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### Permalink

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### Journal

Plant Biotechnology Journal, 21(12)

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### Publication Date

2023-12-01

### DOI




10.1111/pbi.14155

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# Introduction of barnase/barstar in soybean produces a rescuable male sterility system for hybrid breeding

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Received 20 April 2023;

revised 25 July 2023;

accepted 1 August 2023.

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## Summary

Hybrid breeding for increased vigour has been used for over a century to boost agricultural outputs without requiring higher inputs. While this approach has led to some of the most substantial gains in crop productivity, breeding barriers have fundamentally limited soybean (*Glycine max*) from reaping the benefits of hybrid vigour. Soybean flowers self-pollinate prior to opening and thus are not readily amenable to outcrossing. In this study, we demonstrate that the barnase/barstar male sterility/rescue system can be used in soybean to produce hybrid seeds. By expressing the cytotoxic ribonuclease, barnase, under a tapetum-specific promoter in soybean anthers, we are able to completely block pollen maturation, creating male sterile plants. We show that fertility can be rescued in the F1 generation of these barnase-expressing lines when they are crossed with pollen from plants that express the barnase inhibitor, barstar. Importantly, we found that the successful rescue of male fertility is dependent on the relative dosage of barnase and barstar. When barnase and barstar were expressed under the same tapetum-specific promoter, the F1 offspring remained male sterile. When we expressed barstar under a relatively stronger promoter than barnase, we were able to achieve a successful rescue of male fertility in the F1 generation. This work demonstrates the successful implementation of a biotechnology approach to produce fertile hybrid offspring in soybean.

**Keywords:** barnase, barstar, male sterility, hybrid breeding, soybean, *Glycine max*.

## Introduction

Soybean (*Glycine max* (L.) Merr.) is one of the most economically and societally impactful crops in the world, providing a significant percentage of all protein for animal consumption on a global scale and playing key roles in oil production, manufacturing and biofuel applications (American Soybean Association, n.d.). In 2022, 34.9 million hectares of soybean were planted in the U.S., making soybean the second most planted crop after corn, which covered 37.6 million hectares (American Soybean Association, n.d.; Ates and Bukowski, 2023a; Dohلمان and Hansen, 2022). To keep up with the growing demand for soy-based animal feed, the USDA projects that land cultivated for soybean will increase by 19.6% by 2032 (Dohلمان and Hansen, 2022). Given the importance of soybean to global agriculture, advances in soybean productivity could have a transformative impact and promote sustainable agriculture by enabling farmers to produce higher yields on existing land.

Hybrid vigour, also known as heterosis, is the phenomenon in which offspring outperform both their parents and has been used for over a century to increase crop yields, improve abiotic and biotic stress tolerance and enhance the nutritional quality of seeds (Fu *et al.*, 2014; Lippman and Zamir, 2007). Unlike crops that have benefited from hybrid vigour, such as maize, soybean has yet to reap the benefits of a large hybrid breeding program. Based on previous small-scale trials, soybean hybrids made through laborious hand-crossing techniques can produce a 10%–20% increase in yield relative to inbred parents

(Burton and Brownie, 2006; Palmer *et al.*, 2001; Perez *et al.*, 2009). Due to the challenging nature of producing soybean hybrids, these trials are limited to a small number of genotype combinations, indicating that the potential for yield improvement through heterosis is far from exhausted. To capture the benefits of hybrid breeding, the production of high-yielding and commercially available hybrid soybean seeds must be efficient, affordable and compatible with existing farming practices.

One approach to increase outcrossing without emasculating flowers is to block self-fertilization using male sterility (MS). The combination of nuclear male sterility (NMS), cytoplasmic male sterility (CMS) and photoperiod/temperature-sensitive genic male sterility systems with fertility restorer genes have produced promising results in small trials of soybean hybrids (Nadeem *et al.*, 2021; Palmer, 2000; Palmer *et al.*, 2001; Ramlal *et al.*, 2022; Thu *et al.*, 2019). However, multiple drawbacks exist in these systems, making them nearly impractical for commercial hybrid soybean breeding applications. For example, the three-line breeding system requires a maintainer line, results in low genetic diversity occurring from inbreeding CMS lines and requires restrictive growth conditions in the case of photoperiod/temperature-sensitive male sterility systems (Bai and Gai, 2006; Li *et al.*, 2019; Nadeem *et al.*, 2021). Most importantly, many of these male sterile lines are not 100% effective at blocking self-fertilization and often fail to exhibit a full rescue of fertility in the F1 generation, making these existing solutions for hybrid breeding commercially inviable (Bai and Gai, 2006).

An alternative to NMS and CMS is the combination of barnase and barstar; a two-component system in which obligate outcrossing plants are created through the expression of a tapetum-specific cytotoxic ribonuclease (barnase) and its inhibitor (barstar; Hartley, 1989, 1988, 2001). In wild-type post-meiotic anthers, each pollen sac is composed of a ring of endothecium cells, a middle layer and a tapetal layer that surrounds developing pollen grains. The role of the tapetum is to provide nutrients and precursory material to build the outer pollen coat for developing grains. Multiple anther-specific genes were identified from anther cDNA in tobacco, however, the *TA29* promoter is more commonly chosen to induce male sterility due to the protein's simple structure, primary transcriptional level regulation and the constitutive and intense signal seen in the tapetum during stage three of anther development (Beals and Goldberg, 1997; Koltunow et al., 1990; Mariani et al., 1990, 1992; Seurinck et al., 1990; Singh et al., 2015). Targeted expression of *barnase* driven by *TA29* in the tapetum destroys this cell layer, causing male pollen infertility (Bisht et al., 2004, 2007; Denis et al., 1993; Mariani et al., 1992). Fertility is restored in the second generation by crossing barnase-expressing plants with pollen carrying a transgene for the tapetum-specific expression of the ribonuclease inhibitor, barstar (Hartley, 1988, 1989, 2001; Mariani et al., 1990, 1992). This approach has been adopted to create hybrid breeding systems for several crops, including a variety of vegetables and oil seed crops (Colombo and Galmarini, 2017; Jagannath et al., 2002; Mariani et al., 1992; Ray et al., 2007).

In this study, we translated the barnase/barstar male sterility/rescue technology to soybean to test the potential for this biotechnological approach to enable large-scale hybrid breeding in soybean. We generated barnase-expressing lines that consistently exhibited complete male sterility and two different rescue crosses that expressed barstar at equal or higher levels than barnase. We show that a higher dose of barstar relative to barnase is essential for producing a successful rescue of male fertility in the hybrid generation of barnase male sterility lines. This work opens up new avenues for investigating heterosis and advancing the future of breeding in soybean.

## Results

### Designing tapetum-specific promoters to develop a rescuable male sterility system in soybean

To isolate the cytotoxic ribonuclease activity of barnase and rescue activity of barstar to the tapetal cell layer of soybean anthers, we identified two soybean paralogs for the previously characterized tapetum-specific *N. tabacum* gene *TA29* (*A9* in *A. thaliana* At5G07230; Koltunow et al., 1990; Mariani et al., 1990, 1992; Seurinck et al., 1990). *GmTA29a* (Glyma.09G144000) and *GmTA29b* (Glyma.16G197100) are specifically expressed in unopened soybean flowers with *GmTA29a* exhibiting higher expression than *GmTA29b* (Figure S1; Sreedasyam et al., 2022; Valliyodan et al., 2019). To confirm that *GmTA29a/GmTA29b* are specifically expressed in tapetal cells, we generated a dual reporter line with *GmTA29a::tdTomato* and *GmTA29b::ZsGreen* (Figure 1d). In this reporter line, we observed tapetum-specific expression of both fluorophores in the early stages of microspore development (Figure 1e–i), indicating that these *GmTA29* paralogs are appropriate for directing barnase and barstar expression in soybean.

Since barnase is a potent ribonuclease, we decided to take advantage of the differential expression of *GmTA29a/GmTA29b*

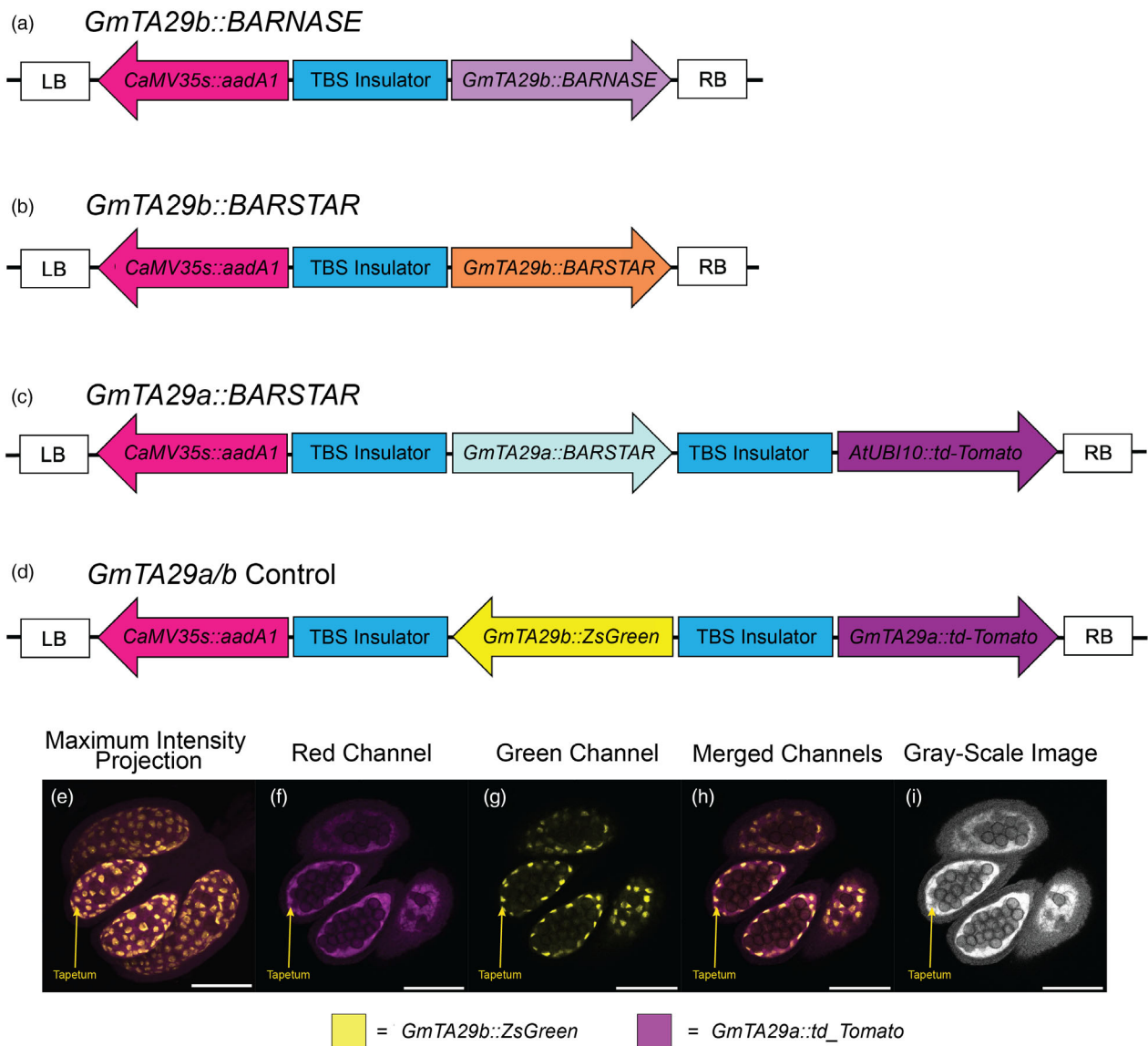
to create a rescue system in which barstar dosage is consistently higher than barnase. We hypothesized that this differential dosage would limit the presence of uninhibited barnase which could negatively impact male fertility in the F1 rescues. To test this hypothesis, we designed one *GmTA29b::BARNASE* expressing construct and two *GmTA29a::BARSTAR* and *GmTA29b::BARSTAR* rescue constructs. In addition, we tagged *GmTA29a::BARSTAR* with an *AtUBI10::td-Tomato* fluorophore to facilitate quick identification of successful crosses onto *GmTA29b::BARNASE* pre-genotyping (Figure 1a–c).

### Tapetum-specific expression of Barnase produces complete male sterility

Of the 13 independent *GmTA29b::BARNASE* events that we generated, 12 exhibited consistent male sterility phenotypes (Figure S2). Wildtype (WT) soybean plants produce both cleistogamous (closed) and chasmogamous (open) flowers at maturity. Interestingly, while there were no other alterations to overall flower morphology and size, we noticed that all 12 of the male sterile barnase events only produced cleistogamous flowers (Figure 2g). To evaluate the effect of barnase expression on stamen and carpel formation, we dissected and imaged flowers at peak reproductive maturity (Figure 2g–k). All the *GmTA29b::BARNASE* male sterile events formed anthers that were misshapen, either opaque white/light yellow or semi-translucent in appearance, and failed to form pollen grains (Figure 2h–k; Figure S2b,e,h,k). In contrast, wildtype (Williams 82) anthers were golden and shedding pollen at maturity (Figure 2b,c). We used propidium iodide (PI) staining and confocal imaging to further investigate the impact of barnase on internal anther organization and identify the cellular basis for male sterility in these events. In WT post-meiotic anthers, we observed pollen grain release from mature pollen sacs, whereas, in barnase-expressing plants, the cavity of the anther lacked microspores and instead exhibited degenerating cellular debris that fluoresced brightly with PI staining (Figure 2c,i).

To investigate whether *GmTA29b::BARNASE* impacted carpel formation, we imaged overall carpel shape and papillar cell morphology using a dissecting microscope and confocal imaging, respectively. Compared to WT, we noticed that mature carpels in barnase-expressing plants tended to curve inward, positioning the stigma towards the centre of the flower where the free stamen is located (Figure 2d,e,j,k; Singh et al., 2007; Talukdar and Shivakumar, 2012). We found this phenotype to be consistent across independent *GmTA29b::BARNASE* transformants (Figure S2c,f,i,l). WT carpels also exhibited slight curvature, however, the stigma tended to face upward in these flowers. Congruent with our findings that the *barnase* plants were completely male sterile, we found zero self-fertilized pods on any of these events (Figure S3). Interestingly, in lieu of fertilized pods, we did observe parthenocarpic pod formation on the *GmTA29b::BARNASE* (Figure S3). These stubby pods were formed as a result of ovule development in the absence of fertilization and could easily be distinguished from viable pods based on their length, presence of unfertilized ovules in place of seeds and greatly reduced size (Figure S3).

Since barstar is an inhibitor of barnase, we did not expect *GmTA29a::BARSTAR* and *GmTA29b::BARSTAR* to differ in phenotype from WT. In line with this hypothesis, the petals, carpels and stamens for these two transgenic lines were indistinguishable from those formed on WT flowers (Figure 2a–

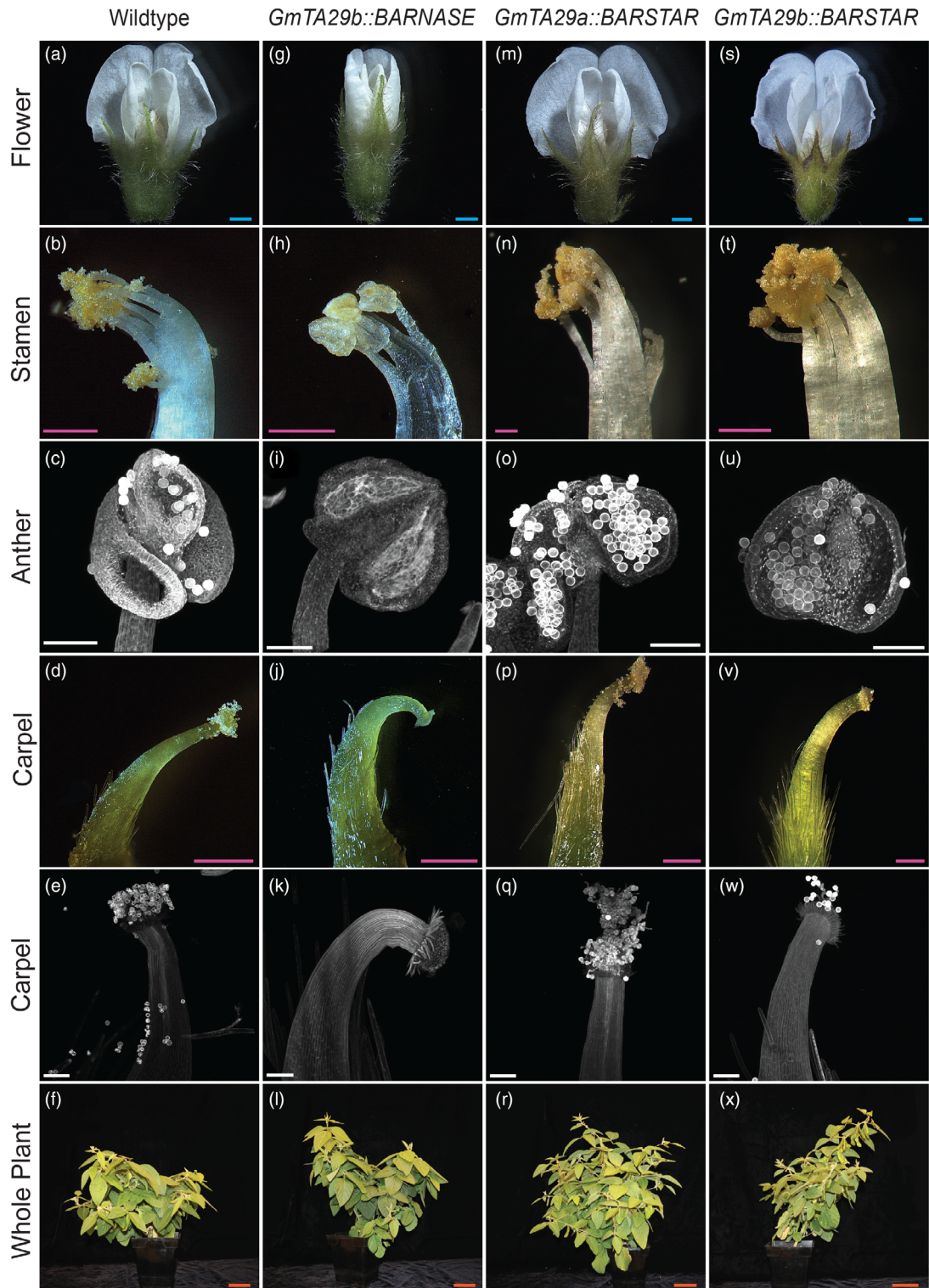


**Figure 1** Construct designs for tapetum-specific expression of *barnase* and *barstar* generate stable and spatially accurate gene expression in soybean anthers. (a, b) Tapetum-specific expression of *barnase* and *barstar* driven by the weaker promoter, *GmTA29b*. *aadA1* is a resistance gene for spectinomycin selection. (c) Construct design for *barstar* expression under stronger promoter, *GmTA29a*. This construct design also includes constitutive expression of the fluorophore, *td\_Tomato*. (d) Construct design for *GmTA29a/b* control line. Anther images of the *GmTA29a/b* control line (e–i) were cleared and imaged with confocal microscopy. Red and green channels were altered in the Fiji software system to project magenta and yellow, respectively. The white scale bar is 100  $\mu\text{m}$ .

e,m–q,s–w). Furthermore, the two *barstar* lines produced numerous fertile pods similar to the WT controls. To test whether *barnase* and *barstar* constructs impacted the overall phenotype of WT, we collected a spectrum of image-based measurements that relate to vegetative health and photosynthetic capacity using a CropReporter™ system (Netherlands Plant Ecophenotyping Center, Wageningen, Netherlands) and corresponding Phenovation Data Analysis software v548. We did not identify any noticeable change in the vegetative health of either the *barnase* or *barstar* transgenic plants relative to WT plants (Figure S4). This data demonstrate that the *barstar* constructs have no measurable impact on plant phenotype, and the *barnase* constructs primarily impacts male reproductive development.

### Rescuing male fertility to generate F1 hybrids

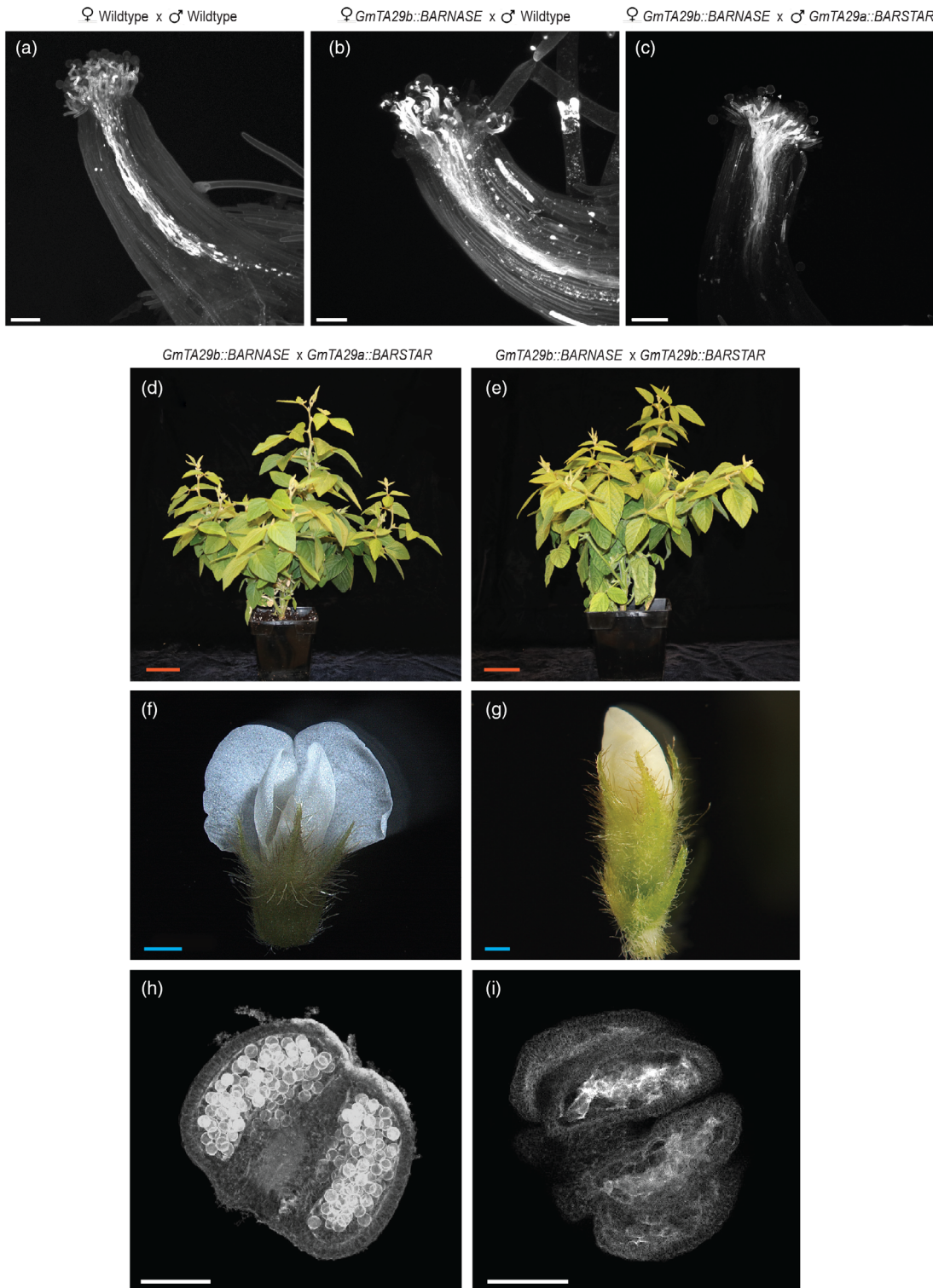
To test whether *barnase*-expressing carpels are receptive to pollen, we crossed WT and *GmTA29a::BARSTAR* pollen onto *GmTA29b::BARNASE* stigmas and used aniline blue staining to track pollen tube elongation. Relative to the WT control, we observed unaltered pollen grain germination and growth on the *GmTA29b::BARNASE* carpels (Figure 3a–c). Our confocal images show that pollen grains were able to adhere to the surface of the stigma, and regardless of the pollen and/or carpel genotypes, we observed comparable levels of pollen tube growth at 24 h post-pollination (Figure 3a–c). This data demonstrate that *GmTA29b::BARNASE* carpels are reproductively viable.



**Figure 2** Flower dissection of *GmTA29b::BARNASE* (T1) displays cleistogamous flowers, male-sterile anthers, and curved carpels compared to wildtype, *GmTA29a::BARSTAR* (T1) and *GmTA29b::BARSTAR* (T1). Flowers (a, g, m, s), stamen (b, h, n, t), and carpel images (d, j, p, v) were dissected and imaged under a dissecting microscope. Whole flower images were captured at peak maturity. Anther (c, i, o, u) and carpel images (e, k, q, w) were stained with PI and imaged using confocal microscopy. Soybean plants (f, l, r, x) were grown under growth chamber conditions and imaged 90 days post-germination. Scale bars for the figure are as follows: the blue bar is 1 mm, the pink bar is 500  $\mu\text{m}$ , the white bar is 100  $\mu\text{m}$  and the orange bar is 100  $\mu\text{m}$ .

As mentioned above, we constructed two barstar lines to test whether a higher dosage of barstar relative to barnase is sufficient to fully rescue male fertility in F1 hybrids. We crossed

three different *GmTA29a::BARSTAR* and two different *GmTA29b::BARSTAR* events onto *GmTA29b::BARNASE* plants, genotyped F1 seedlings for the presence of *barnase*



**Figure 3** *GmTA29b::BARNASE* male-sterile carpels are receptive towards pollination and restore fertility to F1 generation when crossed with *GmTA29a::BARSTAR*. (a–c) Aniline blue images capture pollen tube growth through the style of receptive carpels. *GmTA29b::BARNASE* (T1) and *GmTA29a/b::BARSTAR* (T1) plants were crossed and the F1 (d, e) were grown together under growth chamber conditions, and imaged 90 days post-germination. (f, g) Flower buds were imaged at peak reproductive maturity. (h, i) PI-stained anthers were dissected from flowers and imaged with a confocal microscope. Scale bars for the following figure are as follows: the white bar is 100  $\mu$ m, the orange bar is 100 mm and the blue bar is 1 mm.

and *barstar* transgenes (Table S1), and examined male fertility in these F1 hybrids. In line with our findings for the parental transgenic lines (*GmTA29b::BARNASE*, *GmTA29a::*

*BARSTAR* and *GmTA29b::BARSTAR*), the presence of both transgenes did not affect overall vegetative growth and morphology (Figure 3d,e).

At reproductive maturity, we noticed that, like the *GmTA29b::BARNASE* parental lines, all the *GmTA29b::BARNASE* × *GmTA29b::BARSTAR* F1 flowers were cleistogamous, while *GmTA29b::BARNASE* × *GmTA29a::BARSTAR* F1 plants developed both cleistogamous and chasmogamous flowers, similar to WT plants (Figure 3f,g). To test whether pollen formation was rescued by crossing with either *barstar* construct, we examined PI-stained anthers from our F1 plants using confocal microscopy. While the anther sacs from the *GmTA29b::BARNASE* × *GmTA29a::BARSTAR* crosses were full of pollen, similar to WT anthers, we found that *GmTA29b::BARNASE* × *GmTA29b::BARSTAR* anthers formed zero pollen grains (Figure 3h,i). Instead, flowers from *GmTA29b::BARNASE* × *GmTA29b::BARSTAR* F1s exhibited brightly stained cellular debris inside of the anther sacs, similar to the phenotype that we characterized for *GmTA29b::BARNASE* flowers (Figures 2i and 3i). Moreover, the only pods that were formed on the *GmTA29b::BARNASE* × *GmTA29b::BARSTAR* crosses were parthenocarpic, indicating that the plants were completely male sterile. We, therefore, refer to the crosses with *GmTA29b::BARSTAR* as failed and those with *GmTA29a::BARSTAR* as successful rescues.

Not all *GmTA29b::BARNASE* × *GmTA29a::BARSTAR* crosses rescued pollen formation to the same degree. We observed one *barnase/barstar* combination, *GmTA29b::BARNASE*-event 11 × *GmTA29a::BARSTAR*-event 12, that produced clumpy pollen grains that failed to efficiently release from the anthers (Figure S5e). This partial rescue exhibited both cleistogamous and chasmogamous flowers (similar to the full rescue plants); however, aggravated shaking was required to dehisce and release clumpy pollen from the anthers for imaging (Figure S5e). These partial rescue plants set a mix of reproductive and parthenocarpic pods, however, they formed significantly fewer seeds overall than WT plants (Figure S6). These results indicate that further tuning is needed across the independent *barnase/barstar* lines to ensure consistent rescue phenotypes.

To examine whether successful rescue of pollen formation in *GmTA29b::BARNASE* × *GmTA29a::BARSTAR* plants can be attributed to higher *barstar* expression relative to *barnase*, we performed qRT-PCR for *barnase* and *barstar* on flowers from failed versus successful rescues. We found that F1 plants with rescued fertility exhibited significantly higher *barstar* expression relative to *barnase*, while failed rescue flowers showed no significant differences between *barnase* and *barstar* expression (Figure 4a,b). This data support a model in which higher expression of *barstar* relative to *barnase* is necessary to rescue pollen formation in F1 hybrids.

Next, we tested whether the pollen formed in successful rescue flowers was alive, using a fluorescein diacetate (FDA)/PI pollen viability assay (Figure 4c; Figure S7a-f; Muhlemann et al., 2018). We found that all the successful rescue crosses produced viable pollen; however, overall viability counts for these crosses were significantly lower (3.6% viability on average) than in WT flowers (15.7% on average). Moreover, we noticed that WT anthers released substantially more pollen than the successful rescue anthers (Figure S7g). Despite these differences in pollen counts and per cent viability, we found that the rescue crosses produced sufficient levels of viable pollen to promote self-fertilization and the formation of reproductive seed pods (Figure 4c,d). When comparing WT plants to successful rescue plants (*GmTA29b::BARNASE* – event 13 × *GmTA29a::BARSTAR* – event 12) grown together in growth chamber conditions, the average total seed count of the successful rescue lines was reduced compared to

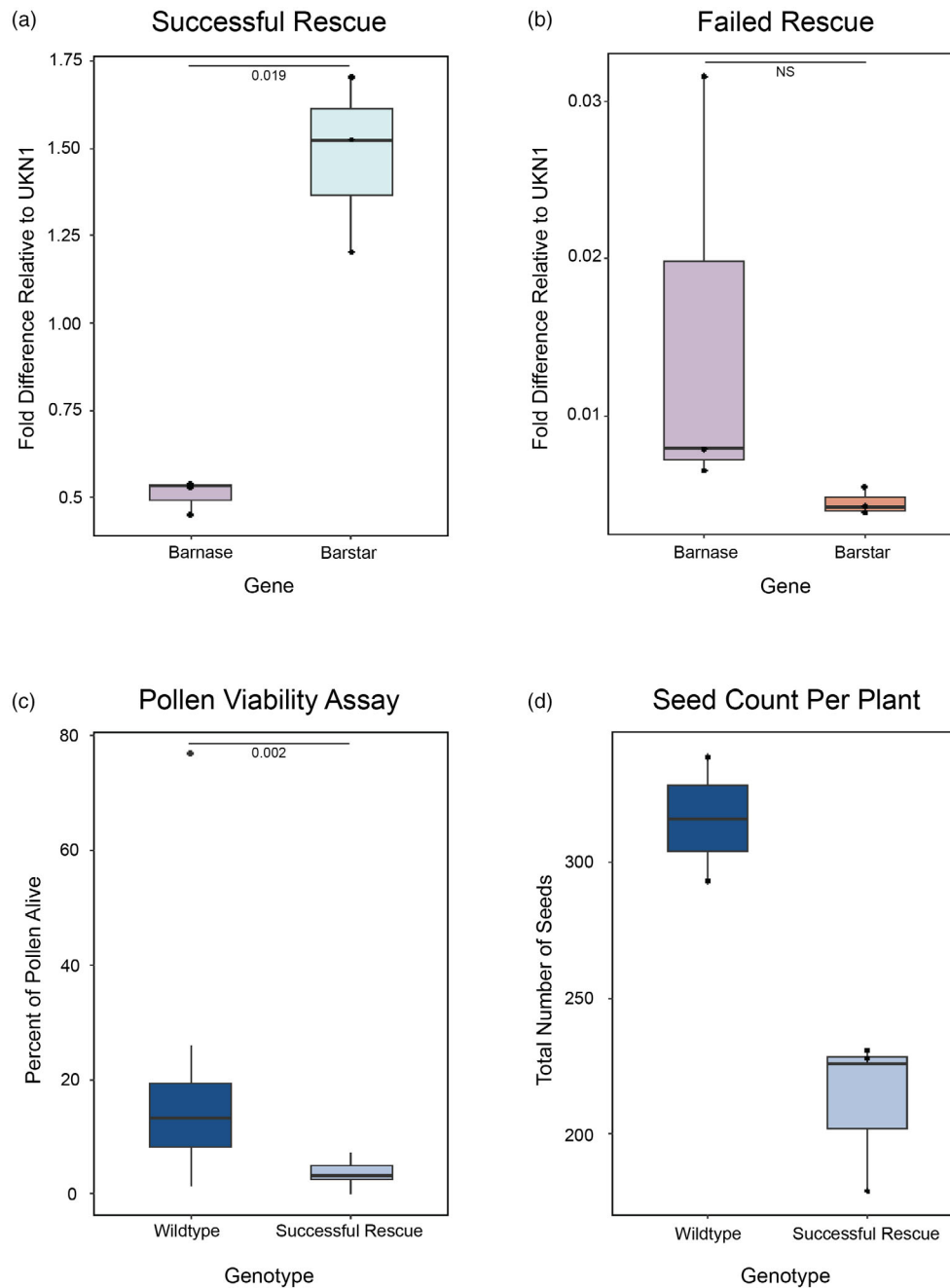
wildtype. These numbers are based on small sample sizes, and future comparisons with higher replication are needed to test the statistical significance of this difference. These results demonstrate that crossing with *GmTA29a::BARSTAR* can restore reproductive fertility to previously sterile plants containing *GmTA29b::BARNASE* (Figure 4d).

## Discussion

Here, we demonstrate that *barnase* and *barstar* can be effectively used to create an obligate outcrossing breeding system in soybean. Notably, we show that a higher dosage of *barstar* relative to *barnase* is required to produce successful male rescue in the F1 generation (Figure 5). *Barnase* is a potent ribonuclease; when expressed in the tapetum it destroys this cell layer and inhibits microspore maturation. We show that cytotoxic ablation of the tapetum using *barnase* is sufficient to produce complete male sterility (Figure 5a). We also demonstrate that male fertility can be rescued in a dosage-dependent manner by expressing the *barnase* inhibitor, *barstar*. While an equal dosage of *barstar* to *barnase* failed to rescue fertility (Figure 5b), when *barstar* was expressed at significantly higher levels than *barnase*, we were able to recover self-fertile F1 hybrids (Figure 5c).

Due to the potent cytotoxic nature of *barnase*, a small fraction of uninhibited ribonuclease is sufficient to induce male sterility. In our model, the dosage of *barstar* inhibitor must exceed that of the ribonuclease to ensure complete inactivation of *barnase*, restoring male fertility (Figure 5c). Even with this successful rescue, we observed some *GmTA29b::BARNASE* × *GmTA29a::BARSTAR* F1 hybrids that only exhibited partial rescue phenotypes with reduced fertility. These partial rescues indicate that further optimization of the relative dosage of *barnase* to *barstar* is needed to ensure full rescue and heightened seed set in the F1 hybrids. Tuning of *barnase* and *barstar* dosage could be achieved by modifying the relative strength of the *GmTA29* promoters through the addition of *cis*-regulatory elements (Bilas et al., 2016), incorporating a two gene – two promoter construct to enhance *barstar* expression (Bisht et al., 2004, 2007; Ray et al., 2007), or identifying new tapetum-specific promoters that can be used to regulate *barnase/barstar* expression. Relative to other major crops, reproductive development in soybean is insufficiently studied, in our opinion. While resources are improving, for example a stage-specific floral atlas is now available (Virág et al., 2022), soybean still lacks a detailed floral organ expression atlas and cell type-specific expression profiling. Such resources are essential for achieving a hybrid breeding system through optimized reproductive rewiring.

To fully realize the potential for hybrid breeding, programmed male sterility/rescue is only part of the solution; we would also need to recruit insect vectors to facilitate outcrossing. Male sterile soybeans are an effective resource to evaluate pollinator–soybean interactions. A multi-year investigation using 21 male sterile lines emphasizes the important effect that environmental conditions and maturity group can have on influencing plant–pollinator interactions (Ortiz-Perez et al., 2006). Another study found that CMS lines grown in the presence of natural and/or introduced pollinators produced significantly higher seed sets than the same lines grown under control treatments (Zhao et al., 2009). This work highlights the effectiveness of combining male sterility with pollinators to produce hybrid seed. Ecological studies clearly indicate that honeybees are the primary pollinators in soybean fields (Blettler et al., 2018; Delaplane et al., 2000). These studies



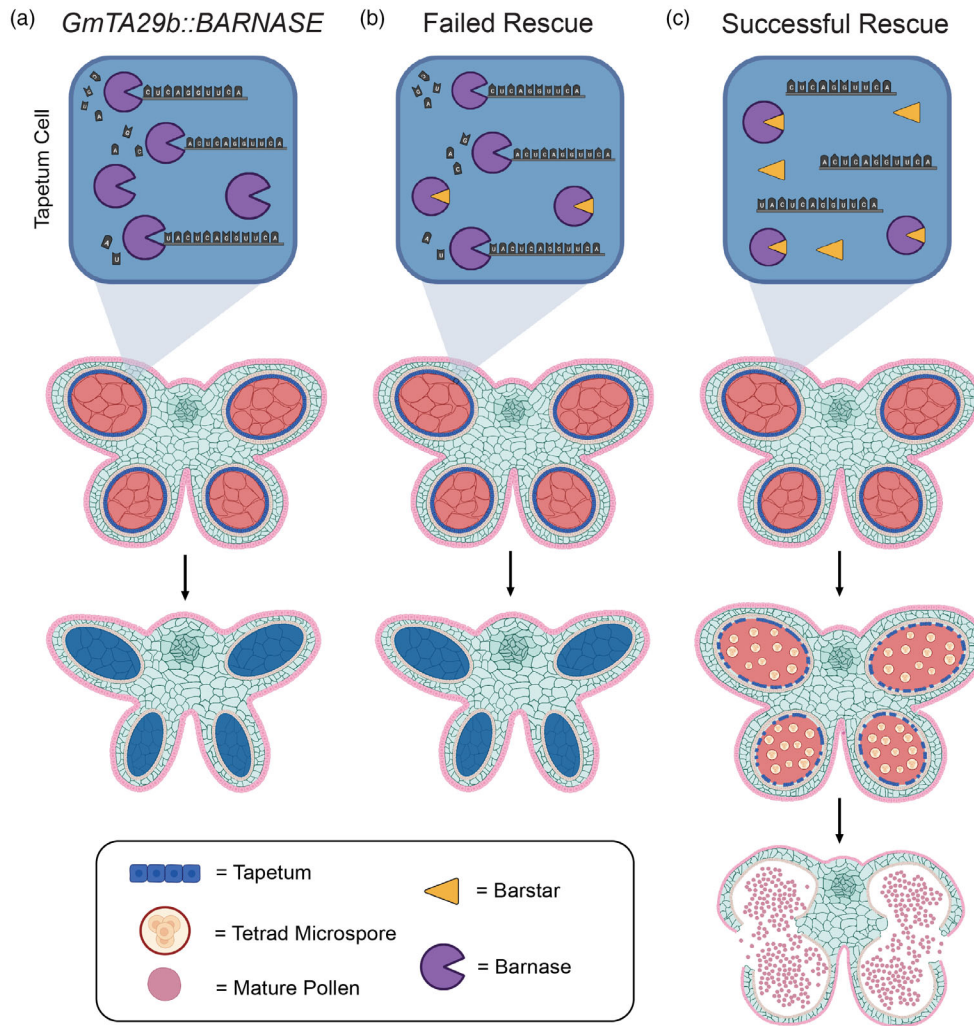
**Figure 4** A higher dosage of barstar contributes to the success of the rescue cross. (a) Boxplot compares qRT-PCR data of *barnase* and *barstar* genes from the successful rescue cross. *Barnase* and *barstar* expression levels are normalized relative to the control, *UKN1*. Bars extending from the boxplot indicate maximums and minimums and the middle line in the box marks the median data point. (b) Boxplot comparing qRT-PCR data of *barnase* and *barstar* genes from the failed rescue cross. (c) A boxplot comparing the per cent of pollen alive per image taken during a pollen viability assay. The plot compares wildtype to the successful rescue for assay images with more than 20 pollen granules (analysed images = 36). (d) A boxplot of total seeds collected per plant taken from wildtype and the successful rescue. Statistics were calculated with the Student's *t*-test.

show that a mutually beneficial interaction exists between bees and soybean fields. Soybean fields provide a source of nectar in mid-western agricultural landscapes (Lin *et al.*, 2022), and in the reverse direction, significant increases in crop yield are associated with the presence of nearby apiaries (Blettler *et al.*, 2018; Erickson, 1975a; Erickson *et al.*, 1978; Garibaldi *et al.*, 2021). This work strongly indicates that honeybees serve as an ecologically beneficial choice for facilitating soybean outcrossing. Building a

better understanding of the floral traits that impact honeybee preferences in soybean is essential to further enhance the use of honeybees as vectors for hybrid breeding.

Furthermore, we currently lack a clear framework for predicting strong heterotic combinations in soybean, which precludes our ability to project the overall benefits that could be derived from applying hybrid breeding to this major crop. This is in large part due to the technical challenges of generating hybrid seeds





**Figure 5** A diagram representation of the effects of *GmTA29b::BARNASE*, *GmTA29b::BARNASE* × *GmTA29b::BARSTAR* (failed rescue), and *GmTA29b::BARNASE* × *GmTA29a::BARSTAR* (successful rescue), respectively. The top row (a–c) depicts barnase, barstar and mRNA interaction inside the nucleus of a single cell in the tapetum. The tapetal cells are enlarged from a digital drawing of soybean anthers containing four pollen sacs. The diagram outlines the development of the anthers under the influence of barnase and barstar expression. (c) The successful rescue mimics wildtype anther growth as it progresses from early anther development, microsporogenesis, and anther maturity. (a, b) Pollen development is halted prematurely when barnase enzymes are uninhibited and the tapetum is destroyed. This figure was created with BioRender.com.

for this species. Indeed, almost all hybrid soybean seed is generated by making crosses by hand, which means that research on heterosis in this crop has been restricted to a small number of crosses with low sample sizes (Burton and Brownie, 2006; Palmer et al., 2001). Though information is limited, existing studies show that particular combinations of inbred parents can lead to significant yield boosts in the F1 generation (Burton and Brownie, 2006; Palmer et al., 2001). However, to date, these studies are limited to narrow geographic regions due to the challenges of generating hybrid seeds. Given that soybean is grown globally across a broad range of environments (Ates and Bukowski, 2023b), current knowledge on hybrid vigor in soybean does not necessarily apply to this crop's expansive geographic range, emphasizing the need for efficient hybrid breeding (Erickson, 1975b). Obligate outcrossing with the *barnase/barstar* lines that we present in this paper provides a new resource that can be used to amplify hybrid seed sets, enabling large-scale trials for heterosis in this major crop.

Beyond the application for large-scale hybridization trials, the *GmTA29::BARNASE/BARSTAR* system removes the need for emasculation and alleviates the difficulty of hand crossing to secure successful F1 hybrids in smaller-scale soybean breeding programs and for trait introgression. Hybrid breeding in soybean has the potential to increase the productivity of one of the most planted and consumed crops in the Americas, yet it has remained largely unexplored. This is in part due to the limitations of current approaches, which have failed to produce reliable obligate outcrossing in soybean. The work we present in this paper provides a key enabling technology to produce obligate outcrossing in soybean.

## Materials and methods

### Construct design and synthesis

Constructs for tapetum-specific expression of barnase/barstar proteins were designed based on sequences from *Bacillus*

*amyloliquefaciens*. Two soybean paralogs for *TA29* were identified using a BLAST search with the orthologous *Arabidopsis thaliana* promoter for A9 (At5g07230) as a query sequence. This query uncovered *GmTA29a* (Glyma.09G144000) and *GmTA29b* (Glyma.16G197100). Expression data for the two soybean *GmTA29* paralogs were downloaded from the JGI Plant Gene Atlas (Sreedasyam *et al.*, 2022; Valliyodan *et al.*, 2019). To ensure the full rescue of barnase with the barstar rescue lines, a relatively weaker expressing *GmTA29b::BARNASE* construct and two versions of barstar constructs, *GmTA29a::BARSTAR* and *GmTA29b::BARSTAR*, were generated (Figure 1a–c; Files S1–S3). In addition, to confirm that *GmTA29a/GmTA29b* are expressed in tapetal cells, a dual reporter construct expressing *GmTA29a::td-Tomato* and *GmTA29b::ZsGreen* in opposite directions with an insulator [transformation booster sequence (TBS) from petunia] between the two reporters was generated (Figure 1d; File S4). All construct sequences can be found in Files S1–S4. Cloning was achieved by the Wisconsin Crop Innovation Center (WCIC) using the Golden Gate MoClo Plant Tool Kit and final vector backbone pAGM4673 provided in the kit (Wisconsin Crop Innovation Center, Madison, WI; Engler *et al.*, 2014).

### Plant transformation

Transgenic soybean lines were generated at the WCIC using a proprietary protocol that involves *Agrobacterium tumefaciens*-mediated transformation of Williams 82 cultured shoot meristems. Positive transformants were selected based on spectinomycin resistance and confirmed with transgene-specific primers. At least eight independent transformants were generated for each construct.

### Genotyping transformants

All plants used in this study were genotyped for transgene inheritance using primers targeted to the *aadA1* antibiotic resistance gene, which was present in all the constructs, and construct-specific targets: *barnase*, *barstar*, *ZsGreen* and *td-Tomato* (Primers, PCR conditions and construct details are in Table S1). Genotyping PCRs were performed using Promega Hotstart Green MasterMix following the recommended conditions from the manufacturer (Promega, Madison, WI).

### Growth chamber and greenhouse conditions

Plants were grown in a greenhouse (daytime temp: 23.9 °C in heating and 26.7 °C in cooling; nighttime temp: 20 °C in heating and 22.8 °C in cooling, light for 14 h) and growth chamber (23 °C, 100% light for 16 h, R/H at 60%) facilities at Cornell University. Plants in Figure S4 were grown in greenhouse conditions at the Donald Danforth Plant Science Center (daytime temp: 25 °C day/23 °C night; 35% minimum humidity); light for 14 h (supplemental light when sunlight is below 400 W/m<sup>2</sup> and shade curtain pulls down to 50% when sunlight is over 900 W/m<sup>2</sup> and pulls down to 100% when sunlight is over 1000 W/m<sup>2</sup>).

### Soybean crosses with *GmTA29b::BARNASE* events

Mature *GmTA29b::BARNASE* flowers were identified based on the emergence of petals from under the sepal whorl. At this stage in normal flower development, the pollen is viable and the stigma is receptive to pollination (Talukdar and Shivakumar, 2012). To uncover the stigma, sepals and petals were manually removed from the *GmTA29b::BARNASE* flowers, and anthers from pollen donors were rubbed directly onto the stigma surface. To increase the probability of fertilization, multiple crosses were made from

the same pollen donor onto each stigma. The F1 hybrids evaluated were generated from crosses between *GmTA29b::BARNASE* (T1) and *GmTA29a/b::BARSTAR* (T1) events.

### Staining and imaging samples

To obtain light microscopy images of whole flowers and individual whorls, soybean flowers were dissected under a Leica M205 FCA fluorescent stereo microscope and imaged with a DMC6200 camera.

For propidium iodide staining of transgenic *barnase* plants, flowers were collected for evaluation of male sterility from T1 *GmTA29b::BARNASE* events. Flowers from wildtype and *GmTA29a/b::BARSTAR* (T1) events were collected and evaluated in comparison to *GmTA29b::BARNASE* flowers. Flowers from T2 F1 hybrids (*GmTA29b::BARNASE* × *GmTA29a::BARSTAR* and *GmTA29b::BARNASE* × *GmTA29b::BARSTAR*) were also collected and evaluated for male-rescue phenotypes. All flowers were processed using the same PI-staining protocol. Samples were fixed in fresh FAA (50% Ethanol, 10%, 37% Formaldehyde, 5% glacial acetic acid) in small glass vials, and vacuum infiltrated until the majority of the samples sunk to the bottom of the vial. The flower samples were transferred to cuvettes and placed in closed cups containing fresh FAA and incubated overnight at 4 °C. The samples were transferred to 50% ethanol at room temperature, and then gradually dehydrated through an ethanol series for 20 min each, followed by incubation in 2 × 100% ethanol for an hour each, and gradually rehydrated (20 min each) to 100% water. Rehydrated samples were rinsed with sterile, autoclaved water twice, and then stained with Propidium Iodide (2 mL of 1 mg/mL PI stock in 100 mL + 0.02% DMSO) for 1 h. Samples were rinsed 2 × 20 min in water, gradually dehydrated to 100% ethanol and then transferred to 50:50 EtOH:methyl salicylate for 2 h, followed by 100% methyl salicylate for 1–2 weeks at 4 °C for clearing. Completely cleared samples were determined based on their translucent appearance and imaged on an LSM880 Confocal multiphoton inverted – i880 Zeiss microscope. Samples were imaged using either 514 or 561 nm excitation, collecting in the 566–718 nm emission range, with laser power ranging from 0.02% to 0.024%.

To image *GmTA29a::td-Tomato/GmTA29b::ZsGreen* dual reporter lines (T1), flowers were cleared following a previously published ClearSee protocol to reduce background autofluorescence (Kurihara *et al.*, 2015). Young soybean flower buds (approximately 3–4 mm in length) were fixed with 4% (w/v) paraformaldehyde for 2 h in PBS under a vacuum at room temperature. The fixed tissue was washed twice with PBS for 1 min each and cleared with ClearSee solution (10% Xylitol (w/v), 15% sodium deoxycholate (w/v), 25% urea (w/v)) at room temperature for 4 weeks under gentle shaking (120 rpm). The clearing solution was changed every other day. After confirming that the tissue was translucent, young buds were imaged using an LSM880 Confocal multiphoton inverted – i880 Zeiss microscope. ZsGreen fluorescence was imaged with a 488 nm laser, collecting in the 493–556 nm emission range, with laser power at 0.025%. td-Tomato fluorescence was imaged with a 561 nm laser, collecting in the 566–691 nm emission range, with laser power at 0.02%. The Fiji software was used to merge image data from td-Tomato and ZsGreen (Schindelin *et al.*, 2012).

### Pollen viability and pollen tube growth assays

Pollen viability assays for *barstar* (T1), WT and *barnase/barstar* rescue crosses were quantified using a previously published

protocol (Muhlemann *et al.*, 2018). Briefly, anthers were dissected during anthesis and placed in a 15-mL conical tube. Pollen was released from the anthers by vortexing. The released pollen was resuspended in pollen viability solution [PVS, 290 mM sucrose, 1.27 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.16 mM boric acid, 1 mM (KNO<sub>3</sub>) containing 0.001% (wt/vol) fluorescein diacetate and 10 μM propidium iodide (stock solutions: 1% (wt/vol) fluorescein diacetate in acetone; 2 mM PI in water)]. The pollen was stained for 15 min at 28 °C and then centrifuged. The PVS-containing FDA and PI was replaced with PVS alone and the stained pollen was placed on a microscope slide and then imaged by confocal microscopy on a Zeiss 880 LSM microscope. The FDA was excited with a 488 nm laser and its signal was collected at 493–584 nm. The PI was excited with a 561 nm laser and its signal was collected at 584–718 nm. Pollen was hand-counted within the Fiji software using the cell counter plugin (Schindelin *et al.*, 2012).

To highlight pollen tube development, a modified protocol for using aniline blue staining was followed (Nasrallah *et al.*, 2002). Carpels from self-pollinated wildtype flowers, *GmTA29b::BARNASE* (T0) x wildtype flowers and *GmTA29b::BARNASE* (T1) x *GmTA29a::BARSTAR* (T1) flowers were harvested and fixed in Farmer's Solution (3:1100% ethanol:glacial acetic acid) 24 h post-pollination. Samples were incubated at room temperature for 5 min and then transferred to 5 M NaOH and moved to 65 °C for 30 min to soften the plant tissue. The samples were carefully rinsed three times in distilled water and then placed in decolorized aniline blue solution [0.1% (w/v) aniline blue dissolved in 0.1 M K<sub>3</sub>PO<sub>4</sub> and decolorized by incubating overnight at 37 °C in the dark]. After 30 min, the carpel was dissected, the trichomes were removed, and the sample was arranged in a drop of aniline blue solution on the microscope slide. The cover slip was added, and pressure was gently applied to squash the tissue. Slides were sealed with clear topcoat nail polish to avoid drying and examined with an LSM880 Confocal multiphoton inverted – i880 Zeiss microscope, exciting with a 405 nm diode laser and collecting in the 415–735 nm emission range, or on an Olympus upright Metamorph microscope using UV fluorescence and a DAPI filter.

### CropReporter™ imaging

Wildtype, *GmTA29b::BARNASE* (T1) and *GmTA29a::BARSTAR* (T1) plants were imaged over the course of multiple days at late vegetative and flowering stages using the CropReporter™ imaging box (Netherlands Plant Eco-phenotyping Centre [NPEC], Wageningen University & Research and Utrecht University). From a top view, the CropReporter™ collected dFv/Fm, F0, Fm, RGB, NIR, CHL and dCHL measurements in dark- and light-adapted plants to calculate the Anthocyanin index ( $(R_{550})^{-1} - (R_{700})^{-1}$ ), the Chlorophyll index ( $(R_{700})^{-1} - (R_{NIR})^{-1}$ ) and the Fv/FM ( $Fv/Fm = Fm - F0/Fm$ ). Fv/Fm was used to determine if a stressor was affecting photosystem II efficiency. The Phenovation Data Analysis software v548 was used to separate the plant from its background and segregate collected data into individual heatmaps for each type of information collected. The heat maps visualized regions of high anthocyanin pigmentation, high photosystem II efficiency and overall plant stress.

### Molecular characterization

For genotyping, DNA was extracted using a modified protocol (King *et al.*, 2014). 400 μL of Edward's Buffer [40% (v/v) 5 M

NaCl and 60% (v/v) extraction buffer (200 mM Tris/HCL pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS)] was added to a 1.5 mL microcentrifuge tube containing a young trifoliate leaf, ground up with a micro pestle and incubated at 60 °C for 30 min. After incubation, samples were centrifuged at 9000 **g** for 5 min. 270 μL of the supernatant was transferred to a new tube and gently mixed with 270 μL of isopropanol. The new tubes were incubated at –20 °C for 15 min, followed by centrifugation at full speed for 5 min. Following centrifugation, the supernatant was removed, and the pellet was washed with 70% EtOH, centrifuged at full speed for 5 min, and decanted. Residual EtOH at the bottom of the tube was removed via micropipette and the tube was left open to dry at 60 °C for 10 min. Once all the ethanol was removed, sterile water was added and the tube was incubated at 60 °C for a final 30 min.

### Quantifying *barnase/barstar* expression

Gene expression was quantified using qRT-PCR. RNA was extracted from young soybean buds using TRIzol (Ambion, Austin, TX), following the manufacturer's instructions for plant extraction. GlycoBlue™ Blue Coprecipitant (Invitrogen, Waltham, MA) was added to each sample during the RNA precipitation step to facilitate recovery of the RNA pellet. The concentration and quality of the RNA were checked using a DeNovix DS-11 FX+ Spectrophotometer/Fluorometer. RNA samples with 260/280 ratios ranging from 1.7 to 2.2 were converted to cDNA using QuantiTect™ Reverse Transcriptase (Qiagen, Hilden, Germany). cDNA concentrations were quantified using a nanodrop and diluted to 1–10 ng of cDNA, and 1 μL of diluted cDNA was added to Power Up™ SYBR Green Master Mix (Applied Biosystems, Waltham, MA) along with gene-specific primers targeting *ZsGreen*, *td-Tomato*, *BARNASE*, *BARSTAR* and a housekeeping gene, *UKN1* (Table S2). The qRT-PCR housekeeping gene, *UKN1* was selected based on a literature review of stable targets used for soybean gene expression experiments (Hu *et al.*, 2009; Jian *et al.*, 2008). Samples were run on a Bio-Rad CFX96 Optics Module with a C1000 Touch Base set at 50 °C for 2 min followed by 95 °C for 2 min for predenaturation. For 39 cycles, the machine was set at 95 °C for 10 s and 60 °C for 30 s to denature, anneal and elongate. Downstream data analysis was performed with an Excel spreadsheet to compute the delta Ct value (normalized to the housekeeping gene, *UKN1*) and Student's *t*-test for significance.

### Acknowledgements

This work was funded by an award from the United Soybean Board 1920-152-0131-D; and a Foundation for Food and Agricultural Research New Innovator in Food and Agriculture Research Award FF-NIA21-0000000017. Imaging data were acquired through the Cornell Institute of Biotechnology's Imaging Facility, with NIH S10OD018516 funding for the shared Zeiss LSM880 confocal/multiphoton microscope.

### Conflict of interest

The Donald Danforth Plant Science Center has applied for a United States Patent (patent pending), titled 'Modification of floral architecture in soybean prevents self-pollination and facilitates outcrossing, enabling the production of hybrid soybean seeds' with B.C.M and M.H.F. as inventors. All other authors have no competing interests.

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- Figure S1** Paralogs of *GmTA29* are differentially expressed in flower buds.
- Figure S2** *GmTA29b::BARNASE* transformation events (T0) result in cleistogamous flowers, sterile anthers, and curved carpels.
- Figure S3** Male sterile *barnase* lines produce parthenocarpic pods.
- Figure S4** No stressors are acting upon *GmTA29b::BARNASE* (T1) and *GmTA29a::BARSTAR* (T1) transformation events.
- Figure S5** *GmTA29b::BARSTAR* is unable to rescue fertility in the F1 generation.
- Figure S6** Partial rescue produces significantly fewer seeds compared to wildtype.
- Figure S7** Successful rescue crosses produce significantly less pollen compared to wildtype.
- Table S1** A table of the primers used for genotyping transgenic lines.
- Table S2** A table listing primers used for qRT-PCR.
- File S1** *GmTA29b::BARNASE* construct.
- File S2** *GmTA29a::BARSTAR* construct.
- File S3** *GmTA29b::BARSTAR* construct.
- File S4** *GmTA29a/b* control construct.

## Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.