were assessed for the effect of carpel removal on expression. Non-orthologous auxin-response factor (*ARF*) genes, *AtARF8* in *Arabidopsis*[29] and *SlARF7* in tomato[30], are negative regulators of fruit initiation. Apple *ARFs* with expression patterns consistent with negative fruit set regulation include *MdARF107* and orthologue to *SlARF7*, *MdARF105*[31]. Of all 33 *ARF* genes in apple, only these two genes were significantly down-regulated in *ag-21* open flowers compared with WT ($p \leq 0.05$, Log2FC ≤ -1) (Supplemental Fig. S4a, b). Gibberellin-signalling *DELLA* transcription factors have also been implicated in fruit set regulation in ovary-derived tomato[32]. Apple has six *DELLA* transcription factors[33] and all were mostly unaffected by carpel removal (Supplemental Fig. S4c).

Reiterating petal whorls seen in RNAi lines were consistent with the known role of *AG* in controlling floral determinacy, and so mRNA-seq data were assessed for effects on meristem maintenance genes. *AG* repression of *WUSCHEL* (*WUS*) is central to the genetic network regulating floral meristem determinacy[34]. One of two *WUS* apple homologues (HF19331) was down-regulated in RNAi lines especially at the open flower stage (Supplemental Fig. S5a). Further meristem maintenance genes, *SHOOT MERISTEMLESS* (*STM*), *POUND-FOOLISH* (*PNF/BLH8*), and *KNOTTED-LIKE FROM ARABIDOPSIS THALIANA 6* (*KNAT6*) were also down-regulated in RNAi lines and again particularly at the open flower stage (Supplemental Fig. S5a). Genes involved in meristem identity such as apple

homologues to *UNUSUAL FLORAL ORGANS* (*UFO*), *CUP-SHAPED COTYLEDON 1* (*CUC1*), *ARABIDOPSIS THALIANA HOMEOBOX GENE 1* (*ATH1*), *APETALA2* (*AP2*), *PROTODERMAL FACTOR 2* (*PDF2*), and *REVOLUTA* (*REV*) were conversely all found to be predominantly up-regulated in RNAi lines (Supplemental Fig. S5b). Interestingly this trend is contrary to that observed in *Arabidopsis ag* mutants at similar late floral stages[10].

Hormone-induced fruit set of *ag* lines yields coreless apples

To test whether fruit development could be triggered in the absence of carpel tissues, hormone-induced fruit initiation was used in place of pollination which had failed to produce fruit in the extreme RNAi lines. In apple, hormone treatments to induce fruit set vary widely between cultivars in terms of timing and number and type of treatments[20,21]. In this study, different regimes were trialled over three years (Table 1). In the first year, auxin and gibberellin applied separately did not yield fruit. In the second year, treatment with gibberellin A4 (GA4) and indole-3-acetic acid (IAA) was sufficient to induce fruit set in 4.5% of cases in control lines and yielded a single fruit in line *ag-05*. Treatment with GA4, IAA and 6-benzylaminopurine (6-BAP) induced fruit set in 6.1% of cases in control lines, and produced one fruit in the extreme line *ag-21*. In year three, to increase incidence, all flowers were treated with the same treatment of GA4, IAA, and 6-BAP with more regularity and for a longer duration, which yielded 63 fruit in partial line *ag-05* and 5 fruit in extreme line *ag-21*.

Fruit by hormone induction in control lines retained locular cavities with remnant integument-derived seed coats present in seed locules (Fig. 4a). In year two, fruit from partial line *ag-05* had a reduced core located more distally with double the normal locule number. Fruit from extreme line *ag-21* had no core tissues (Fig. 4a). Vascular traces in the RNAi lines were similar to controls despite reduction or removal of locular cavities (Fig. 4a). In year three, in order to increase the incidence of fruit initiation, hormones were applied more regularly and over a longer period. In this year, fruit were more irregularly-shaped owing in part to development of fruit tissues downwards along the pedicel beyond the
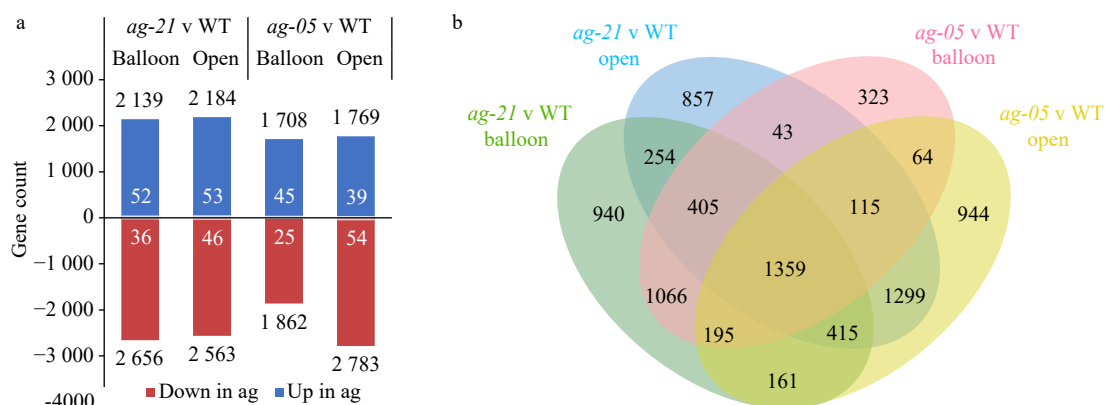

Fig. 3 Comparison of transcriptomes of extreme and partial RNAi apple lines. (a) Significantly differentially expressed genes ($p \leq 0.05$, $-1 \geq$ Log2FC ≥ 1, black data labels) in RNAi lines compared with wild-type (WT) at two floral stages (balloon and open flower) and corresponding proportion of AG direct target homologues (white data labels). (b) Venn diagram showing relationships of differentially expressed genes in terms of floral stage and genotype.

Table 1. Hormone treatments to induce pathenocarpy.

| Year	Conditions of hormone treatment	Line	No. of flowers treated	No. of mature fruit
1	Single 1.5 mM GA4 from -7 to -4 DAFB (no auxin treatment)	ag-05	65	0
	Single 1.5 mM GA4 from -7 to -4 DAFB with daily 5.7 µM IAA from 0 to 1 DAFB, until appearance of yellowing	ag-05	55	0
	Daily 5.7 µM IAA from 0 to 1 DAFB, until appearance of yellowing	ag-05	50	0
2	602 µM GA4, 5.7 µM IAA, beginning -7 DAFB, repeated every 3–4 days for 2 weeks	WT	110	5
		ag-05	110	1
		ag-21	40	0
	602 µM GA4, 5.7 µM IAA, 444 µM 6-BAP, beginning -7 DAFB, repeated every 3–4 days for 2 weeks	WT	115	7
		ag-05	115	0
		ag-21	45	1
	No Hormone control, beginning -7 DAFB, repeated every 3–4 days for 2 weeks	WT	55	0
		ag-05	65	0
		ag-21	40	0
3	602 µM GA4, 5.7 µM IAA, 444 µM 6-BAP, beginning -7 DAFB, repeated every 2 days for 3 weeks, then every 3 days for 1 week.	ag-05	193	63
		ag-21	123	5
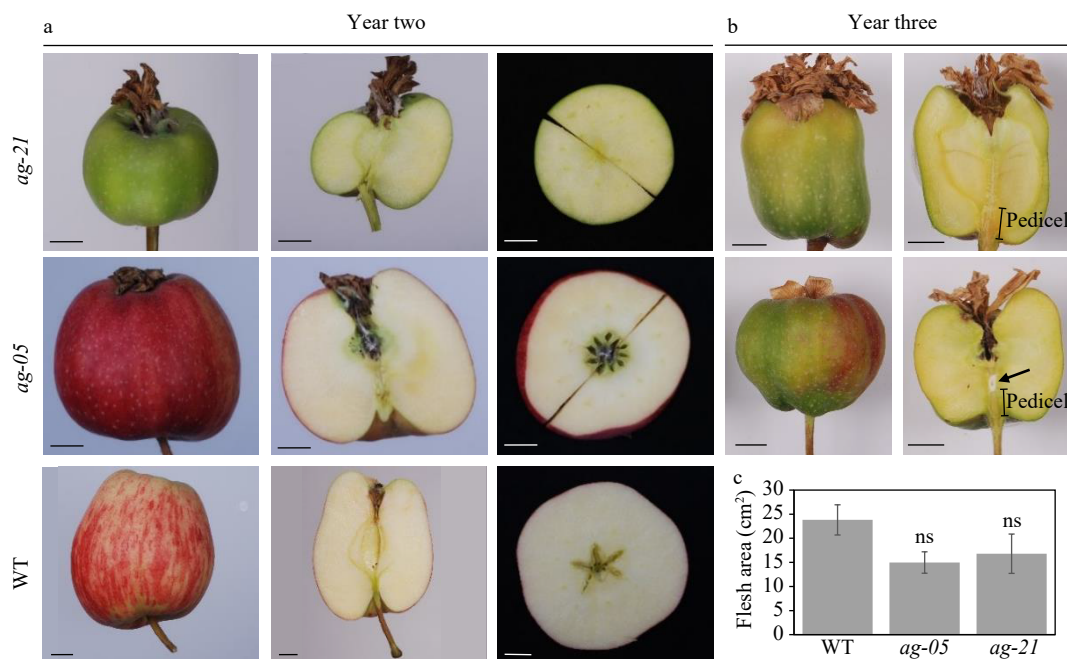
Fig. 4 Mature apples (approx. 120 DAFB) produced by hormone-induction in wild-type and RNAi lines in (a) year two and (b) year three. Classic parthenocarpic fruit phenotype in wild-type, compared with coreless phenotype in *ag-21*. Scale bar, 1 cm. (c) Comparison of flesh area in wild-type and RNAi lines, n = 5 per genotype; Student's t-test, $p \leq 0.05$.

hypanthium. In longitudinally sectioned fruit, more fibrous pedicel-like tissues were visible connecting with central vascular bundles (Fig. 4b). Occasionally, a hole formed in mature fruit in the region where pedicel tissues separated from fruit vascular tissues (Fig. 4b, arrow). Calculation of flesh area in *ag* lines was in addition found to be not significantly different from that of pollinated wild-type controls (Student's t-test, $p \leq 0.05$; Fig. 4c).

Coreless apples undergo fruit ripening

For coreless apples to be acceptable to the consumer it is essential that they undergo fruit ripening, therefore coreless apples were assessed for both competence and degree of ripening. Apple is a climacteric fruit, and so many ripening events respond at varying sensitivities and dependencies to

endogenous auto-catalytic ethylene[35]. Compared with pollinated controls, coreless apples produced a similar amount of ethylene at harvest and during storage as the fruit matured (Fig. 5). The early ripening events such as starch clearance were somewhat more advanced in the RNAi lines than in the wild-type, with near-complete clearance at-harvest. The background skin colour changed from green to yellow but was slightly delayed compared with that in the wild type, and finally both RNAi lines underwent softening (Fig. 5). While these results show that coreless apples were able to undergo a ripening processes a more detailed analysis with larger numbers of fruit are needed to verify the earlier ripening phenotype.
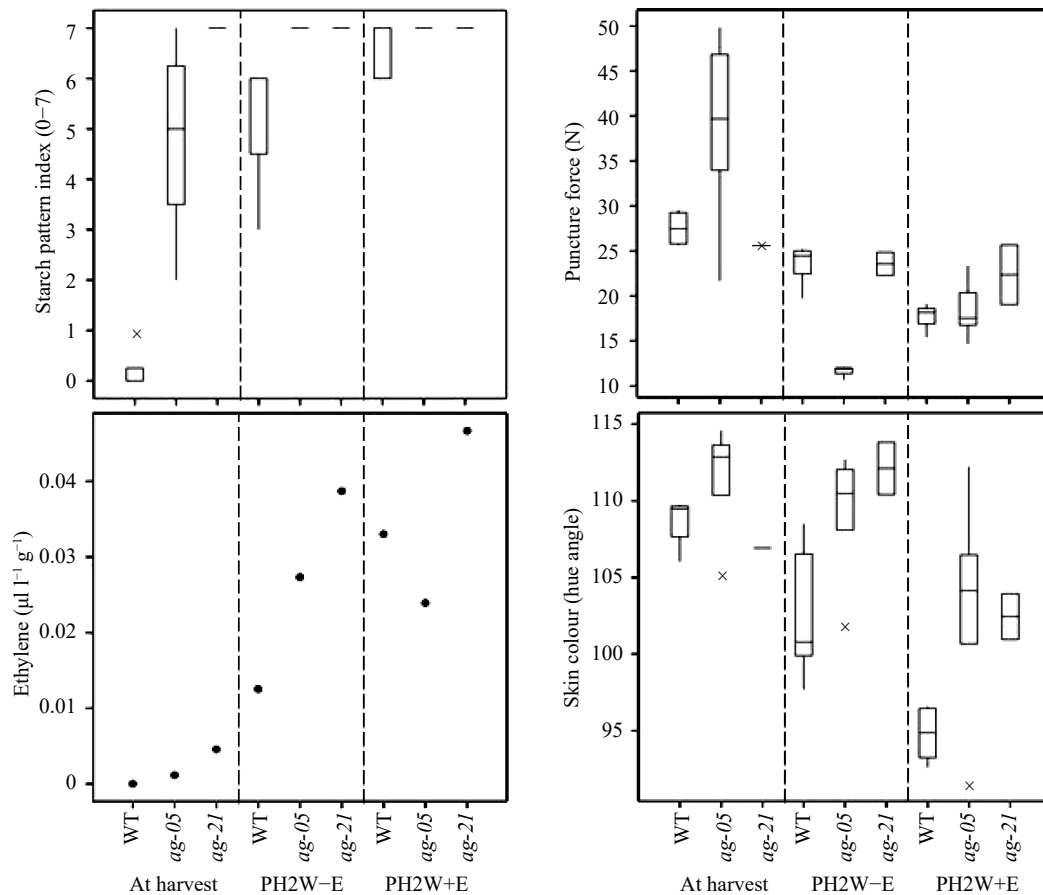
Fig. 5 Fruit ripening in mature pollinated 'Royal Gala' (wild-type; WT) (n = 5 fruit), and RNAi lines *ag-05* (n = 5 fruit) and *ag-21* (n = 1−2 fruit) apples. Starch content on a scale of 1 (high starch) to 8 (no starch), flesh firmness measured by puncture force, emission of ethylene using head space analysis, background skin colour measured by hue angle from green (115) to yellow (95) as observed during ripening. PH2W−E are postharvest fruit ripened for 2 weeks at room temperature in the absence of ethylene, PH2W+E are postharvest fruit ripened for 2 weeks in the presence of 100 ppm ethylene.

DISCUSSION

This study demonstrates the feasibility of generating a coreless pipfruit through the suppression of *AG*-like genes and hormone-induced fruit development. Given that all six genes in the *AG* clade were suppressed in the RNAi lines (more so in the extreme line), there is the possibility that all genes need to be suppressed for complete removal of carpel tissues. This is consistent with previous work in tomato[8] and apple[9] where there appears to be partial redundancy in function within the *AG* clade. Additionally, *SHP*-driven *AG*-independent carpel-development pathways exist in *Arabidopsis* and *SHP* and *STK* are expressed in carpel and carpel sub-structures[12,13]. In apple *ag* lines it was therefore unknown whether suppression of *AG* clade genes was due to direct RNAi regulation or from absence of carpel tissues; especially given there is a direct regulation of *SHP1/2* by *AG*[10,13].

The coreless apple phenotype in line *ag-21* is consistent with core identity and development being determined by *AG*-like clade. Previously, it was shown that apple flesh identity, development and ripening are predominantly directed by E-type *SEP1/2*-like *MADS8/9*[17]. The coreless phenotype is evidence that C-type *AG*-like and E-type *SEP1/2*-like genes act

independently in their facilitation of fruit tissue development. In cherry tomato, elevated expression of *TOMATO AGAMOUS1* (*TAG1*) causes homeotically-normal, mature sepals to undergo ripening, especially when cultured at low temperatures[36], suggesting a role for *AG* in fleshy fruit development in ovary-derived fruit. In apples, suppression of *AG* did not remove fleshy fruit characters, which shows *AG* is not required for flesh identity in apple. Therefore, in tomato, ectopic expression which resulted in fleshy sepals was probably due to ectopic carpel identity later defined by other genes to be flesh competent.

Decoupling of flesh and core development in *ag* lines demonstrates inherent flesh-competency of the hypanthium. This observation aligns with the flesh competency model proposed by Liu, Ma[37]. The apple hypanthium has all three properties the authors propose are needed for flesh competency: one, co-existence with photosynthetic capability; two, ability to promote expression of fruit-related MADS-box genes i.e. *MdMADS8/9*[17]; and three, potential to inactivate auxin/GA signalling repressors, demonstrated here by fruit set with hormone treatment and also suggested by non-elimination of auxin/GA signalling repressors with carpel removal. Typically the sepal is exemplified as a non-competent organ, and strong support exists for the hypanthium being

predominantly sepal-derived[38]. While *AG*-like *MADS15* is not needed for flesh identity, it is expressed in the hypanthium[17,24]. Its role here may be to promote auxin/GA signalling, a role circumvented by exogenous hormones. In accordance with this, direct targets of *AG* include auxin, GA and cytokinin signalling pathway components[10].

Different hormone treatment regimes were trialled over the course of this study, but full development of coreless fruit appeared to require all three fruit-set hormones. Treatments of single hormones in the first year failed, including a staged approach of GA then IAA. Comparison of the need for cytokinin in year two produced inconclusive results in *ag* lines; however, controls had a small increase in yield when cytokinin was included. Treatments with single hormones have had success in other species, such as auxin in citrus and strawberry, gibberellin in grapes, rose and apples, and cytokinin in watermelon, pear, and kiwifruit[19,22]. Apple cultivars have varying requirements[20,21] but usually see improved yields with a combination of all three hormones, and indeed the underdevelopment of ovary tissues in gibberellin-induced 'Honeycrisp' apples was putatively attributed to a lack of cytokinins[22]. In this study, all three hormones together produced coreless fruit. Of note was the need for repeated treatments, when in practice, in the field, fruit set is usually triggered after a single spray[20,22]. The extended treatment regime in year three also altered fruit shape compared with that in year two, highlighting the need to find a minimal optimal dose.

It is hypothesised that seed auxin concentrations regulate fruit development, and the onset of seed dormancy depletes fruit auxins, thereby enabling ripening progression[31,39]. Seedless fruits would therefore be expected to undergo an altered ripening progression compared with seeded fruit. This is indeed the case in avocado[40] and grape[41] which both observe early ripening in seedless or low-seeded varieties. The same appears to occur with coreless apples, providing an opportunity for earlier harvests.

CONCLUSIONS

Fleshy fruit formation and ripening is therefore inherent to the apple hypanthium and is possible in the absence of carpel tissues. The lack of integration of fruit initiation signals normally orchestrated by the carpel can be overcome with exogenous treatment of fruit initiation phytohormones, but ripening signals are able to proceed in the absence of carpel tissue and without exogenous treatments of any kind. This study provides a proof of concept for fruit formation in the absence of carpels and sexual reproduction, thus bypassing the need for pollination and fertilisation altogether.

MATERIALS AND METHODS

Development of transgenic apple plants suppressing *MADS15*

Apple genes homologous to *Arabidopsis AGAMOUS* (*AG*) were identified using BLASTp search against an apple EST collection[42]. The apple MADS-box gene *MADS15*[24] was found to be most similar to *Arabidopsis AG* and was later verified using whole genome data[23]. An RNAi hairpin

construct was created in the binary vector pSAK277[43] with the 35S promoter driving an inverted repeat of the first 430 bp of *MADS15*. Twelve independent lines of transgenic *Malus* × *domestica* 'Royal Gala' were generated using *Agrobacterium*-mediated transformation[44]. Plants were grown in contained glasshouse conditions. Flowers were scored for homeotic changes and harvested for microscopy.

Microscopy

Plant tissue samples were fixed in FAA (4% formalin/50% alcohol/5% acetic acid in water to 100%). Tissues were dehydrated with an ethanol series (50%, 70%, 90%, and x3 100%), cleared with xylene/ethanol (1:1) followed by two changes of xylene (all steps 3 h) followed by three changes of molten wax held at 58–60 °C 12 h per step. All steps were carried out using a Leica TP1020 tissue processor (Leica Microscopy Systems Ltd, Heerbrugg, Switzerland) using both agitation and vacuum cycling. The tissue was embedded in wax blocks using a Leica EG1160 wax embedding station (Leica Microscopy Systems Ltd, Heerbrugg, Switzerland) using Paraplast wax (Oxford Labware, St Louis, MO). Material was sectioned at 15 µm using a Leitz 1512 rotary microtome (Ernst Leitz, Weitzer, Germany) mounted on glass slides and allowed to dry at 42 °C. Sections were dewaxed with two changes of xylene followed by two changes of absolute ethanol (each 5 min) and then stained using the safranin fast green staining method[45]. Sections were observed and imaged using either an Olympus Vanox AHBT3 (Olympus Optical Co. Ltd, Tokyo, Japan) equipped with a CoolSnap digital camera (Roper Scientific Ltd, Tucson, Arizona) or a Leica FLZIII (Leica Microscopy Systems Ltd, Heerbrugg, Switzerland) fitted with an Infinity2-3C digital camera (Lumenera Corporation, Ottawa, ON, Canada).

Hormone-induced fruit set and measurement of ripening

In year one, between -7 and -4 days after full bloom (DAFB) flowers of a single line (*ag-05*) received a single treatment of 1.5 mM gibberellin A4 (GA4) by dipping, followed by no or multiple applications of 5.7 µM indole-3-acetic acid (IAA) by painting onto the hypanthium. In year two, starting at -7 DAFB, hypanthia of whole flowers of three extreme (*ag-05*, *ag-06*, *ag-21*), one intermediate (*ag-11*), and two control lines were painted with either 602 µM GA4 and 5.7 µM IAA, 602 µM GA4, 5.7 µM IAA, and 444 µM 6-benzylaminopurine (6-BAP) or blank solution every 3–4 days for two weeks. In year three, flowers of lines *ag-05* and *ag-21* were treated with 602 µM GA4, 5.7 µM IAA, and 444 µM 6-BAP every 2 days for 3 weeks then 3 days for 1 week over the period from -7 to 21 DAFB (Table 1). Control lines in year two were *ag* lines with wild-type leaf, floral and fruit phenotype and were bagged during hormone treatments. Mature apples were harvested and ripened with 100 µl L⁻¹ ethylene[17]. Fruit were assessed for weight, background skin colour, firmness, and starch clearance as per Johnston et al[35]. For headspace ethylene, fruit were held for 1 h in 2 L headspace tubs, fruit treated with ethylene were aerated for 1 h before being placed in the tubs. A 1 ml headspace sample was extracted and analysed for ethylene using gas chromatography as per Johnston et al.[35]. Flesh area was calculated in ImageJ using five fruit per genotype.

Phylogenetic analyses

Analysis was performed using Geneious 10.0.3 (Biomatters, www.geneious.com). Multiple sequence Geneious alignment using Blosum62 cost matrix was used to align apple protein models based on *Malus × domestica* 'HFTH1' genome[46] (Genome Database for Rosaceae, www.rosaceae.org), the putative hybrid sequence of apple *MxMADS15* (Supplemental Table S1) and *Arabidopsis* protein sequences (TAIR, www.arabidopsis.org). A conserved 240-residue region was extracted from the alignment and used to construct a PHYML phylogenetic tree[47] and bootstrap analysis of 100 datasets.

mRNA sequencing and analysis

Three biological replicates from whole flowers at two stages of flower development (unopened balloon and open flower) from untransformed *Malus × domestica* 'Royal Gala' and two RNAi lines showing partial and extreme *ag*-like phenotypes (*ag-05* and *ag-21*) were harvested and preserved in liquid N_2. RNA was isolated by rapid CTAB extraction[48]. Five µg total RNA was used to construct strand-specific poly-A RNA libraries following Zhong, Joung[49]. Libraries were sequenced by Macrogen using HiSeq2000 sequencing system (Illumina) to produce reads per library ranging from 20–27 million. Reads were processed using FastQC and Trimmomatic, aligned independently with STAR[50] to *Malus × domestica* 'Hanfu' Triple Haploid (HFTH1)[46] and *Malus × domestica* 'Golden Delicious' Double Haploid (GDDH13)[51] genomes and counted with HTSeq. Two apple genomes were used owing to differences in quality of gene model predictions. The statistical software R[52] was used to calculate transcripts per million (TPM) and perform statistical analyses.

Homology analysis was performed with a publicly available BLASTp analysis of HFTH1 to TAIR peptides (Genome Database for Rosaceae, www.rosaceae.org). Keyword analysis was performed using combined lists of TAIR loci with flowering-related GO Biological Processes, GO molecular function, Growth & Development Stages, and Plant Anatomical Entity (Plant Structure) in conjunction with the HFTH1-TAIR BLASTp results.

Data availability

Malus *AG* clade accession numbers and gene model numbers are provided in Supplemental Table S1.

The RNA-seq data can be found in: NCBI project PRJNA357936

Conflict of interest

The authors declare that they have no conflict of interest.

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