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BRIEF COMMUNICATION

Transient RNAi based gene silencing of glutathione synthetase reduces glutathione content in *Camellia sinensis* (L.) O. Kuntze somatic embryos

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Abstract

We report on gene silencing of glutathione synthetase (GSHS) that reduces reduced glutathione (GSH) content in somatic embryos of *Camellia sinensis* L. Using degenerate primers with cDNA of *Camellia sinensis*, a 457 bp *GSHS* gene fragment was cloned through polymerase chain reaction. This fragment was used in making ihpRNA. For this it was cloned in sense at AscI and SwaI and in anti-sense at Bam HI and XbaI restriction sites of pFGC5941 that has chalcone synthase (Chs) intron between SwaI and BamHI restriction sites. Resultant RNAi construct was used for *C. sinensis* somatic embryos transformation through *Agrobacterium*. After 11, 13 and 15 d of transformation, embryo *GSHS* transcript levels and GSH content decreased to a great extent which documented the feasibility of RNAi based gene silencing in *C. sinensis.*

Additional key words: *Agrobacterium*, PCR, RNA interference.

In the post genomic era of plant science, function assignment to each gene of a plant genome is a major challenge in front of plant researchers. Before the invention of RNAi technology, this was carried out by knock-out expression of a gene using transposon/T-DNA insertions and was considered as a useful tool for this type of study (Page and Grossniklaus 2002). This approach is used to a limited extent because of its long time consumption in selecting the lethal mutant and applicable to a few plant species. Also, it is complicated by the problem of genetic redundancy caused by multigene families and polyploidy. In contrast to this, RNA interference (RNAi) is based on sequence-specific RNA degradation.

For RNAi we need to have small fragment of a gene whose function is to be determined and should be cloned in vector in such a way that follows the formation of double-stranded RNA (dsRNA) homologous in sequence to the targeted gene (Baulcombe 2004). So formed dsRNA is treated by the host plant genome as aberrant and is cleaved into further small fragment, known as small interfering RNA (siRNA), by the action of Dicer-like enzymes (Hamilton and Baulcombe 1999, Tang *et al*. 2003). These siRNA (21 - 24 mers) make an association with RNA-induced silencing complex (RISC) and based on sequence complementarity directs the cleavage of endogenous RNA transcripts. In addition to this, siRNA also participates in amplification of silencing signals. They act as primer to the endogenous RNA and convert them to dsRNA by the action of RNA-directed RNA polymerase (RdRP) encoded in the plant genome (Lipardi *et al*. 2001, Sijen *et al*. 2001). Thus, an introduction of few dsRNA molecules are sufficient to inactivate the continuously transcribed target mRNA for long periods of time and therefore, inactivation persists even after cell division, spreads to untreated cells and tissues of plants, and is inherited to subsequent generations. Earlier RNAi has been used successfully for gene functional analysis in various plant systems such as *Petunia hybrida*, *Arabidopsis thaliana*, *Papaver somniferum*, *Torenia hybrida*, *Coffea arabica* and *Oryza sativa* (Stam *et al*. 1997, Chuang and Meyerowitz 2000, Wesley *et al*. 2001,

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Abbreviations: *CsGSHS* - *Camellia sinensis* glutathione synthetase; dsRNA - double stranded RNA; GSH - reduced glutathione; GSHS - glutathione synthetase; RNAi - RNA interference; siRNA - small interfering RNA.

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Stoutjesdijk *et al.* 2002, Allen *et al.* 2004, Fukusaki *et al.* 2004, Lee *et al.* 2004, Ogita *et al.* 2004, Miki *et al.* 2005). The transformation protocols have been well established in coffee at least for two species *Coffea arabica* (Ogita *et al.* 2004) and *Coffea canephora* (Ribas *et al*. 2005) as well in tea (Mondal *et al*. 2001) that has helped us in conducting gene silencing studies in these crops.

In view of its wide application for genome-wide reverse genetics in diverse group of species, here we report for the first time on successful implication of RNAi mechanism in *Camellia sinensis* somatic embryos. Glutathione biosynthetic pathway was suppressed through RNA interference of glutathione synthetase (GSHS). In making RNAi vector, partial sequence of *GSHS* gene were cloned in sense and antisense orientation, harboring intron in between, of pFGC5941 vector. Such gene constructs encode intron-spliced RNA with a self-complementary "hairpin" structure (ihpRNA) that have earlier been shown to induce post-transcriptional gene silencing (PTGS) with almost 100 % efficiency when directed against viruses or endogenous genes and transgenes in plants (Smith *et al.* 2000). Decrease in transcript levels of *GSHS* and GSH contents suggest the RNAi operation in *Camellia sinensis* and can be used for the functional analysis of a gene.

Partial fragment of *GSHS* gene was isolated employing the following strategy. Total RNA was isolated from youngest leaf of *Camellia sinensis* L. grown in Institute tea experimental farm and cDNA was synthesized using reverse transcriptase Superscript^{TM} III (*Invitrogen*, Carlsbad, CA, USA). Resulting cDNA was used to carryout the polymerase chain reactions (PCR) with degenerate primers (forward 5'-GAAGARMGV WAYATGTATG ACC-3' and reverse 5'-GTKCKCAT CAARTAACC ACACTG-3') and a 457 bp glutathione synthetase gene fragment was isolated that was confirmed by sequencing and NCBI blast analysis. For the construction of RNAi vector, AscI and SwaI restriction sites were incorporated using GGCGCGCCGAAGAGA GGTATATGTATG as forward and ATTTAAATCTCT TTGCGGTTTCATAAC as reverse primers while BamHI and XbaI restriction sites were incorporated using GGATCCCTCTTTGCGGTTT CATAAC as forward and TCTAGAGAAGAGAGG TATATGTATG as reverse primers. These two PCR products were first cloned in pGEMT-easy vector (*Promega*, Madison, USA) following the manufacturers instructions and then restrict digested fragments were used for cloning in pFGC5941 (purchased from *Arabidopsis* Biological Resource Centre and DNA Stock Centre of the Ohio State University). The resultant RNAi vector was named as pFGC-*GSHS*.

Somatic embryos derived from de-embryonated cotyledons, obtained from mature green fruits of *Camellia sinensis* cv. Kangra Jat were used for *Agrobacterium* mediated transformation of *GSHS* containing pFGC-*GSHS* RNAi vector as well as pFGC vector alone (used as control). Details on somatic embryos production from cotyledons of tea has been described previously (Mondal *et al*. 2001).

To check the effect of *Agrobacterium* mediated transformation of pFGC-*GSHS* and pFGC in somatic embryos of *Camellia sinensis*, 11, 13 and 15 d after *Agrobacterium* infection total RNA was isolated and used for cDNA synthesis. *GSHS* transcript levels were monitored through PCR using GAAGAGAGGTATA TGTATG as forward and CTCTTTGCGGTTTCATAAC as reverse primers. 26S rRNA based gene primers were used as internal control in the transcript expression studies (Singh *et al*. 2004). GSH was estimated by the recycling method described in previous studies (Volohonsky *et al*. 2002). Reaction was initiated by the addition of glutathione reductase (GR) and absorbance was read at A_{412}. GSH content was calculated from its standard curve.

GSH is one of the most crucial antioxidant compounds and help in maintaining the redox balance inside the cell of living organisms. Therefore it is the key compound for the survival of plants as well (May *et al*. 1998, Noctor *et al*. 2002). GSH is synthesized in two steps enzyme catalyzed reaction where the first reaction is catalyzed by γ-glutamylcysteinyl synthetase (γ-ECS) and the second by GSHS. In this study we isolated a 457 bp gene fragment of *GSHS* and first time used in checking the feasibility of RNAi based gene silencing in *Camellia sinensis*. A simple PCR based technique was used for the isolation of *GSHS* fragment. For this, total RNA isolated from the youngest leaf (bud) was used and first strand cDNA was prepared. cDNA was used in PCR with degenerate primers as detailed in material and methods. A ~450 bp fragment so obtained was cloned in pGEMT vector for its sequence analysis. The sequence analysis of the cloned gene fragment revealed that the cDNA clone is 457 bp long. For homology analysis, *BLAST* searches were conducted using GenBank and this documents that sequence belongs to *GSHS* among diverse group of species.

CsGSHS gene fragment was used in developing hpRNA binary vector for RNA interference study in *Camellia sinensis* somatic embryos. AscI at 5' and SwaI at 3' end of *CsGSHS* gene fragment were incorporated through PCR for cloning the fragment in sense orientation in between the 35S promoter and chalcone synthase (Chs) intron. While XbaI at 5'- and BamHI at 3'-end were incorporated to clone the fragment in antisense orientation (Fig. 1). These two fragments were first cloned individually in pGEMT-easy vector and restrict digested to produce the cohesive terminal ends. These cohesive ended fragments were then cloned into pFGC5941 vector and the resulting pFGC-*GSHS* RNAi vector was used for *Agrobacterium* mediated trans-formation of *Camellia sinensis* somatic embryos. For this we used somatic embryos of *Camellia sinensis* because of two reasons: normally infiltration of leaves is done to carry out such studies but it was difficult to infiltrate the leaves of *Camellia sinensis* as they are very sturdy and thick. Secondly, regeneration of plantlets from somatic embryos takes a long time (Mondal *et al*. 2001).

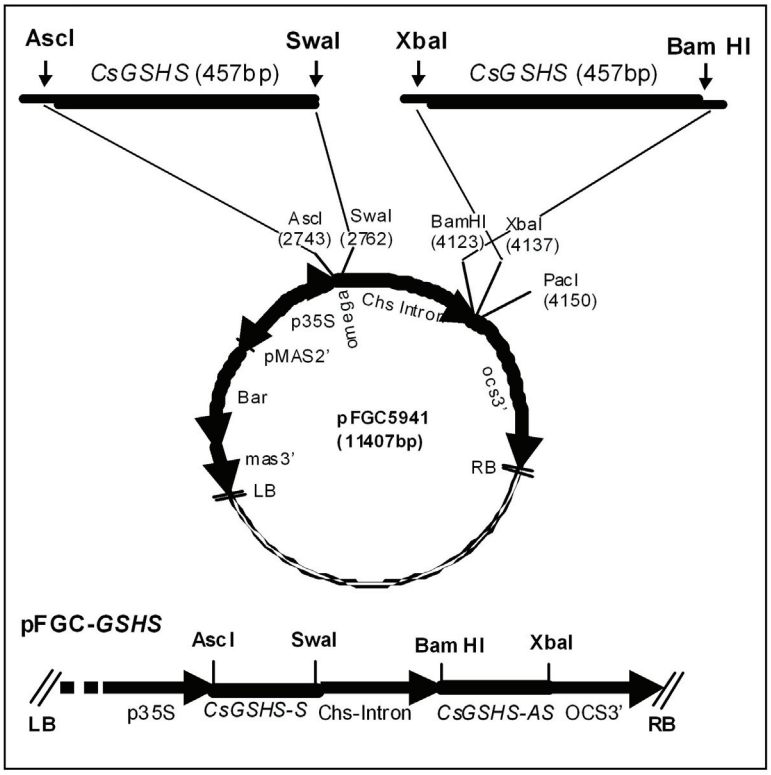

Fig. 1. The construction of RNAi vector of *CsGSHS* gene fragment using pFGC5941 vector. AscI and SwaI restriction sites were incorporated to clone the gene fragment in sense orientation and BamHI and XbaI were incorporated to clone the fragment in antisense orientation. pFGC-*GSHS* harbors Chs intron between the sense and antisense cloned fragments.

Somatic embryos of *Camellia sinensis* were used as plant tissue for RNA interference study. These somatic embryos were developed from mature green fruits of *Camellia sinensis*. Cotyledons of these seeds were first made de-embryo-nated and put into various medium for various time points for the production of somatic embryos as described previously (Mondal *et al*. 2001). *Agrobacterium tumefaciens* strain LBA 4404 containing pFGC-*GSHS* vector was employed for transformation of these somatic embryos as described earlier using p35SGUSINT construct (Mondal *et al*. 2001) and after 11, 13 and 15 d of infection samples were collected for

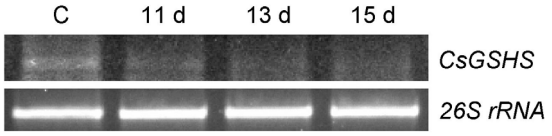

Fig. 2. Relative quantitative RT-PCR of endogenous *GSHS* transcripts targeted by pFGC-*GSHS* and pFGC in somatic embryos of *Camellia sinensis*. C in the figure represents the RT-PCR result of control sample where somatic embryos were transformed with pFGC vector having no *GSHS* insert and after 15 d RNA was isolated. Note the decrease in transcript levels of glutathione synthetase estimated in somatic embryos of *Camellia sinensis* after 11, 13 and 15 d of transformation. Loading control for gel blot analysis is shown by 26S rRNA transcript levels. Experiment was repeated for three times and observed the similar results, shown is a representative picture of one experiment.

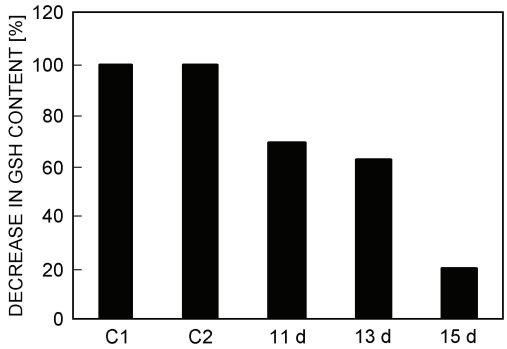

Fig. 3. Decrease in GSH contents measured in somatic embryos of *Camellia sinensis* transformed with a pFGC-*GSHS* and pFGC vector alone (as control, C1). GSH content was also estimated in embryos which were not transformed by either of the vector (as control, C2). Means of three replications. The SE in each case was < 4.

GSHS transcript levels and GSH contents analysis. For control, somatic embryos transformed with pFGC, having no *GSHS* insert, were used for the analysis after 15 d of infection.

We designed the construct in such a way that on its transcription this would produce hpRNA. Transcript of sense and antisense *GSHS* fragment form complementary base pairing and intron Chs form the loop, *i.e*., hair pin type of structure (hpRNA). Smith *et al.* (2000) has documented that this kind of RNAi vector is most

efficient in silencing the function of a particular gene. Here we observed the down-regulation in the *GSHS* transcript levels after 11 d of somatic embryos and declined further on 13th day and almost complete absence of *GSHS* transcript was seen after 15 d of transformation (Fig. 2). Result suggests the successful implication of RNAi technology in tea even with somatic embryos. The efficacy of gene silencing in plants using inverted-repeat transgene constructs encoding hairpin RNA (hpRNA) has been demonstrated (Wesley *et al*. 2001). However, the research on understanding the molecular mechanism of gene silencing is still undergoing. Whether the mechanism of silencing is exactly same in different plants, need much more experimental evidence. This is a useful technology not only for the functional analysis of a gene but also for the improvement of quality of plants and their products.

As we observed the reduction in transcript levels of *GSHS,* decrease in GSH content was expected and therefore was measured in somatic embryos after the similar time interval of 11, 13 and 15 d of infection as was used for transcript levels determination. GSH levels were decreased by 31, 38 and 79 %, respectively, after 11, 13 and 15 d of somatic embryos transformation with pFGC-*GSHS* (Fig. 3). Reduction in GSH content, on *CsGSHS* gene silencing, documents the utilization of gene silencing technology for functional elucidation of various other genes in tea, which has not been reported so far.

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