Down Regulation of Receptor like Kinase Gene in Apomictic *Cenchrus ciliaris*

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Down regulation of receptor like kinase gene in apomictic *Cenchrus ciliaris*

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Introduction

Apomixis is a potential technology for agriculture to fix heterosis or hybrid vigor in hybrid plants. *Cenchrus ciliaris* which is a popular forage crop reproduces predominantly through apomixis and has been used as a model plant to understand genetic and molecular mechanisms controlling apomixis. A partial receptor like kinase gene (*CcRLK*) was isolated from apomictic *C. ciliaris* which showed exclusive expression in the embryo sacs of apomictic flowers compared to sexual embryo sacs during *in situ* hybridization analysis. This gene showed kinase and LRR domains implying its putative role in signaling pathways. In order to understand its functional significance, it was downregulated using RNAi approach.

Keywords: Apomixis, Receptor like kinase, RNA interference, Signaling, Shoot organogenesis

Materials and Methods

RNA was extracted from apomictic *Cenchrus ciliaris* inflorescence at mature embryo sac stage. Specific sense and antisense RNAi primers were designed from the *CcRLK* gene wherein the left primer was from the coding region (290bp) and the right one was from the 3'UTR region (112bp). A fragment of 402bp length from the 3' end of *CcRLK* gene was used for construction. The sense and anti-sense fragments were amplified from pBluescript vector and were cloned into pGEMT vector. Clones having CcRLK hairpin cassette in pHANNIBAL driven by CaMV35S promoter and terminated by OCS terminator were selected. The CcRLK RNAi construct was subcloned in binary vector pCAMBIA 1301 and subsequently used for *Agrobacterium* transformation of *C. ciliaris*. The *Agrobacterium* strain EHA105 containing pCAMBIA 1301 plasmid having RNAi cassette of *CcRLK* gene was used for *Agrobacterium* mediated transformation of *C. ciliaris* geneotype IG-3108 using shoot apex explants. Transformation protocol used was explants were infected for 30 minutes and co-cultivated for 3 days in MS medium containing 400µM of acetosyringone with Agrobacterium at OD_{600nm} 1.0 under a negative pressure of 0.5 X 105 Pa. Putative transformants were maintained on MS medium supplemented with 3mg/l TDZ. Putative transgenic plants were analyzed through PCR using sense and antisense primers as well as by Southern analysis which confirmed the integration of RNAi cassette. To estimate the level of silencing in RNAi plants, semi-quantitative RT PCR was used. *In silico* analysis of partial *CcRLK* gene through GENEVESTIGATER software was used to study the role of gene in reproduction according to rice expression database.

Results and Discussion

A 402bp fragment was cloned by double digestions with *EcoRI-XhoI* and *BamHI-HindIII* both in sense and antisense orientation in pHANNIBAL vector and the CcRLK hairpin RNAi cassette was subcloned into a binary transformation vector pCAMBIA 1301 with the help of restriction enzyme *PstI-SacI*. The positive clones were confirmed by PCR amplification using sense, antisense, GSP and GUS primers. Plant transformation vector pCAMBIA 1301 containing pHANNIBAL-CcRLK RNAi cassette was successfully mobilized into *Agrobacterium* via electroporation. Using optimal transformation procedure, 982 shoot apices were immersed in *Agrobacterium* suspension MS-inf medium. Out of 889 co-culitvated shoot apices transferred to the selection media, 22 survived the selection media and the efficiency of multiplication was 2.52% ±0.62. Among the putative transgenic plants, 13.8%±1.7 were GUS positive, but no GUS activity was observed in untransformed (negative control) plants. Genomic DNA from transgenic plants as well as wild type plant and the pCAMBIA1301 plasmid DNA was digested with *EcoRI* and hybridized with probe of 1029 bp obtained by amplification of pCAMBIA1301 with GUS gene specific primers. Out of 6 transgenic plants, 3 plants ($T_0$1, $T_0$6, $T_0$7) showed single copy, one plant ($T_0$5) showed 2 copies and 2 transgenic plants ($T_0$3, $T_0$4) showed 5 copies (Fig. 1). The expression level of *CcRLK* gene decreased in all the RNAi transgenic plants. Semi-quantitative RT PCR was used to estimate the level of silencing which ranged from 70-90% (Fig. 2) decrease in the level of *CcRLK* transcript. The maximum reduction in gene expression was observed in T3 plant (0.06-fold), followed by T6 (0.07-fold), T5 (0.11-fold) and least in T1 (0.3-fold). On the basis of *in silico* analysis through GENEVESIGATER software, we expect that the targeted gene has an important role in reproduction pathway from development of ovule primordia till fertilization.

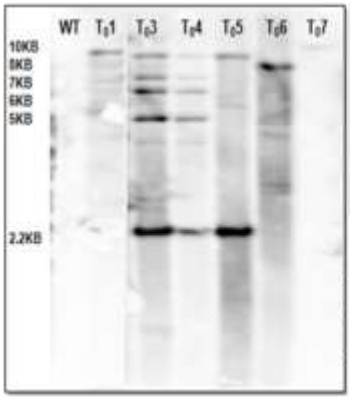

Fig 1 Southern blot analysis of wild type (WT) and RNAi transgenic plants ($T_0$1- $T_0$6) having CcRLK RNAi cassette

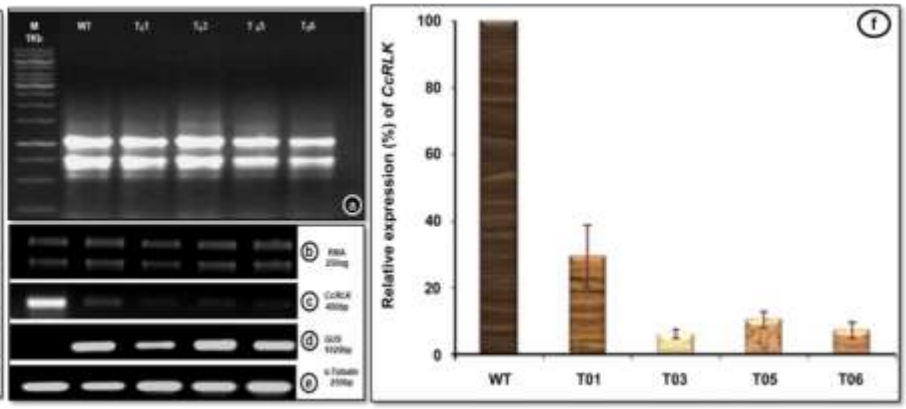

Fig 2 a) Total RNA extracted from leaves of wild type (WT) and transgenic plants ($T_0$1, $T_0$3, $T_0$5 and $T_0$6), b) Semi-quantitative RT PCR analysis of control and RNAi transgenic plants using c) GSP, d) GUS and e) control (α-Tubulin), f) Graphical representation of *CcRLK* gene expression in transgenic RNAi plants ($T_0$1, $T_0$3, $T_0$5 and $T_0$6) relative to wild type (WT).

A high degree of silencing has been obtained with conventional vectors when compared to other silencing techniques like co-suppression or antisense constructs (Wesley *et al.*, 2001). The region selection for targeting gene silencing is important for determining RNAi specificity as vectors harbouring either 3' or 5' end regions of cDNA have been found to be most effective. Therefore, coding region with 3' UTR for *CcRLK* stem region of 100-1,000 nt are the lower and upper threshold limits but usually 200-500 nt long stem region has been used for effective silencing. Therefore, in case of *CcRLK* a 402bp target sequence was selected close to the threshold value. Different RNAi vectors use different spacer sequences like GUS (Chuang and Meyerowitz, 2000), GFP (Piccin *et al.*, 2001) and introns (Wesley *et al.*, 2001). In the present study, pHannibal vector which has a PDK intron. Usually strong promoters like CaMV 35S and rice ubiquitin to drive the hairpin cassette have also impact on the RNAi efficiency. The CcRLK RNAi vector driven by CaMV 35S promoter will have a differential expression of hairpin cassette in the transgenics. Considering all the criteria discussed above, it can be suggested that silencing efficiency of both CcRLK and RNAi vectors can only be determined once they have been transformed in plants and subsequently analysed by gene expression studies. Furthermore, depending on the efficiency of silencing by RNAi vector and role of *RLK* gene in apomixis, transgenic with desired phenotype in knockdown mutants of *C. ciliaris* could be obtained.

To understand the characteristic feature of *CcRLK* gene and its biological function in *C. ciliaris* was thoroughly investigated using web based tool GENEINVESTIGETOR. Like other *RLKs*, expression of *CcRLK* gene was also observed in selective tissue at different developmental stages. It showed comparatively high and constant expression in reproductive organs such as inflorescence, panicle, spikelet, floret, stamen than in other tissues. Although the level of expression of targeted gene was at medium level in all tissues the difference between expression levels in reproductive and somatic tissue was significant. In addition to this, the level of expression was significantly higher from germination to heading stage. On the basis of this analysis, we concluded that the targeted gene plays an important role in the reproduction pathway from the beginning to end *i.e.*, development of ovule primordial till fertilization.

The RNAi construct in pHannibal has CaMV 35S promoter for expression of the hairpin cassette. Therefore, due to the presence of constitutive promoter, the expression of *CcRLK* was reduced significantly even in the leaves of transgenic plants compared to control plants when analysed through RT-PCR. This down-regulation in the expression of *CcRLK* in RNAi transgenic line corroborated changes in growth rate of vegetative tissue, tillering percentage, width of leaves, which indicates RLK's role in vegetative tissues. In maize and Arabidopsis, a predicted receptor kinase named *CR4* was found to be involved in normal differentiation of leaf epidermis (Jin *et al.*, 2000). Further investigations are required to assess the role of *CcRLK* in reproductive tissues and apomixis-related functions in *C. ciliaris. CcRLK* was successfully down regulated in our study as evident from expression analysis using semi-quantitative RT-PCR. Further phenotypic characterizations are under progress to elucidate the gene function in relation to apomixis.

Conclusion

A partial Receptor like kinase gene was downregulated using RNAi technique. The molecular analysis of RNAi plants confirmed silencing of *CcRLK* gene. The rice expression database has indicated significant role for the ortholog of *CcRLK* in reproduction. Due to constitutive effect of silencing, overall growth of RNAi plants in severely affected. The exact function of *CcRLK* is still not clear due to abnormal growth of silenced plants.

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