Chapter 1: Introduction

“True wisdom lies in gathering the precious things out of each day as it goes by.”

- E.S. Bouton
INTRODUCTION

Life cycle of higher plants alternates between a short, haploid gametophyte phase comprising a small number of cells and a long, multicellular diploid sporophyte phase. Transition of sporophyte to gametophyte is initiated when diploid spore mother cells differentiate from the surrounding cells and undergo meiosis to give rise to four haploid spores. In ovules *i.e.* mega/female gametophyte, of the four haploid megaspores, three disintegrate while the fourth one residing towards chalazal end becomes functional megaspore and develops into a mature megagametophyte after three rounds of mitotic divisions followed by cellularization. A typical female gametophyte consists of seven-celled embryo sac that harbours six haploid cells (an egg cell, two synergids, three antipodals) and a diploid central cell. Likewise, the micro/male gametophyte or pollen is derived from anther containing diploid cell (microspore mother cell), which undergoes meiosis to produce haploid microspores in tetrads. These microspores separate and their nuclei undergo an asymmetric mitosis resulting in bicellular pollen and comprises of a vegetative and a generative nuclei. The later undergo one more round of mitosis and forms two sperm cells (Maheshwari 1950; van Went and Willemse 1984; Reiser and Fisher 1993). During its development, the tapetal cells of anther wall undergo Programmed Cell Death (PCD) and produce proteins, lipids, flavonoids, which are deposited on the microspores and forms outer layer of the pollen *i.e.* the exine and trypbine layers (McCormick 1993; Raghavan 1997). All these processes are temporally and spatially regulated and involve complex interplay of several hundred genes (Gupta *et al.* 2007).

In flowering plants, the male gametophyte plays a vital role in plant fertility and crop production through the generation and delivery of the male gametes to the embryo sac for double fertilization. Pollen development can be divided into two major phases, an early phase that comprises microspore and bi-cellular pollen, and a late phase including tricellular and mature pollen. Nearly 12,000 active genes are expressed in the early microspore developmental phase. This number progressively declines to approx. 7,000 in mature pollen (Honys and Twell 2004). The gene-centered knowledge highlights the functional specialization of male gametophyte developmental pathway and offers many new opportunities for the dissection of cellular and molecular processes that control male reproductive success. A number of genes have been identified which are involved in anther and pollen development (Endo *et al.* 2004; Lan *et al.* 2004; Ma 2005; Wang *et al.* 2005) by
following various approaches such as expression profiling, transposon tagging and functional analysis. The use of T-DNA with promoterless reporter gene construct to generate promoter trap lines by its random insertion in the genome followed by screening for tissue specific reporter gene expression can also help to identify promoters involved in developmental processes in specific tissues (Stangeland et al. 2005). Genes have been identified showing spatiotemporal-specific expression within anther have been associated with different steps of pollen development such as cell division and differentiation (Nonomura et al. 2003), tapetum degradation (Jung et al. 2005; Luo et al. 2006; Xu et al. 2006), meiosis related (Yang et al. 2003; Kapoor and Takatsuji 2006), pollen maturation (Park et al. 2005, 2006; Zhao et al. 2006), associated with generative cell formation (Durberry et al. 2005) and expresses at anther dehiscence stage (Zhu et al. 2004). Some genes whose products accumulate abundantly in pollen grains and are involved in germination or pollen tube growth and helps in double fertilization were also have been identified (Golovkin and Reddy 2003; Kaothien et al. 2005). A transcription factor gene (ZmMADS2) from maize, which is required for anther dehiscence and pollen maturation, has been identified (Schreiber et al. 2004).

Promoter is one of the key components for transgenic study. Over the decades, several well-characterized constitutive expression promoters such as Cauliflower mosaic virus (CaMV) 35S gene promoter (Odell et al. 1985) and the maize polyubiquitin1 (Ubi1) promoter (Christensen et al. 1992) have been made available for transgenic studies in crops. Tissue-specific promoters were also isolated to meet the demand for precise control of transgene expression in certain tissues/cells. The endosperm-specific glutelin promoter is isolated and utilized in the production of golden rice for metabolic engineering of Geranyl geranyl pyrophosphate to produce β-carotene (a pro vit-A) in rice endosperm (Ye et al. 2000; Beyer et al. 2002; Paine et al. 2005). The fruit-specific gene expression in transgenic tomato plants has been achieved with the help of AGPL1 gene promoter from Watermelon (Yin et al. 2009). Anther-specific expression promoter can be used in generating male-sterile plants (Mariani et al. 1990; Paul et al. 1992; Luo et al. 2006; Roque et al. 2007; Konagaya et al. 2008). The male sterile plants has been developed by fusing cytotoxic genes such as ribonuclease, barnase etc with anther specific promoters e.g. TA29 promoter from tobacco and PsEND1 Pisum sativum (Mariani et al. 1990, 1992; Gomez et al. 2004; Roque et al. 2007). The PsEND1 anther-specific promoter was used to express cytotoxic gene barnase in anther for the production of seedless and good quality tomato fruits (Medina et al. 2013). Anther specific cell wall-bound invertase (Nin88)
promoter was used for metabolic engineering of pollen development by repressing the activity of invertase thus generating male sterile plants in tobacco, *Arabidopsis* and rapeseed (Hirsche *et al*. 2009; Engelke *et al*. 2010, 2011). Male-sterile plants greatly facilitate the production of hybrids for eliminating the need of manual or mechanical removal of anthers from flowers. Moreover, male-sterile plants prevent transgene flow from genetically modified crops to food crops and wild relatives (Daniell 2002). This helps to cope with the public awareness and fears about the environmental impact of genetically modified plants.

Till now, a few anther and/or pollen specific promoters have been characterized, such as *AtSTP2* and *FKP1* showing maximal expression at pollen tetrad stage of development, *AMS* and *CYP704B2* at uninucleate pollen stage, *AtWRKY34* at pollen maturity and *AtSTP9*, *AtSTP6* and *AtAMTI* at the pollen tube germination stages, etc. has been isolated and characterized from *Arabidopsis thaliana* (Truernit *et al*. 1999; Ishiguro *et al*. 2010; Sorensen *et al*. 2003; Li *et al*. 2010; Honys *et al*. 2006; Schneidereit *et al*. 2003; Scholz-Starke *et al*. 2003; Yuan *et al*. 2009). Identification and characterization of genes that expresses in anther/pollen will provide stage-specific promoters and characterization of its regulatory elements helps to target expression of desirable genes in specific stages of male gametophyte development. There are reports of anther/pollen-specific promoters isolation and characterization from different plant species which has been studied in transgenic systems (van Tunen *et al*. 1990; Twell *et al*. 1991; Eyal *et al*. 1995; Rogers *et al*. 2001; Gomez *et al*. 2004). The activity of male gametophyte-specific promoter *AtSTP2* was observed in pollen grains of all the developmental stages, i.e. transition from pollen tetrad to mature pollen grain to pollen germination (Truernit *et al*. 1999). *FKP1* (*FLAKY POLLEN1*) gene from *Arabidopsis* directed anther-specific expression in the tapetal cells of stage 7 anthers and the expression continued in microspore at stage 9 and further in the mature pollen (Ishiguro *et al*. 2010). An *Arabidopsis* ABC transporter gene (*ABCG26*), conferred strong expression in tapetum and weaker expression in microspores during tetrad stage and early vacuolate stage (Choi *et al*. 2011). A 1,700 bp promoter fragment of a tapetum-specific gene *TAPNAC* conferred maximum expression in tapetum during floral stage 11 (corresponding to microspore development) in *Arabidopsis* (Alvarado *et al*. 2011). Promoter of the *Arabidopsis MS1* gene showed strong expression in tapetum at the stage when microspores releases from tetrad (Wilson *et al*. 2001). Another gene from *Arabidopsis*, *AMS* (*ABORTED
MICROSPORE), showed its maximum expression as a function of GUS activity in the tapetum and haploid microspores during uninucleate microspore stage. Activity was first detected during the microspore release stage (Sorensen et al. 2003). PGA4 (polygalacturonase), an anther-specific gene, related to fruit ripening (Fischer and Bennett 1991), abscission of fruits (Tucker et al. 1984) and microsporogenesis (Allen and Lonsdale 1992) was isolated from Arabidopsis (Torki et al. 1999).

Tissue-specific expression of a promoter is governed by the combined activity of various cis-acting elements present in the promoter/upstream region and the interaction of nuclear proteins (TFs) with these motifs. Identification of these TFs is important because they control the expression of several downstream genes. A search for more anther, pollen and/or ovule specific promoters and cis regulatory elements conferring anther and/or ovule specificity is required which will ultimately help us in the manipulation of male and/or female gametogenesis for biotechnological purposes. A suitable design of combination of cis-acting elements and TFs obtained from these studies can be exploited to modulate gene expression at will (Khurana et al. 2013a).

The gametophyte development involves differential expression of a plethora of genes and its expression is precisely controlled by cis acting regulatory elements. The identification and characterization of promoters from different developmental stages of pollen/male gametophyte will provide a tool to manipulate the genes during particular stage precisely and can be used for development of transgenics with minimized risks of horizontal transfer of genes through pollen to its wild relatives (Prakash et al. 2011). As there are very few anther specific genes are reported during male gametophyte development in Arabidopsis thaliana, more numbers of promoters with specific spatial and temporal activity domains are required for genetic manipulation as they are able to control not only the time and place but also the expression level of specific protein(s). In keeping view of the above discussion, in the present study, it is proposed to identify the promoters involved in male gametophyte developmental process, by generating promoter trap lines of A. thaliana and their characterization. The main objectives set for the present study is as follows.

1. Development of male gametophyte specific promoter trap lines
2. Cloning and functional validation of promoter from the selected trap line