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Development of gene constructs for induction of male sterility and restoration of fertility in safflower

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Abstract
The present work aims at developing plant transformation vectors suitable for induction of male sterility and fertility restoration in safflower. Three genes *i.e*., a mitochondrial, male sterility inducing gene *orf*H522 from PET1 CMS line of sunflower and unedited version of mitochondrial genes, *atp9* (*u-atp9*) and *nad*3(*u-nad3*) from safflower have been cloned under TA29 promoter, in-frame with the *cox*IV pre-sequence, so that the resultant cytoplasmic proteins (ORFH522, u-ATP9 and u-NAD3) would be targeted into mitochondria. *bar* gene that confers resistance against the herbicide basta (phosphinothricin) has been used as selectable marker. All cassettes have been cloned in pCAMBIA0390. To silence the sterility inducing transgenes for restoring the male fertility three different PTGS strategies, that include i) cloning full length antisense version, ii) cloning inverted repeat of complete/selected part of the genes separated by a intron and iii) cloning antisense version of the selected part of *orfH522* upstream of the inverted repeat of a heterologous 3'-UTR separated by an intron have been employed. These gene cassettes have been cloned in pCAMBIA1300. All the developed binary vectors have been mobilized into *Agrobacterium* strain LBA 4404.

Key words: Transgenic male sterility - ablation – PTGS - orfH522 - nad3 - atp9

Introduction

Genetic engineering offers a precise way of manipulating plants to tailor specific traits. The large numbers of transgenics that are being developed bear a testimony to the potential of this new technology. Literature is replete with examples of utilizing this technique for inducing male sterility in plants. In most of these cases, sterility is induced by ablating the tapetal cell layer, a sporogenous tissue that nurtures the pollen mother cells, Tapetal cell layer is essential for normal microsporogenesis as these cells supply the required nutrient for the pollen mother cells in the anther locules. In many male sterility systems, the disruption of differentiation /function of the tapetal layer is the causative factor for non-production of the functional pollen grains. Therefore the tapetal cells have been the targets to produce engineered male sterile plants. With this rationale transgenic male sterile lines were developed in which ablation has been induced in the tapetal cell layer by selectively expressing the cytotoxic genes in tapetal cells using specific promoters. In the first successful example of such kind, a ribonuclease gene from *Bacillus amyloliquifaciens* (*barnase*) was cloned under a tapetum specific promoter TA29. When this gene cassette was introduced into tobacco, rapeseed, lettuce, tomato, cotton, corn, cauliflower and chicory, in each crop the transgenics were male sterile (Mariani *et al.,* 1990, 1992). Subsequently, expression of a ribonuclease inhibitor gene (*barstar*) from *Bacillus amyloliquifaciens* in tapetal cells eliminated the ribonuclease activity and restored fertility in the male sterile lines (Mariani *et al.,* 1992). Using similar rationale and strategies several workers have described the production of transgenic male sterile plants by expression of specific genes, which will disrupt the microsporogenesis (reviewed by Banga *et al*., 2006). In most of these cases the restoration also has been achieved by selectively blocking the expression of the gene causing male sterility (Mariani *et al.,* 1992: Kriete *et al.,* 1996: Schumulling *et al.,* 1993).

Materials and Methods

The present work aimed at developing plant transformation vectors suitable for induction of male sterility and fertility restoration in safflower. Initially all the component sequences (like TA29 promoter, *coxIV* pre-sequence, *orf*H522, u-*atp9* and u-*nad3* and *nos* terminator were amplified using suitable primers) were amplified from respective sources and were cloned in T/A

cloning vector InsT/A (Fermentas, Germany). Later these sequences were cloned in either pUC18 or pRT100 plasmids in proper orientation. The clone (pTON : pUC18 with TA29 promoter-*orfH522-nos* terminator) would serve as a control gene cassette for expressing *orf*H522 without mitochondrial targeting of the protein. The *coxIV* pre-sequence was cloned in between TA29 promoter and *orf*H522 in pTON to realize the complete gene cassettes (pTCON: pUC18 with TA29 promoter-*coxIV* presequence-*orf*H522-*nos* terminator). In these vectors *orf*H522 gene was replaced with the unedited versions of the safflower mitochondrial genes nad3 and atp9 to obtain pTCAN and pTCNN vectors respectively. All the vectors were confirmed by PCR, restriction analysis and DNA sequencing (automated). These gene cassettes were cloned into pCAMBIA0390 vector that lacks plant selection marker so that the appropriate selection gene cassette could be cloned into it subsequently. To enable selection of transgenic plants *in vitro* as well as in the field, bar gene cassette that confers tolerance to the herbicide phosphinothricin (commercial formulation, basta) was cloned in these vectors (Fig.1). To address the effects of constitutive expression of *orf*H522 *v/s* the tapetum specific expression, the gene was cloned under CaMV 35S promoter in pRT100 vector which has CaMV 35S promoter and CaMV poly A signal.

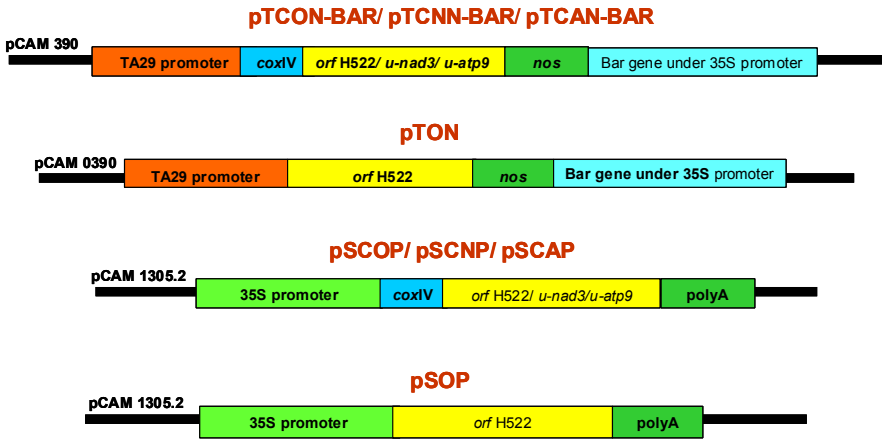

Fig.1. Diagramatic representation of the cassettes developed for induction of male sterility

Restoration of fertility in the male sterile transgenic plants could be achieved by suppressing the expression of the male sterility inducing gene(s). With this basic premise, in our male sterile transgenic plants the restoration could be achieved by silencing the expression of the introduced gene – *orf*H522/*nad3*/*atp9*. For this purpose, the target genes were cloned in the post transcriptional gene silencing (PTGS) vectors that were developed in our laboratory.

Intron interrupted hairpin RNA (ihpRNA) vectors: In this vector, catalase intron sequence has been cloned in the MCS of pRT100. A fragment of the target gene to be silenced was cloned in both antisense and sense orientation upstream and downstream of the catalase intron so that upon transcription from the 35S (constitutive) promoter, the transcript that is produced will have a stem loop structure**.**

SHUTR (Silencing by heterologous 3' untranslated region) vectors: This type of gene silencing called silencing by heterologous 3' untranslated regions (SHUTR) originally proposed by Brummell et al., 2003, has the advantage of ease and rapidity in preparation of the constructs, since a gene of interest can be inserted into a binary vector already containing the promoter and the inverted repeat of the 3' UTR, in a single cloning step, and doesn't require any knowledge of the DNA sequence.

Antisense vectors: Full length genes were cloned in antisense orientation in pRT100 background.

In all the fertility restoration vectors, 35S promoter was replaced with TA29 promoter to ensure better silencing signals in the tapetal cell layer. The confirmed gene cassettes were cloned in binary vector pCAMBIA1300. All the developed male sterile and fertile constructs were mobilized into *Agrobacterium* strain LBA 4404 using freeze thaw method. These cultures were used for transforming tobacco as well as safflower explants. The results obtained with orfH522 constructs only have been reported here. Safflower hypocotyl explants were transformed using an in-house standardized protocol that utilizes thiadiazuron and NAA for induction of the shoots (Rao *et al*. 2007).

Results

The constructs for induction of male sterility were developed as outlined in the materials and methods and they were all mobilized into *Agrobacterium* stain (Harinath, 2004). Similarly, all the strains for fertility restoration were constructed and mobilized into Agrobacterium strain (Reddy 2004). These confirmed strains were used for transformation of both tobacco and safflower.

Putative transgenic shoots were confirmed for the presence of the transgene and they were transferred to the soil. At the time of flowering, the plants were studied for the pollen production. 7/19 tobacco plants carrying the TCON construct were completely sterile while 1/12 transgenic safflower was sterile. These plants did not produce any pollen grains. When the anthers of tobacco plants were analysed using bright field microscopy (Fig 2), it indicated that the tapetal cell layer was ablated completely by the time the anthers reached stage 1 of development. This demonstrated that the ORFH522 resulted in ablation of the tapetal cell layer. The anthers in the sterile plants were recessed below the level of stigma (Fig 3).The sterile tobacco plants were crossed with transgenic plants carrying the restorer gene cassettes. The progeny obtained were all fertile indicating that the post transcriptional gene silencing of the sterility inducing gene led to restoration of fertility (data not shown).

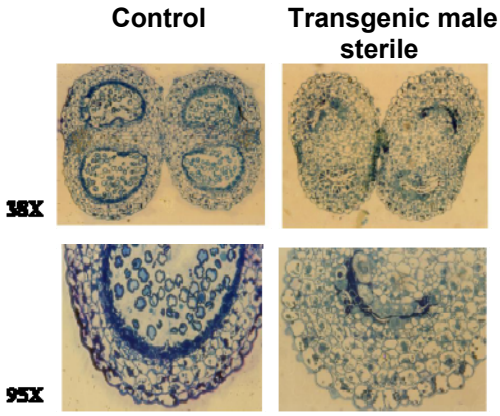

Fig.2. Bright field microscopy of Cross-section of tobacco anthers at stage 1 of flower development

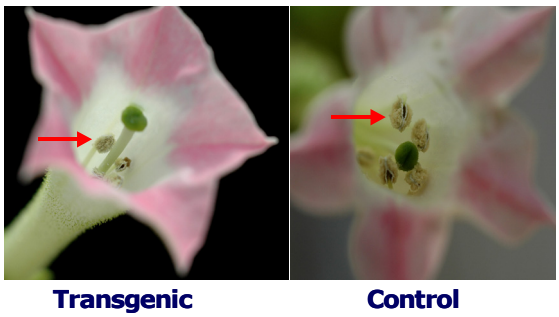

Fig.3. Morphology of the transgenic tobacco flower. Male sterile plants having anthers below the surface of stigma

Discussion and conclusion

The project aims at developing a pollination control system in safflower through the transgenic approach (es) to exploit the substantial heterosis reported in the crop. It was envisaged that the male sterility could be induced by ablating the tapetal cell layer in the anther and fertility in such transgenic lines could be achieved by down regulating the genes used for induction of male sterility. To ablate the tapetum, two strategies were followed: the first aimed at expressing the *orf*H522 gene from sunflower in the tapetal cell layer and the second aimed at expressing the unedited versions of the mitochondrial genes from safflower that undergo extensive RNA editing. In both the strategies the resultant proteins were to be targeted into mitochondria using the signal peptide from yeast. Thus, the vectors for induction of male sterility were to be

constructed by using the tapetum specific promoter TA29, *coxIV* pre-sequence from yeast, *orf*H522/unedited version of mt genes from safflower and the transcription termination signal. This work was on the premise that the promoter TA29 from tobacco as well as the *coxIV* pre-sequence from yeast would work exactly in the same pattern in safflower as has been reported in other crops. To prove this hypothesis, we undertook a preliminary study to establish the tissue specific expression pattern of TA29 promoter and the ability of *coxIV* pre-sequence to target the protein into mitochondria in safflower. To achieve this, suitable constructs were developed and tested in safflower (data not reported) and the results clearly demonstrated that TA29 promoter expressed only in tapetal tissue of safflower and also that COXIV presequence targeted the in-frame protein into mitochondria in safflower. As no information was available on the mitochondrial genes in safflower that undergo extensive editing, two genes, *atp9* and *nad3* were studied to assess the editing status. However, the analysis with safflower *nad3* and *atp9* transcripts indicated extensive editing in both the transcripts (data not reported). Therefore, for the construction of male sterility induction vectors, unedited versions of *nad3* and *atp9* genes were used. Thus male sterility induction vectors have been developed with *orf*H522, *u-nad3* and *u-atp9* genes. To enable the selection of transgenic plants carrying sterility inducing gene, both in – vitro and in the field, bar gene cassette conferring phosphinothricin/ basta resistance was cloned into all the constructs. For fertility restoration vectors, post transcriptional gene silencing approaches have been followed to down regulate the sterility inducing genes. Vectors that could produce intron-interrupted hairpin RNA, silencing by heterologous 3'UTR region and the antisense RNA molecules against the target male sterility inducing gene(s) were constructed. The developed constructs were mobilized into *Agrobacterium tumefaciens* strain LBA4404 and used for transforming tobacco and safflower.

Analysis of the transgenic plants at the molecular and phenotypic level indicated that the expression of ORFH522 in the tapetal cell layer led to male sterility in tobacco. Suppression of this through PTGS approaches restored fertility in the progeny. Thus this report demonstrated that ORFH522 leads to programmed cell death in heterologous systems and also established that this gene alone is enough to induce male sterility. Initial results obtained in safflower with these vectors are encouraging and more number of transgenics are now being raised.

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