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Short communication

Ectopic expression of pigeonpea *Orf147* gene imparts partial sterility in *Cicer arietinum*

Joorie Bhattacharya [a,b], Dumbala Srinivas Reddy [a,c], Kalyani Prasad [a], Rahul B. Nitnavare [d,e], Pooja Bhatnagar-Mathur [f,*], Palakolanu Sudhakar Reddy [a,*]

[a] International Crops Research Institute for the Semi-arid Tropics (ICRISAT), Hyderabad, 502324 Telangana, India
[b] Department of Genetics, Osmania University, Hyderabad, Telangana, India
[c] ATGC Biotech Private Limited, ICCI Knowledge Park (IKP), Genome Valley, Hyderabad, 500078 Telangana, India
[d] Division of Plant and Crop Sciences, School of Biosciences, University of Nottingham, Sutton Bonington LE12 5RD, United Kingdom
[e] Plant Science Department, Rothamsted Research, Harpenden AL5 2JQ, United Kingdom
[f] International Maize and Wheat Improvement Center (CIMMYT), Carretera México-Veracruz 16 Km. 45, El Batán, Texcoco C.P. 56237, Mexico

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ABSTRACT

Orf147, a cytotoxic peptide, has been found to cause cytoplasmic male sterility (CMS) in *Cajanus cajanifolius* (pigeonpea). In our study, *Orf147* was introduced into self-pollinating *Cicer arietinum* (chickpea) using *Agrobacterium*-mediated transformation for induction of CMS. The stable integration and expression of the transgene has been assessed through PCR and qRT-PCR analysis. In addition, phenotypic sterility analysis has been performed, considering developmental parameters like flower development, pod formation and flower drop. Transgene inheritance analysis demonstrates that out of the five PCR positive events in the T_0 generation, two events have segregated according to the Mendelian segregation ratio (3:1) in the T_2 generation. Further, pollen viability test using microscopic analysis confirms the induction of partial CMS in transgenic chickpea. The study holds significant value regarding the heterosis of self-pollinating legumes like chickpea. As a part of the prospect, exploring inducible promoters of species-specific or related legumes would be the next step to developing a two-line hybrid system.

1. Introduction

Efforts to develop a stable hybrid system in legume crops have been going on for the past few years in both the public and private sectors. Owing to their diverse intrinsic values such as high protein content, nitrogen fixation ability and overall resilience, legumes have been explored extensively to further enhance agronomic traits. While hybrid systems have been successfully developed in important legume crops such as pigeonpea (*Cajanus cajanifolius*), research is still lacking in other legumes like chickpea (*Cicer arietinum*). Chickpea is the 3rd largest produced legume globally after common bean and field pea and the largest produced legume in South Asia. India is the largest producer of chickpea contributing a whopping 64% of the total production (Mthulisi and Mcebisi, 2020). In the semi-arid tropical (SAT) region chickpea is the 2nd most cultivated legume and an important source of protein and fibre; therefore, achieving higher yield through hybrid technology is a need of the hour.

Cross-pollinated hybrid crops have been known to exhibit enhanced vigour and yield compared to their parents. This phenomenon referred to as heterosis, has been observed in various naturally cross-pollinating species. Heterosis has also been seen in self-pollinating crops but is comparatively less pronounced than in their cross-pollinating

Abbreviations: AME, Axillary meristem explants; ANOVA, Analysis of variance; cDNA, Complementary DNA; CMS, Cytoplasmic male sterility; CTAB, Cetyl trimethyl ammonium bromide; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GM, Genetically modified; GMS, Genic male sterility; IBA, Indole-3-butyric acid; LSD, Least significant difference; ORF, Open reading frame; PCR, Polymerase chain reaction; qRT-PCR, Quantitative Real time Polymerase chain reaction; SAT, Semi-arid tropical; WT, Wild type.

* Corresponding authors at: Cell Molecular Biology and Trait Engineering, Accelerated Crop Improvement, International Crops Research Institute for the Semi-Arid Tropics, Patancheru - 502324, Hyderabad, Telangana, India (P. Sudhakar Reddy). Principal Scientist & Precision Genetics Lead, International Maize and Wheat Improvement Center (CIMMYT), Texcoco C.P. 56237, Mexico (P. Bhatnagar Mathur).

E-mail addresses: p.b.mathur@cgiar.org (P. Bhatnagar-Mathur), sudhakarreddy.palakolanu@icrisat.org (P. Sudhakar Reddy).

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counterparts. The introduction of heterosis and hybrids in modern agriculture has been a landmark technology (Hochholdinger and Baldauf, 2018). With the increasing demand for food supply globally, hybrid production will serve economic benefits in crop production and land conservation. Male sterility systems have therefore been exploited efficiently to achieve the above needs.

In self-pollinating crops such as maize, rice, cotton, sorghum and a variety of vegetables, male sterile systems aid in hybrid seed production (Eckardt, 2006). The two major kinds of male sterility are genic male sterility (GMS) caused by nuclear genes, and cytoplasmic male sterility (CMS), which is caused by the interaction between nuclear and mitochondrial genes (Chen and Liu, 2014). Cytoplasmic male sterility is governed by genes present in the mitochondria and often interact with other nuclear genes, which suppress their activity. In most cases, the CMS inducing factor is a novel open reading frame (ORF). These ORF's are chimeric and translate into unique proteins which interfere with the mitochondrial function and pollen development (Andrés et al., 2007). These ORF's are directly linked to plant mitochondrial promoter sequences or lie upstream of mitochondrial genes (Chase, 2007). A novel *Orf147* was identified from the A4 cytoplasm of pigeonpea (*Cajanus cajanifolius),* which induced partial to complete male sterility in transgenic progenies of model systems such as *Arabidopsis thaliana* and *Nicotiana tabacum* (Bhatnagar-Mathur et al., 2018). Cytotoxicity and aberrant programmed cell death induced by Orf147 protein could be important for mechanisms underlying male sterility and provides further opportunities for exploring hybrid vigour (Bhatnagar-Mathur et al., 2018). The current study attempts to transgress *Orf147* in self-pollinating related legume, *Cicer arietinum* to develop cytoplasmic male sterile transgenic lines for exploitation of heterosis. The Orf147 protein is cytotoxic, which induces CMS in the model systems (Bhatnagar-Mathur et al., 2018). This study emphasizes the use of the *Orf147* gene to generate cytoplasmic male sterile chickpea by focussing on various developmental stages of flower and pod development in chickpea. The results would be a premise for developing a robust CMS-based hybrid system in self-pollinating legumes like chickpea.

2. Materials and methods

2.1. Agrobacterium-mediated transformation of Orf147 into C. arietinum

The seeds of chickpea variety JG11 (*C. arietinum*) were obtained from the ICRISAT breeding unit, Patancheru, India and used for *Agrobacterium* mediated transformation (protocol developed by Sharma et al., 2006). JG11 has a high transformation efficiency and recalcitrancy (Sadhu et al., 2022) and is a known elite cultivar of chickpea widely studied for different aspects of crop improvement (Thudi et al., 2014). It is early maturing (95–100 days), high yielding (up to 2.5 t ha-1 in rainfed and up to 3.5 t ha-1 under irrigated conditions), having attractive large seed (22 g 100 seed-1), and high resistance to fusarium wilt (<10% mortality). The axillary meristem explants (AMEs) were used to do co-cultivation with *Agrobacterium tumefaciens*. The co-cultivation was carried out with *Agrobacterium tumefaciens* strain C58 carrying the *Orf147* gene by wounding the explant. The emerged shoots after co-cultivation were maintained by sub-culturing on fresh SIM media with low selection pressure (25 mg /L Kanamycin). The healthy shoots were subjected to higher selection pressure (125 mg/L Kanamycin) and placed on filter paper bridges immersed in root induction media with Indole-3-butyric acid (IBA) (1.02 mg/L or 5 μM) hormone. After this, the root primordial begins to appear, and the rooted shoots were placed in Arnon's solution for hardening and acclimatization in glasshouse environment. The hardened plants were transferred to glasshouse after 7–10 days (Sharma et al., 2006).

2.2. Characterisation of transgenics using PCR and qRT-PCR analysis

Inheritance of transgenes was analysed through PCR screening of T_0, T_1 and T_2 generation events. Genomic DNA isolation of 1–2 weeks old fresh leaves (100 mg) of transgenic and control events was performed using CTAB (Cetyl trimethyl ammonium bromide) method. The DNA quality was checked on 0.8% agarose gel. PCR analysis was carried out using *Orf147* specific primers **Supplementary table 1**). The PCR conditions were 94 °C initial denaturation for 5 min, followed by 94 °C denaturation for 30 s for 32 cycles, annealing at 58 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 10 min. The amplified products were then analysed on 1.2% agarose gel through electrophoresis and visualised on a UV transilluminator. The PCR-positive events were selected for qRT-PCR analysis. The total RNA was isolated from young, unopened flower buds (100 mg) of transgenic and control events using RNeasy Plant Mini Kit (Qiagen, Germany) and then cDNA synthesis was done using Thermoscript RT-PCR system (Invitrogen, USA) Kit according to the manufacturer's protocol. qRT-PCR analysis of the selected transgenic events was done using the Realplex Real-Time PCR system (Eppendorf, Germany) and SYBR Green mix (Bioline) in 96 well optical reaction plates (Axygen, USA) sealed with ultra-clear sealing film (Platemax) for 40 cycles using gene specific *Orf147* primers and expression was normalized using *GAPDH* (Glyceraldehyde 3-phosphate dehydrogenase) reference primers (Reddy et al., 2016) Supplementary table 1).. The reaction conditions were 95 °C for 2 min, followed by 40 cycles of 15 s at 95 °C and 30 s at 60 °C with fluorescent signal recording.

2.3. Statistical and phenotypic analysis

Ideally, sterility would lead to inability of the plant to form pods. Taking this into consideration, the following parameters were taken to study the phenotypic changes in T_0, T_1 and T_2 generation as compared to control plants, per day, per event for 120–140 days (4–5 months-1 generation). (i) Number of flowers developed (ii) Number of pods developed (iii) Number of flower drop (iv) Number of pods degenerated. Further, the percentage of each of these parameters was calculated to comprehend the generation-to-generation variation. The data collected for the above parameters in each generation were subjected to analysis of variance (ANOVA) where the mean values in each treatment was compared using least significant difference (LSD) at the 5 % level of significance (P = 0.05) using Genstat version 20 statistical package (VSN, 2009) (**Supplementary table 3 and 5**). The values were means of five replicates per event.

The goodness of fit of the observed segregation ratio for the transgene was tested by PCR against the Mendelian segregation ratio (3: 1) using the chi-square (χ2) test. The χ2 values were calculated using the following formula (Greenwood and Nikulin, 1996).

$$\chi\,\mathbf{2} = \sum (\text{Observed frequencies} - \text{Expected frequencies})^2 / (\text{Expected frequencies})$$

2.4. Microscopic analysis of pollen viability

The pollen from control and transgenic events flower buds was collected on a clean glass slide by gently tapping on the stamen. One drop of 2% acetocarmine solution was added to the pollen and fixed with a cover slip. The fixed pollen was observed under an Olympus Cx41 upright microscope (Olympus Corporation, Japan) driven by the Q-Capture Pro 7 (Version: 7.0.30 Build 4257, 32-bit) software. Images were recorded at 10x, 20x and 40x magnification.

3. Results

3.1. Generating chickpea transgenics and molecular analysis

Putative chickpea transgenics were generated through

Agrobacterium-mediated transformation **(**Fig. 1**)** using the fused gene construct *Orf147-CoxIV* driven by *AtAP3* promoter cloned into pMDC100 plant expression vector followed by mobilization into *Agrobacterium* strain C58, for the expression of cytotoxic *Orf147* conferring sterility in the subsequent transgenics. A total of 6 kanamycin-resistant T_0 transgenic chickpea plants were developed which were further taken up for molecular characterization. Genomic DNA isolations were carried of the same number of putative transgenic plants **(**Supplementary Fig. 1**, A and B)**. Out of the 6, 5 were found to be PCR positive and advanced for T_1 generation **(**Supplementary Fig. 1**, C).** Further, in the T_1 generation, out of the total 21 progenies derived from 5 transgenic events, 15 were found to be PCR positive and advanced for T_2 generation **(**Supplementary Fig. 1**, D)**. Finally, in the T_2 generation, 40 progenies were generated out of which 14 were found to be PCR positive. The PCR positive transgenics were then analysed for significance of event using statistical analysis.

3.2. Phenotypic analysis implies towards partial sterility of Orf147 transgenic chickpea

The vegetative growth of the transgenic and wild type (WT) events was found to be uniform throughout T_0, T_1 and T_2 generation, however reproductive characteristics, such as flower drop and number of pods formed, varies in transgenic and control events (Fig. 2).

3.2.1. T_0 Generation

A total of 6 events were present in the T_0 generation out of which 5 PCR positives (Events: 1, 2, 3, 4 & 5) were obtained. Due to lack of replicates in this generation, statistical analysis is not possible, however, a basic interpretation can be done by taking into account number of pods formed and number of flower drops observed. In the first two events (1 and 2), the percentage of flower drops (41.94 and 40.43% respectively) is greater than that of the control (10.87%), resulting in low percentage of pod formed (54.84 and 48.94% respectively) compared to the control (63.04%). The rest of the 4 events do not show significant differences in the context of pod formation and flower drop in comparison to control (**Supplementary Table 2**).

3.2.2. T_1 Generation

In the T_1 generation, out of the 21 progenies derived from 5 transgenic events, 15 were found to be PCR positive. These were further taken up for data analysis. Among total 5 events, only events 1 (1–1, 1–3, 1–4, 1–5, 1–6, 1–7), 2 (2–1, 2–2) and 3 (3–1, 3–2, 3–3, 3–5, 3–6) were analysed as 4 (4–1) & 5 (5–2) did not have replicates (**Supplementary Table 3**). For all the events, the percentage of flower drops (range: 31.7–45.6%) was significantly higher than the control (6.9%) resulting in lower percentage of pods formed (range: 41.1–52.6%) compared to the control (70.9%) (**Supplementary table 4**). This may imply towards partial sterility.

3.2.3. T_2 Generation

In the T_2 generation, out of the 40 progenies derived from 5 transgenic events, 14 were found to be PCR positive from 4 events, 1 (1–1–1, 1–3-1, 1–4-2, 1–5-1, 1–5–2), 2 (2–1–1, 2–1-2, 2–2–2), 3 (3–5-1, 3–5–2) and 5 (5–2–1, 5–2–2, 5–3-1, 5–4-2) (**Supplementary table 5**) which were then taken up for data analysis. Although, all the 4 events showed significant increase in percentage of flower drops (range: 31.2–45.5%) in comparison to the control (9.8%), only event no. 2 and 5 showed significant decrease in percentage of pod formed (47.4 and 44.3% respectively) in comparison to the control (66.8%). In this generation, the number of flowers developed is comparable and has no difference unlike the previous generation. Event 1 is demonstrating significant difference in number of pod formed (31.4) in comparison to the control (24.5) (**Supplementary table 6**).

However, it is also observed that the number of flowers developed in the transgenic events in T_1 and T_2 generations is greater than that of the control (**Supplementary Table 4 and 6).** In all 3 generations, there is no significant difference observed in percentage of degenerated pods and number of degenerated pods.

The number of individuals screened was much greater in T_2, in which sensitive phenotype was observed. In a few of the events, 50% or > 50% of flower drop was observed which could be correlated to partial sterility conferred due to the cytotoxic nature of *Orf147*. Further, the events were compared with the percentage of pod formation. A higher percentage of flower drop should lead to a lower percentage of pod formation if the

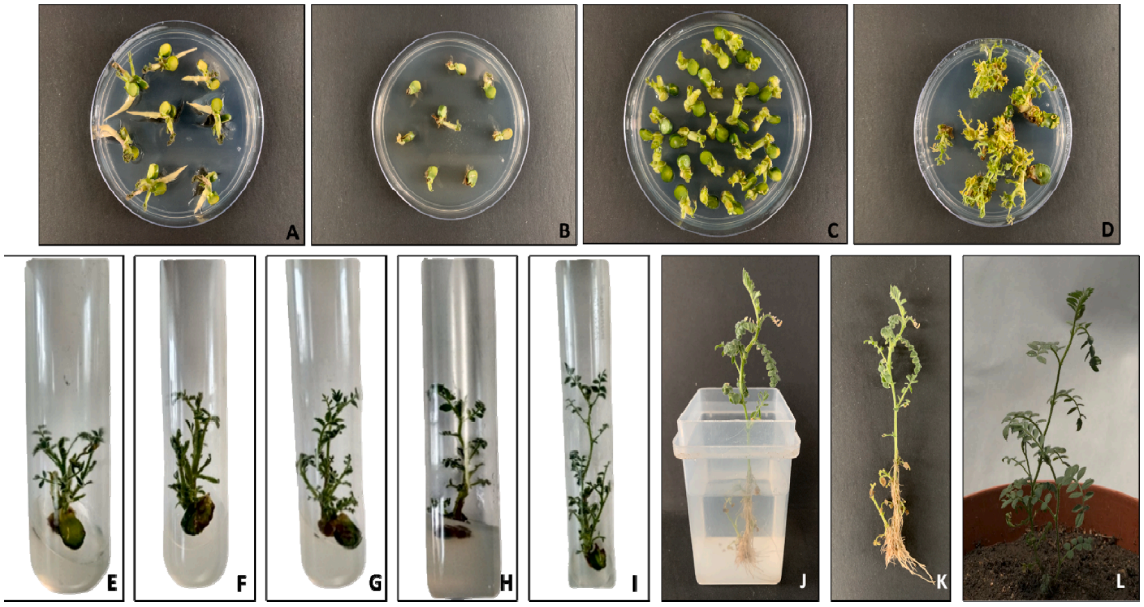

Fig. 1. Development of transgenic chickpea plants via *Agrobacterium*-mediated transformation derived from *in to vitro* germinated chickpea seeds. A) Seedling explant 1 week post germination; B) Axillary meristem explant; C) Post infection with *Agrobacterium* culture showing initiation of shoot buds; D) Infected explants 1 week post infection showing shoot elongation; E) Explants on selection media (BK media + Kanamycin 25 mg/l; F) Selection media (50 mg/l); G) Selection media (75 mg/l); H) Selection media(100 mg/l); I) Selection media (125 mg/l); J) Plant placed in Arnon's solution for root hardening; K) Post hardening showing root system; L) Transplanted chickpea plant growing in glasshouse conditions.

Fig. 2. A) Phenotypic observation showed difference in conversion of flower to pod and pod formation 1: Control, 2: transgenic plants showing visibly lesser pod potentially due to enhanced flower drop. The flower drop was due to the cytotoxic effects of *Orf147* which causes premature tapetal programmed cell death. A semi-sterile phenotype has been observed resulting in poor pod formation and seed set. **B) Depiction of potential flower drop against healthy flower.** While flower drop and eventual inability to form pod has been known to occur due to various reasons, such as high temperature and drought stress, the high expression of *Orf147* transcript in transgenic events, under controlled conditions, was found to induce the high number of flower drop.

causative agent was *Orf147*. In T_1 generation, pod formation of 60–80% was observed in the control wild type plants.

3.3. Segregation analysis

Chi-square test demonstrated that events 1, 2, 3 and 4 in T_1 generation and events 2 and 5 in T_2 generation follow the 3:1 Mendelian ratio of segregation (Table 1).

3.4. Microscopic analysis provides evidence of partial sterility

Acetocarmine staining of pollen in transgenic event showed pollen population containing fully stained viable as well as significant unstained, non-viable pollen. In the control event, however, completely stained viable pollen was observed (Fig. 3**).** This implies towards the partial sterility of transgenic event due to the presence of both viable and non-viable pollen.

3.5. Chickpea transgenics showed elevated expression of Orf147

Previous experiments based on phenotypic data conclusively implied towards the partial development of cytoplasmic male sterility in *Cicer arietinum*. According to the statistical analysis of the transgenic events pertaining to T_1 and T_2 generations, it was observed that event number 1,2,3 and 4 showed significance in T_1 generation while events 2 and 5 demonstrated significant decrease in pod formation along with increased number of flower drops as compared to the control. This could be indicative of partial-sterility induction in the transgenic events.

In order to validate the data set, expression profiles of the transgenic events were evaluated. This was done by carrying out qRT-PCR studies to examine the levels of expression of *Orf147* sterility causing genes in the flower buds of events which were PCR positive along with the events which showed significant results in the statistical analysis. The expression analysis of 26 transgenic events across T_1 and T_2 showed some visibly significant results as compared to the control **(**Fig. 4**).** In T_1 generation, 1–1, 1–3, 1–4 and 2–1 have shown high levels of expression of *Orf147*. All of the PCR positive events showed comparatively high *Orf147* expression as compared to the control. Further in the T_2 generation, 1–1–1, 1–5-2, 2–1–1, 2–2–2 and 5–3-1 have shown relatively higher levels of expression from each event as compared to the control. These events are in correspondence to the phenotypic data analysis which showed that events 2 and 5 in T_2 generation exhibited chi-square values which followed the Mendelian ratio of segregation.

The statistical and phenotypic data in corroboration with expression profiles of the transgenic events, provides basis for our study, wherein, we are trying to induce cytoplasmic male sterility in legume systems.

Table 1
Chi-square analysis of T_1 and T_2 events.

Generation	Event#	No. of PCR positive plants/	No. of plants used in the test	Chi square value
T1	1	7	6	0.43*
	2	3	2	0.11*
	3	6	5	0.22*
	4	1	1	0.33*
	5	4	1	5.33
T2	1	14	5	11.52
	2	6	3	2.00*
	3	10	2	16.13
	4	2	0	6.00
	5	8	4	2.67*

* χ^2 value at 0.05% probability at 1 df is 3.84. Calculated values below 3.84 were non-significant, and the samples fit for 3: 1 segregation ratio.
*Values following Mendelian ratio of segregation.

4. Discussion

Male sterility in chickpea has been reported previously, with the discovery of a monogenic recessive gene, and its induction has been carried out through gametocide. However, the efficiency in such a mechanism for achieving male sterility is tedious in chickpea as a pollen dispersal system is a requisite. Since chickpea is a self-pollinating legume with a cleistogamous flower, induction of male sterility is a problem (Ahmad et al., 2005). The use of CMS system in chickpea has not been demonstrated yet, and alternatives, such as apomixis, for achieving heterosis has been suggested. However, there are no reports of commercial cultivation of hybrid chickpea. Heterosis is central in increasing yield and quality of a crop, however, in self-pollinating legumes like chickpea, the biological infeasibility poses a crucial problem in its exploitation (Yamini et al., 2015). The current study provides important insights into the possibility of development of cytoplasmic male sterile line in chickpea for hybrid production.

The identification of *Orf147* in pigeonpea laid the foundation for our study wherein introgression of the gene into chickpea was done. According to the previous study, *Orf147* induced complete CMS in model systems *viz, A. thaliana* and *N. tabacum* by the T_2 generation. In the T_1 generation, partial sterility was observed wherein the quantity of seed set was lower in the transgenics compared to the control (Bhatnagar-Mathur et al., 2018). An extrapolation of this study was done in related

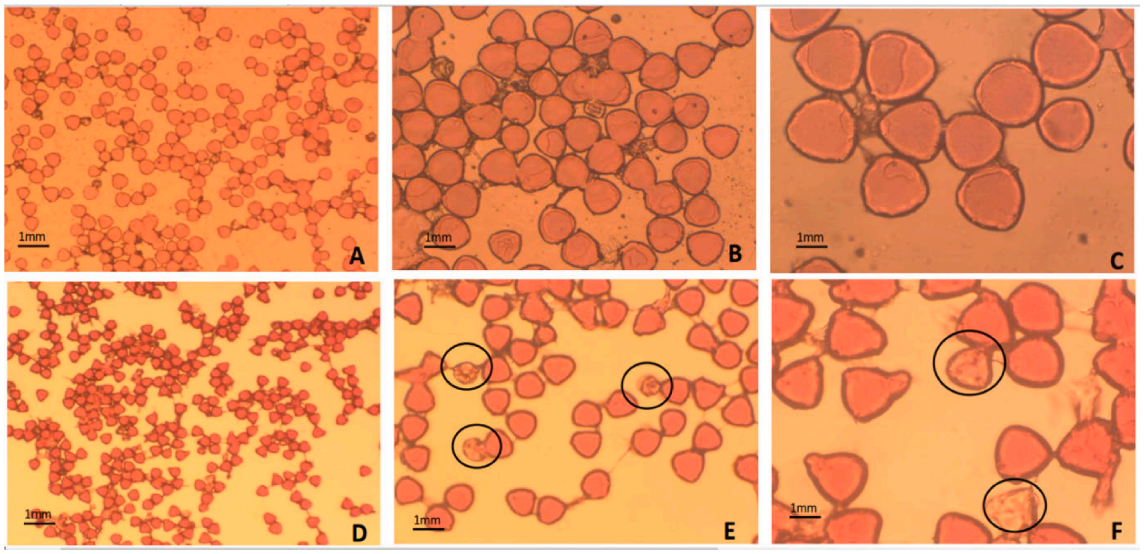

Fig. 3. Microscopic analysis of pollen viability through acetocarmine staining A, B, C: Control, fully stained pollen in 10x, 20x and 40x magnification. D,E,F: Transgenic, showing fully stained and non-stained pollen in 10x, 20x and 40x magnification. The presence of non-viable pollen in the pollen population of transgenic events implies towards induction of partial sterility due to cytotoxic *Orf147*.

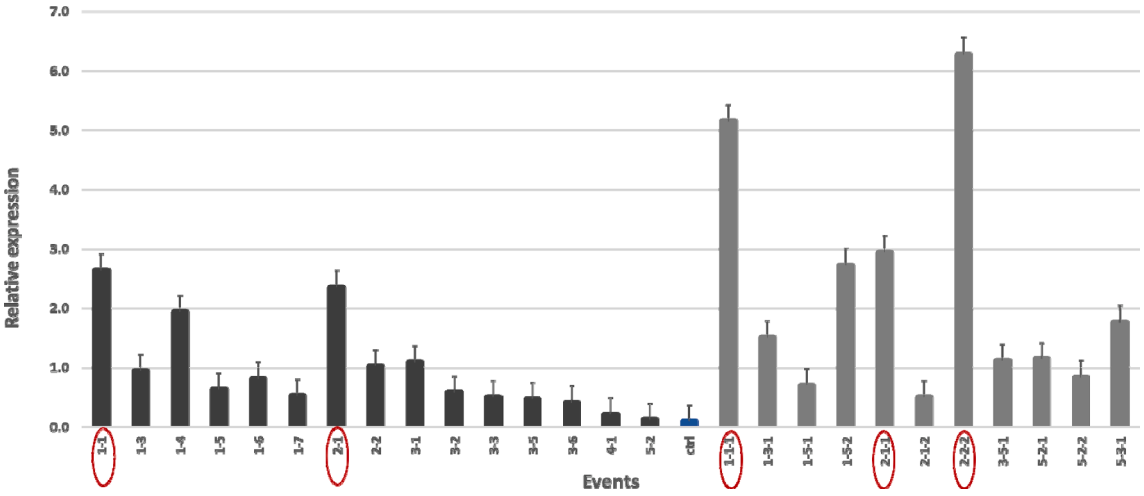

Fig. 4. qRT-PCR profiles of picked chickpea PCR positive transgenics showing various expression levels of *Orf147*. The red circled events are showing highest expression of *Orf147* as compared to the control events. According to the Chi-square analysis, events 1,2,3 and 4 show significance in T_1 generation while events 2 and 5 follows Mendelian ratio of segregation in T_2 generation. All of the PCR positive events demonstrate significantly higher expression as compared to control. Events 2 and 5 of T_2 generation are also showing high expression of *Orf147* transcript which corroborates with the phenotypic data. The enhanced levels of the transcript contribute towards cytotoxic effect of the peptide which eventually hinders seed set as observed in the transgenic events. The dark grey bars denote the T_1 events and light grey bars denote T_2 events while blue bar represents the control event. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

legume, chickpea. The transformants of chickpea were analysed until T_2 generation using PCR which demonstrated the successful transfer of *Orf147* into chickpea. In each generation, the phenotypic traits were observed such a flower development, pod formation and flower drop. Since *Orf147* produces a cytotoxic peptide and is known to inhibit seed set and cause aberrant floral development; flower drop, which is the inability of the flower to convert into a pod, was taken into account. Since the experiments have been carried out in controlled environments, the effect of aberrant temperature and drought conditions can be ruled out as these factors are known to have an impact on chickpea sterility and flower abortion (Kiran et al., 2019).

Phenotypic characteristics such as number of flowers developed, pod conversion and flower drop were assessed in individual PCR positive plants for 120–140 days in each generation in order to determine the phenotypic variations across generations. Complete CMS was not observed in any of the plants across the generations. However, chi square analysis implied towards Mendelian segregation in both the generations and also exhibited significance in two events in T_2 generation and 4 events in T_1 generation. In order to further comprehend the effect of *Orf147* in the transgenic events, the expression levels of the transcript were studied using qRT-PCR analysis. Interestingly, the expression profile showed high levels of *Orf147* expression in these transgenic plants confirming the phenotypic data. In addition to this, pollen viability test was carried out using acetocarmine staining to determine the viable and non-viable pollen. Microscopic analysis demonstrated the presence of both viable and non-viable pollen population in the transgenic events as compared to the control which had distinct viable pollen population.

Since complete CMS was seen in model systems under the exact circumstances, one of the contributing factors which might vary in the

two situations is the promoter. In *Cicer arietinum*, the *Orf147* gene from *Cajanus cajanifolius* in association with signal peptide, coxIV and *Arabidopsis* tapetum-specific promoter, *AtAP3* has been utilized for generation of male sterile lines. While the promoter was efficient in development of male sterile lines in *A. thaliana*, in *C. arietinum* it gave rise to only partially sterile plants. The transformants were observed to give rise to fewer pods as well as degenerate pods with no seeds. This phenomenon with varying levels of male sterility has also been observed in tobacco, wherein the *CaMV35s* promoter fused with sterility conferring glucanase gene showed lowered levels of sterility and also observed fertile phenotypes. Additionally, transgenic plants expressing *glucanase* gene driven by *Arabidopsis* tapetum-specific promoters (*A3* and *A9*) displayed a range of male sterility, exhibited partial sterility with reduced number of pods which were either empty or contained fewer seeds as compared to the wild type. The previous and present studies shown that, male sterility can be acquired in transgenic line only after high levels of accumulation of the concerned protein or at a time in development appropriate to confer male sterility. The specificity of promoter is crucial for development of fully male sterile plants (Worrall et al., 1992). In self-pollinating legumes, therefore, the production of new hybrid cultivars is dependent on efficacy of promoters used. *Pisum sativum ENDOTHECIUM 1* (*PsEND1*), is an anther-specific promoter expressing in the endothecia of pea anther primordium cells, along with *barnase* gene generates male sterile plants (Roque et al., 2019) Further, due to its specific temporal and spatial expression in anther and not in other floral and vegetative organs, they form male sterile plants. While *PsEND1* promoter proved to be efficient in a wide range of plants like *Arabidopsis*, tobacco, oilseed rape as well as tomato, its efficiency was also seen in distantly related crop like *B. napus*. Engineered male sterility using *PsEND1* can help in development of GM crops especially in legumes wherein hybrid vigour is still being continuously explored (Roque et al., 2019). Similarly, various tapetum/anther-specific promoters have been identified from the tapetal cells of tobacco (*Ta29*) (Mariani et al., 1990)i, tomato (*LAT52*) (Twell et al., 1990) Brassica (*BoA3* and *BoA9*) (Konagaya et al., 2008), rice (*Osg1*, *pTAP & OsGEN-L*) (Rao et al., 2018) and utilized for development of male-sterile lines in different crop species. Additionally, in spite of large number of identified promoters in rice, their effectiveness has not been confirmed in other crops (Rao et al. 2018).

In addition to this, the partial sterility observed in *Orf147* transgenic chickpea could be due to the heterozygous nature of plants in the T_2 generation. Introduction of a maintainer line would facilitate maintenance of a homozygous plant, and possibly, in imparting complete sterility to the transgenic chickpea.

5. Conclusion

Targeted engineering of plant genes for achieving heterosis and eventually food security is an effective and efficient approach. However, comprehending the impact of *cis*-regulatory elements, in the promoter region, on introduced genes is extremely important in order to achieve the desired trait. The use of tissue-specific promoters in this case led to induction of cytotoxicity of *Orf147* in chickpea to a certain extent but was unable to induce the complete CMS. Aspects of the study has duly suggested that the *Orf147* system holds great potential in legumes systems. Since successful transformants were obtained having high levels of *Orf147* expression and non-viable pollen has been observed, further studies can be carried out using species-specific promoter for analysing the effects on chickpea. In addition to this, promoter of related legumes such as soybean can also be utilized. Further, crossing experiments with maintainer line to study possibility of complete sterility can also be done.

CRediT authorship contribution statement

Joorie Bhattacharya: Investigation, Formal analysis, Data curation, Methodology, Validation, Writing – original draft. **Dumbala Srinivas Reddy:** Supervision, Data curation, Methodology, Writing – review & editing. **Kalyani Prasad:** Methodology, Formal analysis, Writing – review & editing. **Rahul B. Nitnavare:** Investigation, Formal analysis, Writing – review & editing. **Pooja Bhatnagar-Mathur:** Conceptualization, Supervision, Funding acquisition. **Palakolanu Sudhakar Reddy:** Supervision, Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

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