CHAPTER 1

INTRODUCTION
Introduction

*Cichorium intybus* L. (chicory) is an erect perennial herb, 30-90 cm in height, with fleshy tap root and up to 75 cm length. It is gaining global attention due to its industrial utility as coffee additive and medicinally important phytochemicals (Varotto *et al.*, 2000; Roberfroid, 1996). The plant contains large number of pharmaceutically important compounds, which include sesquiterpene lactones, coumarins, flavonoids, anthocyanins, organic acids and cytokinins (Duke, 1983; Nadkarni, 1976). The tuberous root of this plant contains medicinally important bioactives such as inulin, bitter sesquiterpene lactones, coumarins, flavonoids and vitamins (Varotto *et al.*, 2000). In addition, chicory leaves contain esculin as dominant component, which is a well-known antioxidant (Rastogi *et al.*, 1994; Nadkarni *et al.*, 1976). Recently, esculin has been reported to possess hepatoprotective action against *CCl*_4 induced hepatotoxicity (Naaz *et al.*, 2006). The immune modulator and anticancer activities of *C. intybus* L. are considered to be due to the presence of esculin (Rusu *et al.*, 2005).

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) converts HMG CoA into mevalonic acid (MVA) and is playing a key role in the MVA pathway (Hemmerlin *et al.*, 2003; Hao *et al.*, 2002; Cowan *et al.*, 1997; Choi *et al.*, 1992; Yang *et al.*, 1991) as well as secondary metabolites production (Ram *et al.*, 2010; Schaller, 2003; Lichtenthaler *et al.*, 1997; Stermer *et al.*, 1994). Growth regulators viz. abscisic acid, gibberellins, cytokinins and stress related compounds such as phytoalexins, derived from the MVA pathway contribute to the growth of various species of higher plants in diverse environments. HMGR is a rate limiting enzyme of mevalonate pathway, which contributes carbon for the esculin biosynthesis via shikimic acid pathway (Gaisser and Heide, 1996). It may also influence esculin biosynthesis and yield indirectly, either by exerting its effect on phenylpropanoid pathway via the action of ABA and phytoalexins on phenylalanine ammino lyase (PAL) (Vogt, 2010) or by increasing growth, development and biomass via the effect of growth
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hormones, gibberlins and cytokinins (Chappell, 1995). Therefore, up-regulation of mevalonate pathway by overexpressing HMG CoA reductase transgene driven by 35S promoter may enhance the esculin content and yield of chicory (Hemmerlin et al., 2003; Lichtenthaler et al., 1997; Stermer et al., 1994).

The quality assessment of herbal bioactives has always been a challenging task due to the diversity of the components existing in the plants extracts. The chromatographic methods have become one of the most frequently applied approaches, which can provide the whole profile of the marker compounds as well as the unknown components (Sasidharean et al., 2011; Li et al., 2011; Zhang et al., 2011). Therefore, it is crucial to ensure and validate the quality of herbal drugs through effective phytochemical screening methods. Among the available analytical methodologies, high performance liquid chromatography (HPLC) and its improved modifications are popular methods for the analysis of phytoconstituents in herbal drugs because of their rapidity, accuracy and precision. These analytical methods are also not limited by the volatility or stability of the herbal drugs (Sheu et al., 2011; Baumgartner et al., 2011; Yang et al., 2011; Zhao et al., 2010).

For the introduction (transformation) of a transgene, a suitable tissue culture protocol for regeneration of plant is essential. Moreover, multiplication of clones derived either from mother plant or transgenic plants is also an important need. Various investigators developed different protocols for clonal propagation and multiplication of various plant species and transgenic crops (Ohadi et al., 2011; Shrawat et al., 2011; Subramanyam et al., 2011; Verma et al., 2011; Permiakova et al., 2009; Husaini et al., 2008; Tzfira et al., 2003). In addition, different transformation methods were used by several investigators for stable transformation and expression of transgenes associated with agronomic traits to improve the yield of crops
and secondary plant metabolites contents in the industrial crops including medicinal plants (Dafny-Yelin et al., 2008; Roy et al., 2003).

Till now different gene transfer techniques have been explored such as physical methods like electroporation (Lurquin, 1997), particle bombardment (Kikker et al., 1993), microinjection (Holmberget et al., 1998), chemical methods where, chemicals like PEG, polyvinyle alcohole were used (Swapan et al., 1992; Hain et al., 1985) and biological methods in which *Agrobacterium* species and viruses were used (Subramanyam et al., 2011; Potrykus, 1991). Although many of these conventional methods are effective in cellular gene transfer but their efficacy is compromised over the rapidity e.g. in case of physical methods the excessive use of energy damages the DNA (Oard, 1991; Potrykus, 1991). In recent years with the advent of recombinant DNA technology, the field has evolved over the past few decades, with most gene transfer studies based on the use of *Agrobacterium* and viruses to deliver the gene (Shrawat et al., 2011; Subramanyam et al., 2011; Verma et al., 2011; Permiakova et al., 2009; Husaini et al., 2008; Tzfira et al., 2003). Although these cases are attractive in terms of the scientific strategy of exploiting natural mechanism, but such modes of genetic transformation bear difficulties like poor reproducibility, scale up and the possibility of reversion of an engineered plant to the normal cultivar, particularly in case of viruses (Dafny-Yelin et al., 2008). In addition, the safety risks including insertional mutagenesis and toxicity are also the matter of concern when viral vectors are used (Roy et al., 2003). Consequently, a major focus is now being given to the development and use of alternative synthetic, non-viral vectors for safe and efficient gene delivery systems.

Introduction of gene encapsulated in the nanoparticles is expected to be advantageous as the fast mode of gene transfer in plants that can be achieved at the nano scale level. The
transgene is protected from DNase and cytoplasmic as well as nucleoplasmic environments due to its encapsulation in nanoparticles (Ozbas-Turan et al., 2003; Roy et al., 2003). Moreover, the probability of energy induced DNA damage (commonly seen in case of physical methods) is very much reduced. The small size (1-100nm) of nanoparticles with their unique surface makes them versatile carriers for the cellular delivery of bioactives and transgenes.

Although, very few nanotechnology based methods were reported for the genetic transformation, but they seems to be effective in terms of time consumption and DNA protection (Jun et al., 2005). Calcium phosphate nanoparticles are of interest for many biochemical and biomedical applications due to their good biocompatibility and bioactivity. Calcium phosphate material based gene transfer relies profoundly on the fact that the divalent metal cations, such as Ca$^{2+}$, Mg$^{2+}$, Mn$^{2+}$ and Ba$^{2+}$ can form ionic complexes with the helical phosphates of DNA (Ohadi et al., 2012). Calcium phosphate forms complexes with the nucleic acid backbone and thus may impart a stabilizing function to certain DNA structures. The plant cell wall is the main barrier for the entry of a foreign gene. The particles found entry through cutting surfaces of the explants. The complexes can then be carried across the cell membrane mediated by endocytosis. The released plasmids enter the nucleoplasm and get integrated into the genomic DNA by non-homologous recombination (Naqvi et al., 2012).

Application of nanotechnology as non-viral/non-bacterial strategy for gene transfer in plant is little explored. With the best of our knowledge till now, calcium phosphate nanoparticles have not been used in plant genetic transformation.

Keeping these parts in view, we have undertaken this study with the following objectives:

1. Establishment of efficient genetic transformation and regeneration protocols for *Cichorium intybus* L. involving recombinant HMG CoA reductase gene tagged with
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CaMV 35S promoter, employing conventional (*Agrobacterium tumefaciens*) and novel (calcium phosphate nanoparticles) gene delivery systems.

2. Selection and molecular characterization of putative transgenic chicory plants harboring recombinant HMG CoA reductase gene.

3. Assessment of transgenic plants for accumulation of bioactive compound, esculin, total soluble protein and chlorophyll contents.