Chapter 1

Introduction
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The plants had been hyped as potential bioreactors for pharmaceutically and commercially valuable protein production as early as about two decades ago (Thanavala et al., 2006; Schillberg et al., 2003). Plants offer safe and cost-effective production of recombinant proteins on an agricultural scale using available agronomic infrastructure, while eliminating risks of product contamination with endotoxins or human pathogens (Spök and Karner, 2008; Fischer and Emans, 2000; Sorrentino et al., 2009). However, the market scenario of commercial molecular farming product is far from being satisfactory. The effective use of plants as bioreactors depends mainly on high level accumulation of proteins during the life of transgenic plants and in subsequent generations (De Wilde et al., 2000). Unfortunately, in many cases, the introduced transgenes are either not expressed or the expression level is too low even in most favorable environment.

Various factors such as copy numbers, RNA silencing, position effects, somaclonal variation and regulatory sequences are the potential hurdles to achieve desirable and predictable transgene expression in GM plants (Butaye et al., 2005). Among these factors, RNA silencing poses a serious problem to achieve desirable and predictable transgene expression in GM plants (Fagard and Vauchere, 2000). Moreover, RNA silencing plays a dominant role in the establishment of transgene expression variation. Many laboratories referred transgene silencing as a major commercial threat and it hampers utilization of plants as protein factories (Alvarez et al., 2008). Thus, RNA silencing has become a major impediment for production of commercial GM crops with stable transgene performance.

In plant, RNA silencing serves as an adaptive immune response that restricts accumulation or spread of inducing viruses (Waterhouse et al., 2001). As a counter defensive mechanism, viruses encode proteins known as suppressors that suppress RNA silencing at different stages of the pathway (Li and Ding, 2006). More than fifty such suppressors have been characterized from plant viruses and a few of them are known from insect as well as animal viruses. Most
of the suppressors act using different mechanisms and no ‘suppression motifs’ have yet been found in these molecules. One example of such viral suppressors of RNA silencing includes the AC2 protein of Mungbean yellow mosaic India virus, MYMIV, a member of the Begomoviridae genus of the Geminiviridae family (Karjee et al., 2008; Singh et al., 2007a).

Molecular farming technology requires high-level expression of transgenes in plants. However, plants perceive transgenes like virus molecules and endeavour to build a defence mechanism against invaded transgene(s) like VIGS (virus-induced gene silencing). However, viral suppressors can overcome this RNA silencing barrier and recently, these suppressors have been explored to ameliorate low transgene expression in plant transformation technology. The use of such viral suppressors has proven to be an efficient strategy to inhibit the negative influence of RNA silencing on transgene expression in transient assay system (Voinnet et al., 2003; Mallory et al., 2002). However, exploitation of such viral suppressors from plant DNA viruses to boost transgene expression in stable transgenic system is not yet reported. Interestingly, it was found that ectopic expression of MYMIV-AC2 suppressor can efficiently reverse GFP expression from its pre-established silenced state in a transient system (Karjee et al., 2008). Based on this fact the present study was undertaken to exploit the silencing suppressor activity of MYMIV-AC2 to increase the transgene expression in stable transgenic tobacco, as a model system. Besides this, it is important to investigate into the mechanistic insight of suppressor activity of MYMIV-AC2 in RNA silencing pathway. Thus, the present work was taken up with the following objectives.

1.1 Objectives

A. To establish the proof of molecular farming principle with MYMIV-AC2, we used a reporter GFP based transgenic model system and the following specific objectives were taken up.

1. Development of MYMIV-AC2 transgenic homozygous lines (i.e. T2 generation).
2. Development of transgenically silenced GFP homozygous lines (i.e. T2 generation).

3. Introduction of MYMIV-AC2 suppressor on the background of transgenically silenced GFP lines by adopting two approaches:
   i. Crossing between homozygous MYMIV-AC2 lines and homozygous GFP silenced parental lines to obtain the F1 hybrids.
   ii. Transformation of MYMIV-AC2 on the background of transgenically silenced GFP explants to generate T0 double transgenics.

4. Analysis of GFP expression and reduction of silencing in F1 hybrids and T0 generation of double transformed lines.

5. Analysis of GFP expression in cellular level e.g. in leaf protoplasts.

B. To validate the molecular farming principle with other genes, we took two endogens. *Phytoene desaturase* (*PDS*) endogene of tobacco was chosen as its silencing leads to a readily visible phenotype and another endogene of tobacco *topoisomerase II* (*TOPOII*) was also chosen as it is an essential ubiquitous enzyme involved in controlling DNA topology during multiple processes of DNA metabolism. Therefore, to validate the molecular farming principle with these two endogens, the following objectives were taken up:

1. Development of transgenically silenced *PDS* lines by using RNAi approach.

2. Introduction of MYMIV-AC2 on the transgenically silenced *PDS* lines by crossing between MYMIV-AC2 and *PDS* silenced lines.

3. Analysis of F1 hybrids to examine the *PDS* expression or reversion.

4. Secondly, a low expressing *TOPOII* transgenic tobacco line was used for crossing with MYMIV-AC2 and the obtained F1 lines were analyzed to examine the expression of *TOPOII* in F1 lines.
C. The mechanism of RNAi suppression by MYMIV-AC2 is still obscure. Hence, it is important to discover the suppression mechanism of MYMIV-AC2 protein. In our lab, the 'Phage display' analysis showed that RdR6 protein might be an interacting partner of MYMIV-AC2 in RNA silencing pathway. Moreover, the ectopic expression of MYMIV-AC2 mimicked the knockdown of RdR6 activity in planta. Therefore, to gain mechanistic insights of the MYMIV-AC2 suppression activity in RNAi pathway, we studied the interaction between MYMIV-AC2 and plant RDR6 protein. To meet this goal, the following objectives were taken up:

1. To fish out the full length RdR6 genes from Arabidopsis and Nicotiana.
2. To express AtRDR6 and NtRDR6 in vitro.
3. To study the interaction of MYMIV-AC2 with RDR6 through co-immunoprecipitation.