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ABSTRACT

The present investigation was carried out with the aim of developing constructs and using them for the development of transgenic male sterile and restorer lines in safflower and tobacco. The premise for the research project was that if orfH522, the mitochondrial gene known to cause male sterility in PET1-CMS line of sunflower, is nuclear expressed in the tapetal cell layer and the resultant protein ORFH522 is targeted into mitochondria in a heterologous system, it should lead to male sterility providing an empirical proof that ORFH522 is sufficient to induce male sterility.

In the first phase, efficient plant regeneration and genetic transformation protocols were established in safflower. Among the several media tried, for establishing regeneration system, high shoot induction response from seedling explants was obtained on MS medium supplemented with TDZ @ 1.0 mg l\(^{-1}\) + NAA @ 0.2 mg l\(^{-1}\) followed by that on TDZ @ 0.5 mg l\(^{-1}\) + NAA @ 0.2 mg l\(^{-1}\). Shoots emerged as clusters within 2 weeks. Root explants gave maximum regeneration frequency on MS medium with TDZ @ 0.5 mg l\(^{-1}\) + NAA @ 0.2 mg l\(^{-1}\) while the hypocotyls gave the maximum frequency with TDZ @ 1.0 mg l\(^{-1}\) + NAA @ 0.2 mg l\(^{-1}\). Rooting of the obtained shoots was obtained on MS medium supplemented with NAA @ 0.5 mg l\(^{-1}\) followed by phloroglucinol @ 1 mg l\(^{-1}\) + IBA @ 2 mg l\(^{-1}\). Agrobacterium-mediated transformation using constructs harboring reporter (gus) and selectable marker (hpt) genes was also optimized for safflower. Among different explants tried, hypocotyls gave higher transformation frequency (21.7% to 37.5%) followed by roots (13.8% to 22.2%) at the bacterial concentration of 0.1 O.D. Vacuum infiltration of the explants with the bacterial culture was better compared to shaking the explants with the culture for 30 minutes. Kill curve analysis indicated that 10 mg l\(^{-1}\) of hygromycin and 1.0 mg l\(^{-1}\) of phosphinotricin were effective concentrations for selecting the transformed shoots. The hypocotyl explants showed shoot emergence
within three weeks after co-cultivation and the shoots were elongated on selection medium supplemented with BA @ 0.5 mg l⁻¹ in the next 30-40 days. The elongated shoots of 4-5 cm length were rooted on the rooting medium. Molecular analysis (i.e., PCR analysis) to confirm the presence of transferred genes was carried out with gene specific primers. This analysis proved that the putative transgenic shoots carried the introduced genes.

In the next phase, constructs developed previously at DOR using the orfH1522, for inducing transgenic male sterility and for restoring fertility were suitably modified and utilized in the present study. Towards the former, two constructs were developed, orfH522 gene was cloned under tapetum specific (TA29) promoter either with or without the mitochondrial target sequence encoding the first 25 amino acids of COXIV pre-sequence from yeast in the binary vector pCAMBIA0390. The bar gene cassette, that confers resistance to the herbicide phosphinotricin, was cloned into these vectors resulting in two plant transformation vectors pBin-TCONBAR and pBin-TONBAR. Vectors for restoration of fertility based on post-transcriptional gene silencing (PTGS) approach were developed such that they could down-regulate the expression of orfH522 gene thereby restoring fertility. Three strategies of PTGS, viz., antisense RNA, intron-interrupted hairpin RNA (ihp-RNA) and silencing by heterologous 3'untranslated region (SHUTR), were adopted for developing the gene constructs. All the three types of vectors either under 35S promoter (porfAP, porfihp and porfSHUTR) or under TA29 promoter (porfTAP, porfTihp and porfTSHUTR), were developed. Thus, a set of six gene constructs was developed for fertility restoration and these cassettes were cloned into the pCAMBIA1300 binary vector. All the confirmed binary vectors for both sterility induction and fertility restoration were mobilized into Agrobacterium strain LBA4404.

The two constructs for induction of male sterility viz., LBA4404: pBin-TCONBAR and LBA4404: pBin-TONBAR as well as four constructs for
restoration of fertility *viz.*, LBA4404: pBin-orfihp, LBA4404: pBin-orfSHUTR, LBA4404: pBin-orfTihp and LBA4404: pBin-orfTSHUTR were used to develop transgenic safflower and tobacco plants. Transgenic plants obtained were confirmed for the presence of the transgene by PCR and Southern analysis, for transgene expression by RT-PCR, for induced programmed cell death by DNA fragmentation, ablation of tapetal cell layer by histology studies and sensitivity test to phosphinothricin or hygromycin (depending on the vector used) by leaf assays. The plants and their flowers were observed for the presence of any morphological differences as well as for pollen fertility. Tissue culture derived shoots of safflower were lanky and they did not produce many flowers indicating the inherent problems of *de novo* regenerated shoots. Among hundreds of plants transferred to pots only 13 plants survived till flowering. Among them, one plant was sterile as it produced no pollen grains and also, there were three plants, which produced less than 10% pollen compared to the control plants, and were designated partial steriles.

Among several independent tobacco transformants, obtained with each of the gene cassettes, one third of the transgenics (6/17) with TCONBAR were completely male sterile while more than 10 independent transformants obtained with TONBAR construct were all fertile indicating that only when ORFl1522 was targeted into mitochondria it led to male sterility. The male sterile plants were morphologically similar to fertile plants except that anthers remained below the stigmatic surface at anthesis. RT-PCR analysis of the sterile plants confirmed the anther specific expression of *orf*I1522 and bright field microscopy demonstrated the ablation of tapetal cell layer. Premature programmed cell death was confirmed by DNA fragmentation and was observed at meiotic stage in the anthers of sterile plants. The stable inheritance of induced male sterility trait was confirmed in test cross progeny.

Transgenic tobacco plants obtained with the restorer constructs were analyzed to select the ones that carried single copy insert. Such plants
were selfed and in the progeny the homozygous plants were selected and crossed with the sterile plants to study the efficacy of the restorer constructs in restoring the fertility.

Hybrid plants were analyzed for the restoration of fertility by observing for the pollen production, pollen staining and pollen germination. All the plants were fertile and produced pollen grains indicating the restoration of fertility. Molecular analysis clearly proved that the plants segregated for the sterility cassette in the expected 1:1 ratio while all the plants carried the restorer cassette. When such hybrid plants were analyzed for the presence of orfH522 transcripts using RT-PCR, the transcripts were detectable in the plants restored using SHUTR vector while there was no detectable transcript in the plants carrying ihp-RNA silencing vector. This indicated that intron-interrupted hairpin RNA (ihp-RNA) mediated gene silencing was more effective compared to silencing by heterologous 3'UTR (SHUTR).

The present investigation has provided, for the first time, the proof-of-concept for the utility of orfH522 for induction of male sterility in heterologous systems. However, in case of safflower due to problems of acclimatization of transgenic shoots, many plants could not be advanced to flowering stage and therefore the utility of the developed constructs could not be assessed thoroughly in safflower. As a future line of work, more number of transgenic safflower plants needs to be produced and analyzed for each of the constructs to confirm the results obtained as well as to validate the utility of the constructs with higher confidence limits. The stability of the transgene(s) and their co-segregation with the intended phenotype will have to be established using both genetic and molecular analyses. These aspects shall form the future line of work.

This could lead to development of a complete pollination control system, not only in safflower, but also in other crops.