MOLECULAR CHARACTERIZATION OF 3-HYDROXY-3-METHYL GLUTARYL COA REDUCTASE *(hmgr)* GENE FROM SOLANACEOUS PLANTS

By

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THESIS

submitted in partial fulfilment of the requirement for the degree of

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DECLARATION

I hereby declare that this thesis entitled **"Molecular characterization of 3-hydroxy-3-methyl glutaryl CoA reductase (*hmgr*) gene from solanaceous plants"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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Dedicated to My
Loving Parents
&
Sister

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Abbreviations

AA	- Arachidonic acid
ABA	- Abscisic acid
b	- Bases
BLAST	- Basic local alignment search tool
bp	- Base pair
cDNA	- Complementary DNA
CTAB	- Cetyl trimethyl ammonium bromide
DAP	- Days after planting
DMAPP	- Dimethyl allyl dphosphate
DMSO	- Dimethyl sulphoxide
DTT	- Dithiothreitol
EDTA	- Ethylene diamine tetra acetic acid
g	- Gram
GSP	- Gene specific primer
HMGR	- Hydroxy methyl glutaryl CoA reductase
HR	- Hypersensitive resistance
IPP	- Isopentenyl diphosphate
IPTG	- Isopropyl thio galactoside
kb	- kilo base
MEP	- Methyl erythritol pathway
ml	- Milli litre
mM	- Milli molar
mRNA	- messenger RNA
MVA	- Mevalonic acid
NCBI	- National Centre for Biotechnology Information
ORF	- Open reading frame
PCR	- Polymerase chain reaction
RACE	- Rapid amplification of cDNA ends
rpm	- Revolutions per minute
RT- PCR	- Reverse transcriptase polymerase chain reaction

SDS	- Sodium dodecyl sulphate
Snhmgr	- *Solanum nigrum* hmgr
Sthmgr	- *Solanum torvum* hmgr
Sxhmgr	- *Solanum xanthocarpum* hmgr
TE	- Tris EDTA
UPM	- Universal primer mix
UV	- Ultra violet
v/v	- Volume per volume
w/v	- Weight per volume
µg	- microgram
2,4 –D	- 2,4 Dichloro phenoxy acetic acid

Introduction

1. INTRODUCTION

Cultivated plants are susceptible to a variety of pests and diseases. However many indigenous weed flora are often resistant to them. This is due to their ability to produce some defence chemicals. Crude extracts from fruits and roots of *Solanum xanthocarpum,* a solanaceous plant, can control larvae of important vectors of malaria and dengue (Singh and Bansal, 2003). *Solanum nigrum* , another member of the same family is reported to have molluscicidal activities. At present, the snail control is through chemical molluscicides with high risk of health problems and environmental pollution. Hence the molluscicidal activity of *Solanum nigrum* can be efficiently used to control snails in an ecofriendly way.

Most of the biochemical compounds, which perform such defence oriented functions are produced through plant mevalonate pathway. 3 -hydroxy 3-methyl glutaryl CoA reductase (HMGR, EC 1.1.1.34) is a key enzyme involved in the mevalonate pathway leading to the biosynthesis of isopentenyl di phosphate (IPP), the universal precursor for terpenes. (McGarvey and Croteau, 1995). It has been reported to be the rate-limiting enzyme in sesquiterpene and triterpene biosynthesis. In plants, mevalonate is the general precursor of various identified isoprenoid compounds. Isoprenoids are the largest family of natural products, which can play numerous vital roles in basic plant processes including respiration, photosynthesis, growth, development, reproduction, defence and adaptation to environmental conditions.

These compounds are involved in various biological processes including the synthesis of membrane sterols, plant growth regulators (cytokinin, abscisic acid, gibberellins and brassino steroids), photosynthetic pigments (carotenoids, chlorophylls and phylloquinones), defence compounds against pathogen attack (phytoalexins) and some important pharmaceutical agents such as taxol and gingkolides.

The enzyme HMGR has differential expression during flower and fruit development of *Bixa orellana.* It is a tropical plant with high content of bixin, which is

a carotenoid pigment produced in seeds. Treatment with inhibitors of HMGR can produce inhibition of cell growth and loss of cell viability. Reduced microsomal activity of HMGR results in the appearance of small fruit phenotype in avocado. HMGR is strongly induced in seedlings and inflorescence of rice during the early development stage. HMGR expression in tomato is high during fruit maturation and increases strongly during ripening in parallel with the accumulation of lycopene. Loss of function of HMGR in *Arabidopsis* leads to dwarfing, early senescence, male sterility and reduced sterol levels.

It is reported that induction of HMGR is essential for the synthesis of antimicrobial and pathogen responsive isoprenoids in potato. HMGR is also involved in the production of phytoalexins and other defence related compounds in many plants like rice, tobacco, potato, sweet potato and pepper.

Genes encoding this enzyme have been isolated from many plants like *Arabidopsis*, potato, tomato, tobacco, pepper, rubber, mulberry and rice. HMGR is encoded by a small multi gene family in all plants that have been examined so far, ranging from two genes in *Arabidopsis* to four genes in tomato. Of the two *Arabidopsis* HMGR genes, *hmg* I may involve in the house keeping functions. This observation was based on its broad expression detected in all parts of the plant.

Genetic transformation with this gene has been reported to impart resistance to insects and to fungal pathogens. If this gene can be cloned from any of the solanaceous plants, it could later be used for genetic transformation of crop plants for improving their tolerance to biotic stress and for increasing the yield parameters.

Hence this study was undertaken to characterize HMGR gene from solanaceous plants. The objective of this study is isolation and characterization of the gene encoding HMGR from four solanaceous plants viz., *Solanum xanthocarpum, Solanum nigrum, Physalis minima* and *Solanum torvum.*

Review of Literature

2. REVIEW OF LITERATURE

Plants produce a variety of isoprenoid compounds which are mainly involved in plant defence and stress tolerance. HMR is the major rate-limiting enzyme in this pathway. In the present study an attempt has been made to isolate and characterize the gene encoding HMGR from solanaceous plants, *Solanum xanthocarpum* Schrad. & Wendl. (Yellow berried night shade), *S. nigrum* L. (black night shade), *Physalis minima* Linn. (wild cape goose berry) and *S. torvum* Sw. (turkey berry). A comprehensive review of the previous research studies related to the topic has been done in accordance with the objectives of the present study. The contents of this chapter are presented below under the following heads:

2.1 Economic importance of solanaceae family

2.2 Medicinal and insecticidal properties

2.3 Isoprenoid synthesis

2.4 Effect of *hmgr* on growth and development

2.5 Role of hmgr in plant defence mechanism

2.6 HMGR activity in animal cells

2.7 HMGR protein structure

2.8 HMGR in different crops

2.9 Characterization of *hmgr* gene

2.10 Regulation of gene expression

2.1 ECONOMIC IMPORTANCE OF SOLANACEAE FAMILY

The Solanaceae is a cosmopolitan family containing many essential vegetables and fruits such as potato, tomato, brinjal, paprika, chillies, green and red peppers and cape gooseberries, as well as ornamentals such as *Petunia, Schizanthus* and *Lycium* species. It also contains tobacco *(Nicotiana* spp.) and many other plants of both poisonous and medicinal value such as belladonna or deadly nightshade *(Atropa belladona* L.), stramonium *(Datura stramonium* L.) and black henbane *(Hyoscyamus niger* L.) (Decoteau, 2000).The family is composed of approximately 90 genera and

between 2000 and 3000 species, and is widely distributed throughout tropical and temperate regions of the world, with centers of diversity occurring in Central and South America and Australia (Khush and Birhman, 1993). Though the species are distributed throughout the world, they occur in their greatest concentrations in tropical and warm temperate regions.

Solanum is the largest and the most complex genus in Solanaceae. The generic name *Solanum* is generally considered to be derived from the Latin word *solamen,* and refers to the quieting or sedative effects associated with many of the member species.The genus has more than 1500 species, many of which are economically important throughout their cosmopolitan distribution (Mitra *et al.*, 1990). They include food plants like potato *(S. tuberosum* L.), brinjal *(S. melongena* L.) and the lulo or naranjilla *(S. quitoense* Lam.); horticulturally useful plants include the winter cherry *(S. pseudocapsicum* L.) and jasmine nightshade *(S. jasminoides* Paxt.) (Chadha, 1993) and species cultivated for their pharmaceutical use like bitter sweet *(S. dulcamara* L.) and *S. viarum* Dun., both used as sources of corticosteroids.

2.2 MEDICINAL AND INSECTICIDAL PROPERTIES

2.2.1 *Solanum xanthocarpum*

Gupta (1994) investigated medicinal properties of *Solanum xanthocarpum.* Saponins isolated from *S. xanthocarpum* produced augmentation of anti-allergic activity in the lung tissues and proved to be anti-asthmatic.

Fonghua *et al.*, (2000) studied the biological toxicity of plant molluscicide from *S. xanthocarpum* to target snail and non- target organisms. The results revealed that the source is cheap, effective, environmentally acceptable and toxic to both amphibious and fresh water snails in the endemic areas. Insecticidal and larvicidal properties have been reported to be associated with *S. xanthocarpum* against vectors of

malaria and dengue fever (Singh and Bansal, 2003). Results of the experiments conducted by them envisaged larvicidal property in both fruit and root extracts of *S. xanthocarpum.*

It has been shown that *S. xanthocarpum* is highly resistant to shoot and fruit borer. Multiple resistance to all the major biotic stresses was found to be associated with the plant (Sebastian, 2000). Many plant breeding programmes use *S. xanthocarpum* as a source of resistance.

2.2.2 *Solanum nigrum*

Ahmed *et al.* (2000) have studied molluscicidal properties of *Solanum nigrum.* The results revealed that the toxicity of *S. nigrum* increases during warm season. Helmy *et al.* (2000) showed that water extract of leaves of *S. nigrum* has molluscicidal and cercaricidal activities.

Scholte (2000) screened ninety accessions of non-tuber bearing solanaceous plants for resistance to potato cyst nematodes. Two *S. nigrum* varieties showed full resistance to *Globodera rostochinensis* and a high level of resistance to *G. pallida Solanum nigrum* is reported to be associated with resistance to *Phytophthora infestans* (Polkowska-Kowalczyk, 2004). In breeding for resistance to late blight, an economically important disease affecting potatoes, it can be used as a source of durable resistance (Zimnoch-Guzowska *et al.*, 2003).

2.2.3 *Physalis minima*

Preparations from *Physalis minima* can be used as tonic, diuretic, laxative and are useful in inflammations. The fruit is considered to be a tonic, diuretic and purgative. They are juicy, mildly astringent and sweet with a pleasant blend of acid. This plant is highly resistant to insect pests and diseases. (Parmar and Kaushal, 1982).

Hairy root cultures of *Physalis minima,* developed using *Agrobacterium rhizogenes* mediated transformation has produced solasodine glycoside (Putalun *et al.*, 2004).

2.2.4 *Solanum torvum*

Thangavelu *et al.* (2004) reported that plant extracts of *Solanum torvum* can act against the banana fungal pathogen *Colletotrichum musae.* The extract was found to be very effective in reducing the disease incidence, better than treatment with the fungicide benomyl. The extracts also significantly increased the shelf life of bananas, particularly their green life, in all the cultivars tested.

Solanum torvum shows resistance against bacterial wilt and can be used as a root stock for grafting susceptible brinjal varieties (Rahman *et al.*, 2002). Brinjal cultivars grafted on *S. torvum* rootstocks are resistant to root-knot nematodes (Garibaldi *et al.*, 2005).

2.3 ISOPRENOID SYNTHESIS

Plants synthesize a myriad of isoprenoids, which play an essential role in various cellular functions such as photosynthesis (carotenoids, side chain of chlorophyll), respiration, membrane architecture (sterols), chemical signaling, regulation of growth and development (gibberellins, cytokinins, abscisic acid, brassinosteroids) and defence against pathogen attack (Mc Garvey and Croteau, 1995). (Fig. 1). They play important roles in electron transport chain (quinones), in sub cellular targeting and regulation (prenylation of proteins), and as plant defence compounds as well as attractants for pollinators (monoterpenes, sesquiterpenes, and diterpenes) (Bach, 1995). Despite their wide diversity of structures and functions, all the isoprenoid compounds are derived from the universal precursor isopentenyl diphosphate (IPP) and its isomer dimethyl allyl diphosphate (DMAPP) (Newman and Chappell, 1999).

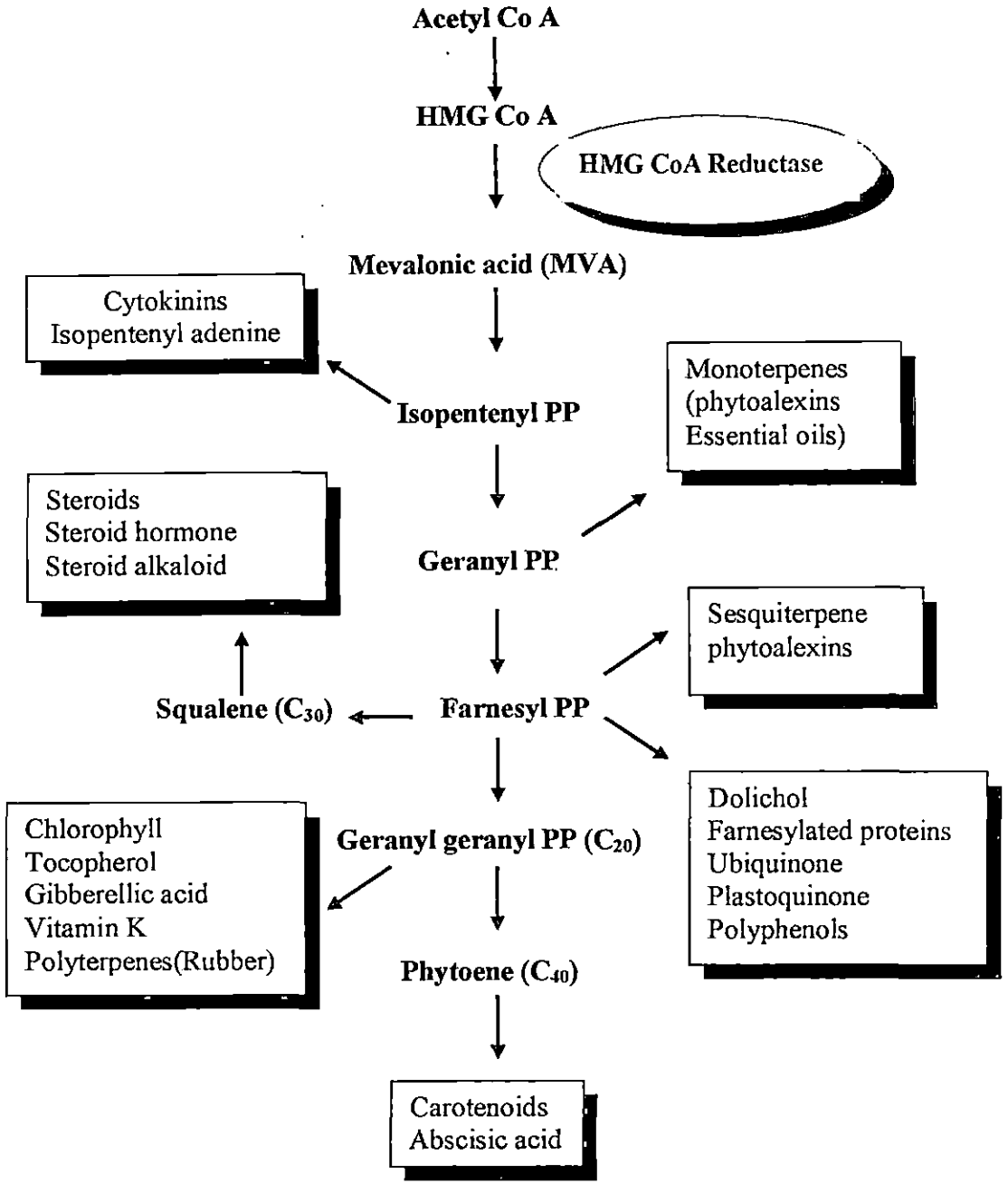

Fig. 1. Diagrammatic representation of plant mevalonate pathway

In higher plants two biosynthetic pathways are responsible for the synthesis of these precursors; the classical mevalonate pathway and the recently discovered methyl erythritol phosphate (MEP) pathway (Rohmer *et al.*, 1993; Lichtenthaler, 1999). The mevalonate pathway operates in the cytosol, whereas the non-mevalonate MEP or Rohmer pathway is confined to plastids (Rohmer, 1999; Kuzuyama and Seto, 2003). The sesquiterpenes in chamomile and lima beans are synthesized as a result of interaction between the cytosolic and plastidial pathways (Piel *et al.*, 1998; Adam *et al.*, 1999). This suggests that the pathways cooperate in the biosynthesis of certain metabolites, even though the sub cellular compartmentation allows both pathways to operate independently in plants. (Kasahara *et al.*, 2002; Nagata *et al.*, 2002).

It has been reported that the cross talk between the pathways of isoprenoid biosynthesis occurs unidirectionally from plastids to cytosol. Experiments conducted in *Arabidopsis thaliana* seedlings and snapdragon flowers have confirmed the above-mentioned interactions (Laule *et al.*, 2003; Dudareva *et al.*, 2005).

The mevalonate (MVA) pathway is responsible for the synthesis of sterols, certain sesquiterpenes, and the side chain of ubiquinone (Arigoni *et al.*, 1997; Disch *et al.*, 1998). Mevalonate biosynthesis appears to be essential for cell-cycle progression and viability of plant cells (Hemmerlin and Bach, 1998; Hemmerlin *et al.*, 1999). In contrast, MEP pathway is involved in providing the precursors for monoterpenes, certain sesquiterpenes, diterpenes, carotenoids, and the side chains of chlorophylls and plastoquinone (Cunningham and Gantt, 1998; Lichtenthaler, 1999).

During the course of evolution, higher plants have maintained both the pathways. With the exception of some species of actinomycetes studied so far, other organisms use either the MEP pathway, for example green algae and many bacteria, or the mevalonic acid pathway, such as archaebacteria, certain eubacteria, fungi and animals (Kuzuyama and Seto, 2003).

Mevalonate biosynthesis is a complex process involving a series of three enzyme-catalysed reactions. Three molecules of acetyl-CoA are converted into one molecule of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by the sequential action of two enzymes: acetoacetyl CoA thiolase (AACT; EC 2.3.1.9) (Clinkenbeard *et al.*, 1973) and 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS, EC 2.3.3.10), (Clinkenbeard *et al.*, 1975).

The combination of these two enzymes favours the overall reaction from acetyl-CoA to HMG-CoA, which is the substrate of NADPH-dependent, membrane-bound HMG CoA reductase (HMGR, EC 1.1.1.34) (Bach *et al.*, 1991). HMGR is the major rate-limiting enzyme in the isoprenoid biosynthesis pathway. Intermediates in this pathway serve as end products essential for normal cell growth and function (Masferrer *et al.*, 2002).

2.3.1 Sterols

Sterols, which are members of the vast family of isoprenoids, are essential molecules for all eukaryotes. In addition to their widely recognized roles as architectural components of cell membranes and their importance for regulating membrane fluidity and permeability, sterols are also able to modulate a variety of metabolic and ontogenetic events. This is particularly true for cholesterol, the most notorious sterol, whose signaling functions in cell division, cell growth, cell death and various developmental processes have been extensively studied in mammals (Edwards and Ericsson, 1999).

In comparison, such a wealth of information about plant sterols is still far from being available (Hartmann, 2004). Animals and fungi usually synthesize a major sterol end product – cholesterol and ergo sterol, respectively – whereas, plants produce a bewildering array of sterols, with sitosterol, stigma sterol and 24-methylcholesterol as the most represented compounds.

In a tobacco mutant callus, containing up to ten fold more sterols than the wild genotype, HMGR activity is increased by a factor of approximately three (Gondet *et al.*, 1992). Tobacco plants over expressing HMGR has been shown to produce higher amounts of sterol intermediates and endproducts (Schaller *et al.*, 1995.) These results confirm the key regulatory role of HMGR in sterol biosynthesis.

Josekutty (1998a) selected variant cell lines of *Solanum xanthocarpum* resistant to mevinolin, a specific inhibitor of HMGR, using the cell plate technique. A selected cell line exhibited more than two fold increase in sterol content and three fold increase in steroidal alkaloid solasodine.

The enzyme HMGR catalyzes the formation of mevalonate which is a reaction common to both sterol and sesquiterpenoid biosynthesis (Chappell *et al.*, 1989). Chappell (1995) reported that cell division activity requires a significant level of sterol biosynthesis. Decrease in sterol level causes dwarfing, early senescence and sterile phenotype. It has been proposed that the distribution of auxin is altered in these mutants because the changed membrane sterol levels leads to a variation in the distribution of membrane proteins (Willemsen *et al.*, 2003).

Sterols are important not only for structural components of eukaryotic cell membranes but also for biosynthetic precursors of steroid hormones. In plants, the diverse functions of sterol derived brassinosteroids in growth and development have been investigated rigorously. Both sterols and brassinosteroids are active regulators of plant development and gene expression (He *et al.*, 2003).

Sterol biosynthesis and accumulation have been studied using molecular genetic tools (Benveniste, 2004). The characterization of the enzymatic components of plant sterol biosynthesis, phenotypic description of a set of *Arabidopsis thaliana* sterol mutants and the identification of aspects of growth and development influenced by

sterols have been a very fruitful area of research in recent years. The overall data obtained in this field have shown an essential role of sterols at the cellular level in hormone signaling, organized divisions and embryo patterning (Schaller, 2004).

2.3.2 Pharmaceuticals

Some important pharmaceutical agents such as taxol (Wani *et al.*, 1971) and gingkolides (Schepmann *et al.*, 2001) are produced from isoprenoid compounds. Taxol is a diterpene derived from *Taxus* spp and is an effective cancer drug used widely in the treatment of a variety of cancers (Goodman and Walsh, 2001).

Studies conducted by Lansing *et al.* (1991) and Zamir *et al.*(1992) showed that when supplied with labelled mevalonate, *Taxus* plants could produce high rates of radio actively labeled taxol, indicating that taxol was produced from mevalonate which in turn was produced by HMGR.

2.4. EFFECT OF HMGR ON GROWTH AND DEVELOPMENT

Korth *et al.* (1997) reported that members of specific HMGR-encoding gene subfamilies are involved in flower development in potato. Later works have shown the accumulation of HMGR protein in apical meristematic tissue of *Solanum tuberosum* suggesting post-translational regulation of potato HMGR affected by the plant development. The *hmg* 2 transcript accumulates developmentally in young flowers, and in mature sepals and ovaries, whereas transcript for *hmg*3 accumulates in mature petals and anthers (Korth *et al.*, 2003).

Genschik *et al.* (1992) showed that HMGR is expressed in freshly isolated mesophyll protoplasts of *Nicotiana sylvestris* cells during transition from G0 to G1 phase as well as in actively dividing cell suspension cultures and in apexes. This strongly confirms the role of the gene in the cell cycle. *Arabidopsis* plants containing

hmg1 mutation exhibited dwarfing, early senescence, male sterility and reduced sterol levels (Suzuki *et al.*, 2004).

Mevinolin, a specific inhibitor of HMGR blocked root growth in etiolated seedlings of radish. It has been shown that radish seedlings treated with the fungal metabolite mevinolin, exhibited a drastic decrease in root elongation (Bach, 1985). Northern blot analyses of HMGR in *Taxus media,* a gymnosperm, demonstrated that the expression of the gene influenced the growth and development of needles and stem, as well as taxol biosynthesis (Liao *et al.*, 2004).

Mevalonate serves as a precursor in the biosynthesis of natural rubber which is cis-1, 4 poly isoprene of high molecular weight (Westall, 1968). HMGR has been reported to be present in the pelleted portion of centrifuged latex in *Hevea braziliensis* and has been implicated to be membrane bound (Sipat, 1982a; 1982b). The enzyme activity and rubber content of latex are positively correlated suggesting that the enzyme regulates the synthesis of rubber in the latex (Suvachittanont and Wititsuwannakul, 1995).

2.4.1 Role in fruit development

In tomato HMGR is encoded by four different *hmgr* genes, out of which, *hmg*1 is highly expressed during the early stages of fruit development, when sterol biosynthesis is required for membrane biogenesis during the cell division and expansion (Narita and Gruissem, 1989). The *hmg*2 expressed is not detectable in young fruits, but is activated during fruit maturation and increases during ripening, in parallel with accumulation of lycopene (Gillaspy *et al.*, 1993).

In developing young fruit, arachidonic acid (AA) blocked fruit growth, inhibited *hmg*1 and activated *hmg*2 expression. These results are consistent with other reports indicating that *hmg*1 expression is closely correlated with growth processes

requiring phytosterol production. In mature green fruit, AA strongly induced the expression of *hmg*2 and lycopene accumulation before the normal onset of carotenoid synthesis and ripening. The induction of lycopene synthesis was not blocked by inhibition of HMGR activity using mevinolin, suggesting that cytoplasmic HMGR is not required for carotenoid synthesis. These results are consistent with the function of an alternative plastid isoprenoid pathway (Rohmer pathway) that appears to direct the production of carotenoids during tomato fruit ripening (Rodriguez-Concepcion and Gruissem, 1999).

The tomato fruits treated with AA remained small and weighed 50 per cent less than control fruits. The reduction in *hmg*1 transcript levels and the concomitant growth inhibition after AA treatment suggests that full *hmg*1 expression is required during fruit development.

The inhibitory effect of AA on fruit growth is similar to that caused by mevinolin (Narita and Gruissem, 1989). However in contrast to mevinolin, which caused complete inhibition of HMGR enzyme activity, AA reduced *hmg*1 mRNA levels but induced *hmg*2 transcripts, which are normally undetectable in young fruits (Gillaspy *et al.*, 1993).

2.4.2 Role in seed development

It is known that following fertilization, rapid cell divisions occur throughout the endosperm to about 12 to 13 days after pollination (DAP). The mitotic activity in the central region of the endosperm peaks at about eight to ten DAP, rapidly declines about 12 to 13 DAP, and then ceases, while kernel development continues (Kowels and Philips, 1985). The activity of HMGR is very high in 12 DAP in parallel with high mitotic activity. At 13 DAP, the HMGR decreases sharply, possibly marking the transition from cell division to differentiation. The HMGR activity increases in the endosperm during the periods when rapid cell divisions are occurring. This occurs

before maximum zein protein accumulation (Bewley and Marcus, 1990; Ober *et al.*, 1991).

Although many compounds are potentially required during seed development, little is known about the seed's specific isoprenoid requirements. Bach (1987) reported that ABA is one of the many end products of the isoprenoid biosynthetic pathway, requiring MVA as a specific precursor. It is known that the isoprenoid ABA is absolutely required in maize seeds to control or maintain aspects of seed dormancy, germination and water relations (Gage *et al.*, 1989; Belefont and Fong, 1991).

There is evidence that isoprenoid compounds are necessary during seed development and many functions to regulate specific developmental processes. These compounds include membrane sterols and ABA (Gage *et al.*, 1989). In addition, synthesis of other isoprenoids necessary for early germination events may also occur during seed development (Bewley and Marcus, 1990).

2.4.3 Role in cell division

The activity of HMGR has been investigated in a variety of young plant tissues (Bach *et al.*, 1990; Ji *et al.*, 1992). Moore and Oishi (1993) observed high levels of embryo specific HMGR activity during the earlier phase of seed development, a time period during which rapid cell divisions occur. The high levels of HMGR activity correlate with the requirements in dividing cells for isoprenoid compounds such as sterols and electron transport chain components.

This pattern of HMGR activity suggests that HMGR may be regulated in the embryo during early seed development when rapid cell divisions and differentiations are occurring (Randolph, 1936; Kiesselbach, 1949). There are evidences for the high levels of embryo HMGR in mammalian systems (Quesney-Huneeus *et al.*, 1979) and in yeast (Basson *et al.*, 1986). This may reflect the need for steroids, which are

believed to be essential for plant cell division (Grossman *et al.*, 1985; Haughan *et al.*, 1987). Studies of HMGR activity in meristematic maize tissues indicate that HMGR is critical for cell division and cell growth (Ji *et al.*, 1992).

2.4.4 Relation of HMGR with growth hormones

Many *in vitro* studies have revealed that HMGR is relatively abundant in rapidly growing tissues such as immature anther and apical dome. The reason for the elevated level of HMGR mRNA in meristematic tissue is unknown; however, it may be partially attributed to the presence of auxin (2,4-D) in the medium, which generally is required for sustained cell growth and division of plant tissue in culture.

It has been reported that HMGR is essential for cytokinin biosynthesis in tobacco Bright Yellow-2 cultured cells and inhibition of HMGR reduces cytokinin content and cell proliferation activity (Crowell and Salaz, 1992; Laureys *et al.*, 1998; Miyazawa *et al.*, 2002). *Arabidopsis* seedlings grown in the presence of lovastatin display inhibited cell elongation and reduced expression of many cell elongation related genes (Suzuki *et al.*, 2003).

Cytokinins, a class of hormones synthesized from IPP and DMAPP in the mevalonate pathway are strong antagonists of senescence (Gan and Amasino, 1995; Ori *et al.*, 1999). Quantification of endogenous cytokinins demonstrated that *hmg*1 mutation causes the reduction in cytokinin levels.

Brassinosteroids including brassinolides are also produced by the MVA pathway. *Arabidopsis* plants containing *hmg*1 mutation showed brassinosteroid deficiency and reduced cell elongation (Fujioka *et al.*, 1997). It resulted in dwarf phenotypes derived from reduced sterol production.

Cowan *et al.* (1997) investigated the effect of isoprenoid growth regulators on avocado fruit growth and mesocarp HMGR activity. Both normal and small fruit phenotypes were used to probe the interaction between end products of isoprenoid biosynthesis and the activity of HMGR in the metabolic control of avocado fruit growth. Application of mevastatin, a competitive inhibitor of HMGR reduced the growth of normal fruit and increased the mesocarp ABA concentration.

The arrest of cell growth by inhibitors of HMGR has been demonstrated using cell cultures from *Nicotiana tabacum* (Crowell and Salaz, 1992; Morehead *et al.*, 1995; Hemmerlin and Bach, 1998), *Catharanthus roseus* (Imbault *et al.*, 1996), *Solanum xanthocarpum* (Josekutty, 1998b) and *Lycopersicon esculentum* (Jelesko *et al.*, 1999). Sub-lethal doses of mevastatin retards cell growth while higher doses result in loss of cell viability and cell death (Mitchell and Cowan, 2003).

2.5 ROLE OF HMGR IN PLANT DEFENCE MECHANISM

Studies about HMGR activity have been conducted in different tissues such as pea seedlings (Brooker and Russel, 1975) and sweet potato roots (Suzuki and Uritani, 1976) .The experiments conducted by Oba *et al.* (1985) have shown that HMGR in potato tubers is the rate-determining enzyme in the formation of defence compounds as it has been shown for cholesterol biosynthesis by Slakey *et al.* (1972) and for furanoterpene biosynthesis by Suzuki *et al.* (1975).

There is an increase in HMGR activity prior to the accumulation of sesquiterpenoid phytoalexins in elicitor treated plants.(Chappell and Nable, 1987; Vogeli and Chappell, 1988). However data from experiments involving elicitor treated potato tuber discs and tobacco cell suspension cultures suggest that regulation of other enzymes of the mevalonate pathway may also play an important role in the synthesis of sesquiterpenoid phytoalexins.

The activity of HMGR is detected in control or non-elicited tissue of potato tuber (Stermer and Bostock, 1987) and tobacco cell suspensions (Chappell and Nable, 1987) despite the absence of detectable phytoalexin accumulation. In elicited tobacco cell suspension cultures, the induction of HMGR activity was rapid and transient; enzyme activity returned to the level of non-elicited control tissue by the time the sesquiterpenoid biosynthesis rate was maximal.

The rapid and transient kinetics of induction of HMGR gene can be contrasted with the much slower and persistent induction of other rice defence genes. Such kinetics recommends the HMGR promoter for use as an inducible promoter in engineered resistance. Its rapid rate of induction could be important in outpacing pathogen penetration, while its transient nature of induction could serve to minimize selection pressure placed on the pathogen (Weissenborn *et al.*, 1995).

It has been reported that steroid glycoalkaloids are produced after slicing and incubating potato tubers. (Allen and Kuc, 1968; Ishizaka and Tomiyama, 1972). The increased activity of HMGR after slicing participates in the biosynthesis of steroid glycoalkaloids. A large amount of steroid glycoalkaloids accumulated in sliced potato tuber tissue, but infection of the sliced tissue by *P. infestans* reduced the accumulation of steroid glycoalkaloids and induced accumulation of sesquiterpenoids such as rishitin. Kuc *et al.* (1976) showed that pathogen infection shifted the mevalonate pathway from steroid glycoalkaloid to sesquiterpenoid syntesis.

Choi *et al.* (1994) used gene specific probes to study different *hmgr* genes (*hmg1 and hmg2*). They showed that methyl jasmonate could induce *hmg*1 expression and glycoalkaloids, produced after wounding, could reduce the abundance of *hmg*2 transcripts. Thus arachidonate and jasmonate responsive pathways are distinct in relation to *hmgr* gene expression and isopernoid product accumulation.

Potato cultivars infected with *Phytophthora infestans* isolates exhibited high level expression of *hmg*2 transcripts. The induction of *hmg*2 was first observed in uninoculated leaflets close to the inoculated leaflets, then in uninoculated leaflets of leaves adjacent to the inoculated leaf and finally in local inoculated leaflets. The stronger expression of the gene in proximal and distal leaflets, as compared to the local site of inoculation suggests the translocation of signals from this site to healthy parts of the plant (Wang *et al.*, 2004).

2.5.1 Defence elicitors

Chappell and Nable (1987) reported that tobacco cell suspension cultures do not produce extra cellular sesquiterpenoids until stimulated with fungal elicitors. Upon addition of elicitor to a culture, sesquiterpenoids began appearing in the media five to six hours after initiation of the treatment and reached the maximum 10 to 15 hours later. Changes in the enzyme activities of HMGR with respect to time after fungal elicitor addition to cultures have been reported (Vogeli and Chappell, 1988). The peak of HMGR activity was reached within four to six hours.

Many compounds isolated from microbial preparations, fungal cell walls, infected plant material, or plant cells treated with digestive enzymes function as elicitors to trigger host response. These elicitors have been instrumental in molecular analyses of defence related gene activation involved in host resistance (Lamb *et al.*, 1989; Dixon and Harrison, 1990). Phytoalexins have been linked to the localized cell death characteristic of hypersensitive resistance (HR) response (Snyder and Nicholson, 1990).

Yang *et al.* (1991) have shown that wounding triggers an increase in *hmg*2 mRNA levels in potato and tomato with kinetics typical of many defence related genes. Treatment with elicitors or inoculation with the soft rot bacterium *Erwinia carotovora* triggers a significantly greater induction of *hmg2* mRNA than wounding

(Park *et al.*, 1992). In contrast, tomato *hmg1* expressions is not induced by defence elicitors, but is elevated in tissues undergoing cell division and thus may be associated with sterol biosynthesis.

In potato tubers, *hmg2* and *hmg3* are similarly activated by wounding and elicitor treatments. Unlike *hmg2* and *hmg3, hmg1* is suppressed by elicitor (Choi *et al.*, 1992). Thus *hmgr* gene appears to show complex defence related regulation in both tomato and potato. In tomato, the *hmg2*:GUS expression obtained in excised leaves inoculated with *Erwinia carotovora* spp. *carotovora* and intact hypocotyls of seedlings inoculated with the fungal pathogen *Rhizoctonia solani*. In both interactions, GUS activity was highly expressed in the host cells directly surrounding the site of inoculation and resulting lesion.

The strong induction of *hmg*2 expression and HMGR activity in tomato by the fungal elicitor AA is due to the result of cellular defence response. The *hmg*2 expression is induced not only by elicitors but also by direct pathogen inoculation (Cramer *et al.*, 1993; Weissenborn *et al.*, 1995).

The pattern of AA-induced *hmg2* mRNA accumulation in tomato fruit was very similar to that of the corresponding *hmg2* gene in potato (Choi *et al.*, 1992) and to that of other transcriptionally regulated defence genes in plants (Matton and Brisson, 1989; Koch *et al.*, 1992). The *hmg*2 activation could be a more general response to cellular disintegration, in particular during fruit ripening and in plant defence.

The gene *hmgr*1 isolated from rice showed expression at low levels in both vegetative and floral organs. In rice plants, the gene expression is not induced by wounding, but is strongly and rapidly induced in suspension cells by a fungal cell wall elicitor from the pathogen *Magnaporthe grisea,* causal agent of rice blast disease. This

suggests that *hmgr*1 is important in the induction of rice phytoalexin biosynthesis in response to pathogen attack (Nelson *et al.*, 1994).

Potato tubers synthesize anti fungal sesquiterpenoid phytoalexins in response to fungal infection or arachidonic acid elicitation and toxic steroid glyco alkaloids in response to wounding. The activity of HMGR has been shown to increase rapidly in response to these stimuli. HMGR levels increased 30 fold following arachidonic acid treatment and 15 fold following wounding (Bianchini *et al.*, 1996).

2.5.2 Role in phytoalexin production

In rice, two types of diterpene isoprenoid phytoalexins, momilactones (Cartwright *et al.*, 1981) and oryzalexins (Kodama *et al.*, 1992; Sekido *et al.*, 1986) have been isolated and characterized. Both have been shown to be effective in inhibiting *in vitro* growth of the fungal pathogen *M. grisea.*

The *hmg2* gene was coordinately and sequentially regulated for the biosynthesis of defence related sesquiterpene phytoalexins in pepper (Ha *et al.*, 2003). The expression of *hmg2* in pepper was rapidly induced within one hour in response to a fungal pathogen and continuously increased up to 48 hours. Wound and elicitor mediated induction of *hmg*2 is generally transient; RNA analyses suggest that *hmg*2 transcript levels peak between 9 and 24 hours and are quite low by 48 hours (Yang *et al.*, 1991).

Sesquiterpenoid phytoalexins such as rishitin accumulate in the necrotic potato tuber tissue infected by incompatible races of *Phytophthora infestans* (Tomiyama *et al.*,1968; Sato *et al.*,1968; Varns *et al.*,1971). Production of phytoalexins is also induced by treatment of potato tuber slices with a hyphal wall component of *P. infestans* (Lisker and Kuc, 1977; Henfling *et al,* 1980; Sakai *et al.*, 1981). Enzymological studies conducted in sweet potato roots have shown that

HMGR is the key enzyme in the biosynthesis of phytoalexins. (Ito *et al.*, 1979; Oba *et al.*, 1982).

2.5.3 Role in controlling parasitism

Parasitisation by *Orobanche aegyptiaca* in tobacco induces expression of *hmg2,* a defence related isogene of HMGR. *Orobanche* is one of the most destructive weeds because they extract water and photosynthates directly from crop plants, causing significant reductions in crop yield and quality (Sauerborn, 1991).

These parasitic weeds are difficult to control because they are closely associated with the host root and are concealed underground for most of their life cycle. Applying herbicides to the subterranean parasite is also difficult, and is further complicated by the lack of herbicide selectivity between host and *Orobanche* (Foy *et al.*, 1989).

Transgenic tobacco plants expressing a construct containing 2.3 Kb of the tomato *hmg* 2 gene promoter fused to the GUS reporter gene were parasitized by *O. aegyptiaca.* Expression of the *hmg*2:GUS gene construct was detected within one day following penetration of the host root by the *O. aegyptiaca* radicle and was localized to the region immediately around the site of parasite invasion. This expression continued and intensified over the course of *O. aegyptiaca* development.

In addition, the *hmg*2:GUS expression was induced by secondary parasitization, where secondary roots of *O. aegyptiaca* contacted the host root at a distance from the primary attachment site. Such secondary attachments have been reported in *Orobanche* and have been the subject of detailed anatomical studies (Dorr and Kollmann, 1975; Kuijt, 1977). This GUS expression was specific to plants containing *hmg*2: GUS construct and was not observed in control plants (Westwood *et al.*, 1998).

The wounding by excision, insect predation and elicitor treatment also triggered induction of the *hmg*2:GUS construct. The *hmg*2 is activated by such a diversity of pathogens in both compatible and incompatible interactions; it serves as an interesting and promising candidate conferring resistance.

2.5.4 Role in biocontrol

When attacked by herbivorous insects or mites, some plant species emit mixtures of volatile compounds dominated by terpenoids. These compounds attract carnivorous arthropods that prey on or parasitize herbivores and so reduce further damage. This fascinating defence strategy offers a new, environmentally friendly approach to crop protection. HMGR plays a major role in terpenoid composition and synthesis. By the genetic manipulation of existing rate of terpenoid emission and composition, attacked crop plants can be enabled to attract enemies and reduce additional herbivory, without compromising the other modes of defence (Degenhardt *et al.*, 2003).

2.5.5 Nematode induced expression

Terpenoid phytoalexins are toxic to nematodes.and are involved in various plant- nematode interactions (Mahajan *et al.*, 1986; Zacheo and Bleve-Zacheo, 1988). Transgenic tomato seedlings containing the *hmg2*: GUS gene were inoculated with second stage juveniles of *Meloidogyne incognita* and *M. hapla.* No GUS activity was seen in root tips of uninoculated seedlings or in infected roots within the first 48 hours after inoculation. However, once feeding and galling was initiated, high levels of GUS activity were observed localized to the galling tissue. This result suggests that *hmg*2 may be a nematode response gene (Cramer *et al.*, 1993).

2.6 HMGR ACTIVITY IN ANIMAL CELLS

In animal cells the enzyme is found almost exclusively in the microsomes (Bucher *et al.*, 1960; Siperstein and Fagan, 1966). It has been extensively characterized in mammals in which it catalyses the major rate limiting step in cholesterol biosynthesis. The HMGR activity is controlled through the complex processes at both transcriptional (Osborne *et al.*, 1985; Reynolds *et al.*, 1985) and translational level (Chin *et al.*, 1985; Gil *et al.*, 1985). Animal HMGR is encoded by a single gene (Istvan and Deisenhofer, 2000).

Mevinolin, a metabolite isolated from the ascomycete, *Aspergillus terreus* (Alberts *et al.*, 1980) is a potent cholesterol-lowering agent in animals (Kroon *et al.*, 1982) and in man (Tobert *et al.*, 1982). A number of HMGR inhibiting compounds commonly referred to as statins are used to lower serum cholesterol level by targeting HMGR. The statins occupy a portion of the binding site of HMG CoA, thus blocking access of this substrate to the active site (Istvan, 2002).

In insects, HMGR is involved in the production of juvenile hormones (Feyereisen and Farnsworth, 1987). Genomic and cDNA clones have been isolated from *Drosophila* (Gertler *et al.*, 1988), yeast (Basson *et al.*, 1988), sea urchin (Woodward *et al.*, 1988) and man (Goldstein and Brown, 1990). HMGR from animal system has been shown to be under developmental control. In sea urchin HMGR activity has been shown to increase 200 fold in the embryo during a period of extensive cell proliferation (Woodward *et al.*, 1988).

2.7 HMGR PROTEIN STRUCTURE

HMGR polypeptides can be divided into four domains. (i) N, the N terminal, (ii) TM, the transmembrane domain, (iii) L, the linker sequence which is defined as the region between TM and the start of the sequence conserved among animal and plant HMGRs and (iv) C, the cytoplasmic domain which carries the catalytic site. The

TM and the C domains are well conserved at the amino acid level among plant HMGRs. The main difference between plant and animal HMGRs is the presence of two trans membrane domains in the former case and seven in the latter case (Chin *et al.*, 1984). It has been shown in hamster (Liscum *et al.*, 1985) and in yeast that these domains are involved in anchoring the enzyme to endoplasmic reticulum membrane.

Other important features of the primary amino acid sequence are the conservation of glycine, cysteine and histidine residues. Cystein residues are important in the active site, since HMGR is known to require high concentrations of thiol reducing agents for its activity. One of the conserved histidine residue gets protonated during the conversion of HMG CoA to mevalonate (Liscum *et al.*, 1985). Glycine residues might be important for the correct maintenance of secondary structures (Caelles *et al.*, 1989).

2.8 HMGR IN DIFFERENT CROPS

Hevea HMGR has been implicated to be a membrane bound enzyme (Sipat, 1982b) present in the pelleted portion of the centrifuged latex that requires NADPH and thiol compounds for its activity (Sipat, 1985). The laticifer specific *hmg1,* which is involved in rubber biosynthesis, is probably unique to *Hevea* and does not possess a corresponding member in other plants that do not produce rubber.

The co-expression of *Hevea* HMGR and tobacco sterol methyl transferase I (SMT1) under control of both constitutive seed specific promoters resulted in increased accumulation of total sterols in seed tissue by 2.5 and 2.1 fold respectively (Holmberg *et al.*, 2003).

Annatto (*Bixa orellana*), is a tropical plant of great agro industrial interest because of the high content of bixin , a carotenoid pigment produced in seeds and which is widely used in the food industry. A fragment of 503 bp of the *hmg* gene

derived from genomic DNA was obtained using the polymerase chain reaction (PCR) and used as a probe to analyze the *hmg* copy number and expression pattern. Southern blot analysis suggested that *hmg* is encoded by a multigene family. Among the different plant tissues, the highest specific enzyme activity was observed in seeds, particularly at the immature stage when it was 3.6 fold higher than that in the mature seed (Narvaez *et al.*, 2001).

In apple, oxidation products of the sesquiterpene, 2-farnesene are thought to induce necrosis of cell layers just beneath the fruit skin, leading to development of scald symptoms. As a part of an effort to devise a molecular genetic strategy for controlling this storage disorder, two different cDNA fragments designated as *hmg*1 and *hmg*3 has been cloned from mRNA obtained from peel tissue of apple (Pechous and Whitaker, 2002).

Studies conducted in rubber have shown that reduction of HMG Co A in latex requires NADPH, as in yeast and liver (Linn, 1967). *Hevea* calmodulin can activate HMGR in latex (Wititsuwannakul *et al.*, 1990). The *hmg*2 expression in *Arabidopsis thaliana* is restricted to meristematic (root tip and shoot apex) and floral (secretory zone of the stigma, mature pollen grains, gynoecium, vascular tissue and fertilized ovules) tissues (Enjuto *et al.*, 1995).

The promoter region of tomato *hmg*2 has been analyzed using the transient expression of *hmg*2-luciferase fusions in red fruit pericarp. The mRNA for *hmg*2 accumulates to high levels during fruit ripening, in a pattern that coincides with the synthesis of the carotenoid lycopene. Unlike most promoters, the region that is upstream of the *hmg*2 TATA element is not required for high-level expression (Daraselia *et al.*, 1996).

2.9 CHARACTERIZATION OF *hmgr* GENE

Two genes of *hmgr* have been reported in yeast (Basson *et al.*, 1988) and in *Arabidopsis thaliana* (Bach,1987) while only one form is known to be present in mammals.(Chin *et al.*, 1984; Luskey and Stevens, 1985). A 2.4 kb *hmgr* mRNA trancscript was identified in *Hevea* and *Arabidopsis* while a 3.0 kb transcript has been reported in tomato (Narita and Gruissem, 1989). In plants, HMGR is usually encoded by a multi gene family (Stermer *et al.*, 1991). In *Hevea brasiliensis*, HMGR is encoded by a small gene family comprising of three members, *hmg1, hmg2* and *hmg3* (Chye *et al.*, 1992).

The occurrence of more than one form of HMGR in plants is not unexpected as it has been previously suggested that sub cellular compartmentation of different forms of the enzyme occurs (Brooker and Russell, 1975). Mitochondrial HMGR has been identified in sweet potato (Suzuki and Uritani, 1976) and plastid and microsomal HMGR in pea seedlings (Wong *et al.*, 1982).

There are reports about the presence of two *hmgr* genes in *Arabidopsis* (Bach, 1987) and rice (Nelson *et al.*, 1994), one to two genes in tobacco (Genschik *et al.*, 1992), three genes in rubber (Chye *et al.*, 1992) and potato (Choi *et al.*, 1992), four genes in tomato (Park *et al.*, 1992) and wheat (Aoyagi *et al.*, 1993). In most plants the gene contains four exons with three intervening introns. The intron-exon junctions appear to be at identical sites in all of the plant genes.

Genomic southern analysis using specific probes has confirmed the presence of at least two *hmgr* genes in apple. The two genes are differentially expressed during low temperature storage and in response to ethylene with *hmg1* being expressed constitutively and *hmg2* being relatively more sensitive to developmental stimuli and ethylene (Rupasinghe *et al.*, 2001).

Molecular characterization of *hmgr* has been done in *Catharanthus roseus* (Maldonado-Mendoza *et al.*, 1992), *Camptotheca acuminata* (Maldonado-Mendoza *et al.*, 1997), cotton (Scott *et al.*, 1999), mulberry (Jain *et al.*, 2000), melon (Kato *et al.*, 2001), pepper (Ha *et al.*, 2003) and sweet potato (Kondo *et al.*, 2003). Ha *et al.* (2001) isolated *hmg2* gene by RT-PCR and by screening of rice genomic and cDNA library. The transcripts were constitutively detected in all parts of the rice plant. The gene *hmgr* has been cloned and characterized from the Zygomycete, *Phycomyces* spp.. Single copy of the gene is present in the genome (Ruiz-Albert *et al.*, 2002).

Studies conducted in *Cathranthus roseus* have shown that HMGR has significant role in the synthesis of mono terpene components of indole alkaloids. A 444 bp fragment was generated from C. *roseus* and used as a probe to screen a *C. roseus* seedling cDNA library. The hydropathy profile of the predicted sequence indicates the presence of two hydrophobic domains, which appear to be typical of other plant HMGRs. (Caelles *et al.*, 1989; Chye *et al.*, 1991).

Three kinds of cDNA clones encoding HMGR were isolated from Korean red pepper (*Capsicum annuum*) and the *hmg2* gene was especially obtained from a cDNA library constructed with *Phytophthora capsici* infected pepper root RNAs. The *hmg*2 encoding 604 amino acid peptide had typical features as an elicitor induced isoform among HMGRs on its gene structure and had a predicted amino acid sequence homology (Ha *et al.*, 2003). Girija *et al.*(2005) have characterized a 501 bp sequence of HMGR from *Piper colubrinum,* which is known to be tolerant to foot rot disease. Specific degenerate primers were used to amplify the gene from cDNA.

2.10 REGULATION OF GENE EXPRESSION

The diversity of isoprenoid compounds and presence of multi genes suggest that multiple pathways exist for the biosynthesis of IPP. The biosynthesis of different isoprenoid compounds like chlorophylls and natural rubber, which occurs in

specialized cell types further, suggests that HMGR genes may be expressed differentially. Yang *et al.* (1991) used a tomato *hmg* probe to show the presence of two *hmg* genes in potato, one isogene is induced by wounding while the other is induced by pathogen challenge.

Examination of the *hmg 3* promoter sequence upstream of the transcription site showed the absence of a typical TATA box. Promoters of many house keeping genes including hamster *hmg* (Reynolds *et al.*, 1984) and human *hmg* (Luskey, 1987) also lack a TATA box suggesting that *Hevea hmg3* is also a constitutively expressed gene. However GC rich hexanucleotide sequences (CCGCCC or GGGCGG) and multiple transcription start sites which are detected in hamster *hmg* and human *hmg* are not found in *Hevea hmg* (Chye *et al.*, 1992).

Arkhipova and Ilyin (1991) have reported a specific class of RNA polymerase II promoters, which lacks both TATA box and GC rich region. HMGR involved in rubber biosynthesis (*hmg1*) can be induced by ethylene treatment. It has been proved that ethephone (2 chloro ethane phosphonic acid) which generates ethylene *in vivo* can stimulate latex yield when applied on trunks of rubber trees.

In *Arabidopsis* genome, the two encoded proteins (*hmg1* and *hmg2*) have the same structural organization and intracellular location. But the expression profiles of these genes are different. The *hmg*1 mRNA can be detected in all *Arabidopsis* tissues (Enjuto *et al.*, 1994). But *hmg*2 is expressed exclusively in meristematic and floral tissues (Enjuto *et al.*, 1995).

Addition of calcium to cell cultures of potato results in a decrease in HMGR protein accumulation, whereas EGTA, a chelator of calcium, causes an increase in levels of HMGR observed on immunoblots. It has been shown that potato HMGR is subjected to many of the same post transcriptional regulatory mechanisms as have been shown in animal and yeast systems (Crane and Korth, 2002).

2.10.1 Regulation by light

In vivo activity of HMGR has been shown to be light regulated in maize leaf tips, pea, radish and potato tuber tissue (Gray, 1987; Stermer and Bostock, 1987; Ji *et al.*, 1992). Moore and Oishi (1993) demonstrated a two to four fold increase in the activity in etiolated maize shoots indicating a light dependent inhibition of HMGR activity. The microsomal HMGR activity is also lower in roots grown under light conditions. Work on HMGR in potato showed that the amount of enzyme activity can be reduced 50 per cent in several minutes by white light suggesting rapid post translational control. A likely candidate for such control is phosphorylation or dephosphorylation of HMGR, a mechanism shown to regulate HMGR activity in pea (Russell *et al.*, 1985).

In *Arabidopsis*, *hmg*1 is actively expressed in leaves and light grown seedling and it represents the major HMGR transcript (Caelles *et al.*, 1989). Studies conducted on various plant HMGR promoters revealed the light dependent regulation of HMGR promoters (Jain *et al.*, 2000; Learned, 1996). Seetha *et al.* (2005) have cloned the partial promoter region of *Andrographis paniculata hmgr,* which showed the presence of light regulatory *cis* acting elements.

In wheat, three HMGR isozymes have been examined to study developmental regulation. Two genes were found to be light regulated. (Aoyagi *et al.*, 1993). The expression of *hmg1* is suppressed by light. Enjuto *et al.* (1994) reported that HMGR mRNA accumulates in dark grown *Arabidopsis* plants. Learned and Connolly (1997) showed that the *hmg*1 promoter is differentially responsive to light in different organs and promoter activation by light deprivation is confined primarily to immature leaves. In contrast, expression of the *hmg*1 gene in roots is confined to the elongation zone and is not responsive to illumination.

Materials and Methods

3. MATERIALS AND METHODS

The study entitled 'Molecular characterization of 3-hydroxy-3-methylglutaryl CoA reducatse (*hmgr*) gene from solanaceous plants' was carried out in the molecular biology laboratory of the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara from May, 2004 to September, 2005. The materials used and the methodologies adopted in this study are described below.

3.1. ISOLATION OF GENOMIC DNA

The four selected solanaceous plants were raised at College of Horticulture (Plate 1). Genomic DNA was isolated from leaf tissue of the four solanaceous plants, *Solanum xanthocarpum, S. nigrum, S. torvum* and *Physalis minima* following modified Doyle and Doyle (1987) method.

3.1.1 Reagents

1. Extraction buffer (4X)
2. Lysis buffer
3. Sarcosine (5 %)
4. TE buffer
5. Ice-cold Isopropanol
6. Chloroform-Isoamyl alcohol (24:1 v/v)
7. Ethanol 70 per cent (v/v)

(Chemical compositions of reagents are given in Annexure I)

3.1.1 Procedure

1. Leaf sample weighing 0.5 g was freeze powdered in liquid nitrogen and ground with 6 ml of 1X extraction buffer, 50 µl β mercapto ethanol and a pinch of sodium metabisulphate using an autoclaved mortar and pestle.
2. To the fine paste material, 6 ml lysis buffer and 1 ml sarcosine were added and contents transferred to a 50 ml centrifuge tube.

Solanum xanthocarpum

Solanum nigrum

Physalis minima

Solanum torvum

Plate 1. Solanaceous plants selected for the study

3. The tubes were incubated in a water bath at 65° C for 10 minutes, with intermittent shaking
4. The tubes were removed from the water bath and equal volume of chloroform:isoamyl alcohol mixture (24:1 v/v) was added and mixed by gentle inversion.
5. Centrifugation was carried out at 10,000 rpm for 10 min at 4°C.
6. The clear aqueous phase was transferred to a fresh tube.
7. Ice-cold isopropanol, 0.6 volumes was added and after gentle mixing, kept in-20°C deep freezer for 30 minutes for complete precipitation of DNA.
8. DNA was pelleted at 10,000 rpm for 10 min at 4°C.
9. The pellet was washed with 70 per cent (v/v) ethanol by centrifuging at 10,000 rpm for 10 min at 4°C.
10. The supernatant was discarded and the pellet air-dried.
11. Dried pellet was dissolved in 100 µl of TE and stored at – 20°C until further use.

3.2 AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis was performed based on the method described by Sambrook *et al.* (1989) to check the quality of DNA and also to separate the amplified products.

3.2.1 Materials

1) Agarose : (Genei, Low EEO)
2) 50X TAE buffer (pH 8.0)
3) Electrophoresis unit, power pack, casting tray, comb.
4) 6X Loading/Tracking dye
5) Ethidium bromide solution (stock 10mg/ml; working concentration, 0.5µg/ml)
6) UV transilluminator (Herolab^{R})
7) Gel documentation and analysis system (Alpha imager TM 1200)

(Chemical compositions of the buffer and dyes are given in Annexure II)

3.2.2 Procedure for casting, loading and running the gel

1. Four hundred ml of electrophoresis buffer (1x TAE) was prepared to fill the electrophoresis tank and to prepare the gel.
2. The open ends of the gel-casting tray were sealed with a cellophane tape and placed on a perfectly horizontal leveled platform.
3. Agarose (1.0 per cent (w/v) for genomic DNA and 0.8 (w/v) for PCR) was added to 1X TAE, boiled till the agarose dissolved completely and then cooled to lukewarm temperature. Ethidium bromide was added to a final concentration of 0.5 μg / ml as an intercalating dye of DNA, which will help in its visualization in UV rays.
4. It was then poured into the gel mould, the comb placed properly and allowed to solidify.
5 After the gel was completely set (30-45 minutes at room temperature), the comb and cellophane tape were carefully removed.
6. The gel was placed in the electrophoresis tank with the wells near the cathode and submerged in 1X TAE to a depth of 1 cm.
7. A piece of cellophane tape was pressed on a solid surface and 1 μl 6X loading buffer was dispensed in small quantity on the tape. A quantity of 3-5 μl of DNA was added to each slot (in the case of PCR products, 10.0-15.0 μl) mixed well by pipetting in and out for 2 to 3 times. Then the mixture was loaded in the wells, with the help of micropipette. Appropriate molecular weight marker was also added in one of the wells.
8. The cathode and anode were connected to the power pack and the gel was run at a constant voltage of 60 volts.
9. The power was turned off when the tracking dye reached at about 3 cm from the anode end.

3.2.3. Gel photodocumentation

The DNA bands separated by electrophoresis were viewed and photographed using Alpha Imager TM 1200 documentation and analysis system.

3.3 QUANTIFICATION OF DNA

The quantification of DNA was carried out using UV spectrophotometer (Spectronic Genesys 5). Fifteen µl of DNA sample was diluted to 1.5 ml with double distilled water. The absorbance was measured at 260 nm and 280 nm. The ratio of OD_{260}/ OD_{280} values was calculated to detect the purity of DNA. Quantity of DNA present in the pure sample was calculated using the following formula.

OD_{260} = 1 is equivalent to 50 µg double stranded DNA /ml sample.

Quantity of DNA present in the sample = OD_{260} x 50 x dilution factor (µg/ml)

3.4 AMPLIFICATION OF THE *hmgr* GENE

3.4.1 Primer designing

1. Complete cDNA or mRNA sequences of the *hmgr* gene reported for solanaceous plants (potato, nicotiana, and capsicum) were downloaded from NCBI Genbank (http://www.ncbi.nlm.nih.gov).
2. Open reading frames of the sequences were found out using 'ORF Finder' tool offered by NCBI (http://www.ncbi.nlm.nih.gov/orf).
3. Multiple sequence alignment of ORFs (potato, nicotiana and capsicum) was done using ClustalW 1.83 (www.ebi.ac.uk/clustal)
4. Based on the homology, conserved boxes of 18-24 bases were selected throughout the sequence.
5. The forward and reverse primers were selected from conserved boxes in such a way that
   a) Conserved boxes have GC content not less than 50 per cent
   b) Melting temperature (T_m = 4GC + 2AT) ranged between 60^0C and 70^0C.
   c) The distance between the primers ranged from 500 to 1000 base pairs.
6. For designing primers, the sequence of the forward primer was taken as such and for the reverse primer, the reverse complementary sequence was taken.

Accordingly two pairs of forward and reverse primers were designed and synthesized by Bangalore Genei.

3.4.2 PCR reaction

Polymerase chain reaction was carried out using the *hmgr* specific primers

3.4.2.1 Composition of the reaction mixture for PCR (25.0 µl)

a) Genomic DNA (25 ng) - 1.0µl
b) 10X Taq assay buffer - 2.5µl
c) dNTP mix (10 mM) - 1.0µl
d) Forward primer (2.5 pM) - 1.0µl
e) Reverse primer (2.5 pM) - 1.0µl
f) Taq DNA polymerase (0.3 U) - 2.0µl
g) Autoclaved distilled water - 16.5µl

25.0µl

The reaction mixture dispensed in 0.2 ml tubes was given a momentary spin for thorough mixing of the cocktail components and 15 µl mineral oil was added to prevent evaporation during the reaction. The PCR tubes were then placed in a thermal cycler (PTC-100^{TM} Programmable Thermocycler, MJ Research).

3.4.2.2 Thermal cycler programme

The following programme was set in order to amplify *hmgr* gene from the template DNA.

Step 1: 94 °C for 2 min - Initial denaturation

Step 2 : 94 °C for 1 min - Denaturing
Step 3 : 58 °C for 1 min - Annealing
Step 4 : 72 °C for 2 min – Extension
} 30 cycles

Step 5 : 72 °C for 5 min - Final extension
Step 6 : 4 °C for infinity to hold the sample.

3.5. GEL ELUTION OF PCR AMPLIFIED FRAGMENTS

Products obtained in different PCR reactions were loaded separately on 0.8 per cent (w/v) agarose gel and desired band in each case was eluted using Perfectprep^{R} Gel Cleanup Kit (Eppendorf AG, Germany) (Procedure followed as per the manufacturer's guide lines).

1. DNA fragment of interest was excised from the gel using a sterile, sharp scalpel while avoiding much exposure to UV on a transilluminator.
2. Gel slice was weighed in a colourless 1.5 ml micro centrifuge tube.
3. A three-gel volume of the binding buffer (w/v) was added and the gel mixture incubated at 50°C for 10 min until the gel slice was completely dissolved. Tube was briefly vortexed every 2-3 min during incubation to help the gel dissolve.
4. Once the gel slice was completely dissolved, one gel volume of isopropanol was added and mixed briefly by inversion
5. A spin column was placed in a 2 ml collection tube
6. 800 µl of the sample was loaded to the spin column that was assembled in the 2 ml collection tube, centrifuged for 1 min and the filtrate was discarded. If samples were larger than 800 µl, they were reloaded and centrifuged again.
7. 750 µl of diluted wash buffer was added to the spin column and centrifuged for 1 min at 10,000 rpm. The filtrate was discarded and spin column was again placed on the collection tube.
8. Column was again centrifuged for 1 min at 10,000 rpm to remove any residual wash buffer.
9. Spin column was transferred to a fresh collection tube and 30 µl of elution buffer was added to the centre of the spin column, centrifuged for 1 min at 10,000 rpm.
10. Eluted DNA fragments were checked on 0.7 per cent (w/v) agarose gel and stored at -20 °C.

3.6. DNA CLONING

3.6.1 Ligation

The eluted product was ligated in pGEMT vector (Fig. 2) using pGEMT Easy Vector System (Promega Corporation, USA) (Procedure followed as per the manufacturers protocol).

1. Reaction mixture was prepared as described below.

2X rapid ligation buffer	- 5.0 µl
pGEMT easy vector (50ng)	- 1.0 µl
PCR product	- 1.0 µl
T4 DNA ligase (3 units/µl)	- 1.0 µl
Deionised water	-2.0 µl
	10.0 µl

2. The reaction mixture was incubated for one hour at room temperature. Then it was kept at 4 °C overnight.

3.6.2 Preparation of competent cells

Competent cells for plasmid transformation were prepared following the protocol of Mandel and Higa (1970).

1. Three ml of sterile LB broth (tryptone 10 g; yeast extract 5 g and sodium chloride 10g, pH 7.0 in one liter water) was inoculated with a single colony of *Escherichia coli* DH5α cells and incubated overnight at 37 °C in a shaker at 160 rpm.
2. The overnight grown culture was transferred aseptically to 50 ml of sterile LB broth kept at 37 °C on a shaker set at 160 rpm until OD_{600} reaches 0.4-0.5 (3-3.5 h only).
3. The cell suspension was transferred to 50 ml sterile ice-cold centrifuge tubes and kept on ice for 20 min.
4. The cell suspension was centrifuged at 3,500 rpm for 10 min at 4 °C.

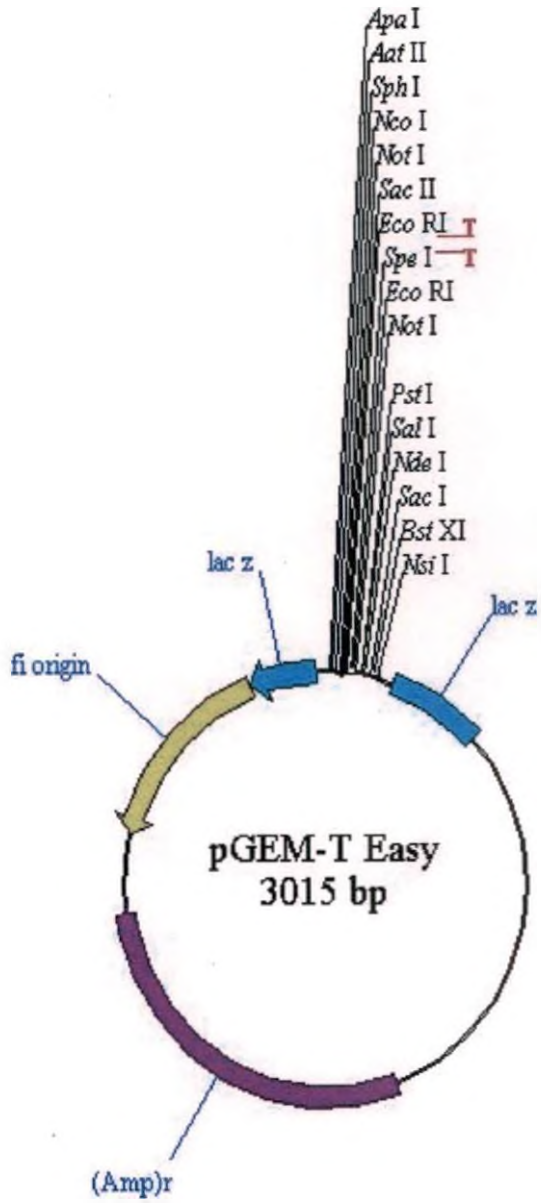

Fig. 2. pGEM-T Easy vector (Promega) used for cloning PCR products. The lac Z region and multiple cloning sites are shown in the figure.

5. The supernatant was carefully discarded and the pellet resuspended in 10 ml of sterile ice-cold 100 mM $CaCl_2$.
6. The tubes were kept on ice for 20 min.
7. The cell suspension was centrifuged at 5,000 rpm for 10 min at 4 °C.
8. The supernatant was carefully discarded and the pellet resuspended gently in two ml of sterile ice-cold 100 mM $CaCl_2$.
9. The tubes were kept on ice for 18 hours.
10. A volume of 400 µl chilled 100 per cent glycerol was added and mixed well using a chilled sterile pipette tip.
11. 100 µl aliquots of resuspended cells were dispensed into pre-chilled tubes and then stored at -70 °C.

The competence of the cells prepared was confirmed by transformation using a plasmid containing ampicillin resistance marker. The cells were plated on a plate containing LBA+ 50 mg l^{-1} ampicillin. Transformed cells harbouring the plasmid alone grew in the presence of ampicillin.

3.6.3 Cloning of ligated DNA into competent cells

1. The vial containing competent cells was thawed on ice.
2. The ligated product was added to the competent cells, contents mixed gently and kept on ice for 40 minutes.
3. Tube was rapidly taken from ice; heat shock was given at 42 °C exactly for 90 seconds without shaking and placed back on ice for 5 min.
4. Under sterile conditions, 250 µl of LB broth was added and the tube was inverted twice to mix the cells and LB broth.
5. The tube was incubated at 37 °C for 1 hour with shaking.
6. 50, 100 and 200 µl aliquots of the transformed cells were plated on LB/ ampicillin (50 mg l^{-1}) plates layered with IPTG (6 µl) and X-gal (60 µl). (Stock: Ampicillin-5 mg/ml in water; IPTG-200 mg/ml in water; X-gal-20 mg/ml in DMSO) and incubated overnight at 37 °C.
7. The recombinant clones were selected based on blue-white screening.

3.6.4 Isolation of recombinant plasmid DNA

Plasmid DNA was isolated from single white colonies using alkaline mini-prep procedure as given by Birnboim and Doly (1979).

3.6.4.1 Reagents

Solution I (Resuspension buffer)

Solution II (Lysis buffer)

Solution III (Neutralisation buffer)

(Chemical compositions of the solutions are given in Annexure III)

3.6.4.2 Procedure for plasmid isolation

1. Cells were pelleted from overnight grown recombinant *E. coli* culture containing plasmid DNA by centrifugation at 12,000 rpm for 1 min at 4 °C.
2. 100 µl of ice-cold solution I was added to the bacterial pellet and resuspended.
3. To the above, 200 µl of freshly prepared lysis buffer was added, mixed gently by inverting the tube five times.
4. 150 µl of ice-cold solution III was added to the tube, mixed well and kept on ice for 5 min.
5. The contents were centrifuged at 12,000 rpm for 5 min at 4 °C and the pellet was discarded.
6. To the supernatant, 0.6 volume of ice-cold isopropanol was added and kept at room temperature for 5 min.
7. The contents were centrifuged at 12,000 rpm for 5 min at 4 °C and the supernatant was discarded.
8. The pellet was rinsed with 1 ml of 70 per cent (v/v) ethanol and mixed gently.
9. The tube was centrifuged at 12,000 rpm for 10 min at 4 °C
10. The supernatant was discarded and the pellet air-dried for 10 minutes.
11. Pellet was finally dissolved in 30 µl TE buffer.

3.7. CONFIRMATION OF PRESENCE OF INSERT

3.7.1 PCR amplification of recombinant plasmid DNA

Polymerase chain reaction was carried out as described in Section 3.4.3. except that, the recombinant plasmid DNA isolated by alkali lysis method was used as template in place of plant genomic DNA. The PCR products were analyzed on 0.8 per cent (w/v) agarose gel as described in Section 3.2.

3.7.2 Restriction digestion of recombinant plasmid DNA

1. Purity of the recombinant plasmid DNA was checked on 0.8 per cent (w/v) agarose gel as described in Section 3.2. before proceeding to restriction digestion.
2. The reaction mix was prepared as given below in a sterile 0.2 ml microfuge tube.

Plasmid DNA	- 10 µl
Eco RI	- 2 µl
Restriction buffer	- 2 µl
Water	- 6 µl
	20 µl

3. The reaction components were mixed by gentle tapping at the bottom of the microfuge tube and gathered at the bottom by gentle spinning.
4. The tubes were incubated at 37 °C for two hours.
5. The reaction was arrested by adding 0.5 M EDTA to the tubes.
6. The digestion was confirmed by running the digest in 0.8 per cent (w/v) agarose gel as described in Section 3.2.

3.8 SEQUENCING OF DNA CLONES

The inserts were sequenced at DNA Sequencing Facility, University of Delhi South Campus (UDSC), New Delhi, using T_7 and SP_6 universal primers.

3.9 THEORETICAL ANALYSIS OF SEQUENCE

The nucleic acid as well as deduced protein sequences were analyzed by various online algorithms for structural prediction, phylogenetic relation with other published *hmgr* genes and their predicted protein sequences and various other parameters. The cloned and sequenced PCR products were analyzed by online BLAST (http://www.ncbi.nlm.nih.gov/blast/ ; Altschul *et al.*, 1997). Nucleic acid and protein sequences of other *hmgr* genes were obtained from NCBI (http://www.ncbi.nlm.nih.gov/). Alignments of sequences were carried out using 'Clustal W 1.83' (www.ebi.ac.uk/clustal; Thompson *et al.*, 1994). Phylogenetic tree was constructed using the 'Phylogram' tool.

The other nucleotide sequence analysis tools used were 'Genscan' (www.genes.mit.edu/genscan/; Burge and karlin, 1997) and nucleic acid tools of 'Biology Workbench'(http://seqtool.sdsc.edu/). Protein sequences were analyzed using 'MOTIF' (http://motif.genome.jp/), InterProScan (www.ebi.ac.uk/InterProScan/), Conserved Domain Database search (Marchler-Bauer and Bryant, 2004), Kyte and Doolittle hydropathy plot analysis (http://occawlonline.pearsoned.com; Kyte and Doolittle, 1982), Amino acid tools of 'Biology Workbench'(http://seqtool.sdsc.edu/) and domain structure prediction (www.biochem.ucl.ac.uk/bsm/cath/).

3.10 ISOLATION OF FULL-LENGTH GENE

Isolation of full-length gene was carried out using BD SMART™ RACE cDNA Amplification Kit provided by BD Biosciences Clontech, CA (Procedure followed as per the manufacturer's protocol).

3.10.1 RNA isolation

Total RNA was isolated from tender leaves of *S. xanthocarpum* by single step RNA isolation method (Chomczynski and Sacchi, 1987).

3.10.1.1 Requirements for RNA isolation

The mortar, pestle, microtips and microfuge tubes used for RNA isolation were treated with 0.5 M NaOH solution for three hours and washed well using Milli Q water to remove the traces of NaOH solution. Then they were autoclaved.

Solanum xanthocarpum plants were treated with three different concentrations of ABA to induce HMGR mRNA. The concentrations used were 10 mg l^{-1}, 100 mg l^{-1} and 1000 mg l^{-1}. Wilting of leaves was observed after four hours. Then the leaf samples were taken for RNA isolation.

3.10.1.2 Procedure for RNA isolation

1. Leaf tissue weighing 0.1 g was ground with liquid nitrogen
2. One ml Trizol reagent was added to the homogenate and the sample was kept at room temperature for five minutes.
3. The sample was transferred to a 1.5 ml tube and centrifuged at 12,000 rpm for 10 min at 4 °C.
4. The supernatant was transferred to a new tube and 0.2 ml chloroform was added. The contents were mixed by inversion and the tube was centrifuged at 12,000 rpm for 15 min at 4 °C.
5. The upper aqueous phase was transferred to a new tube and 0.5 ml isopropanol was added.
6. The tube was kept at room temperature for ten minutes and then centrifuged at 12,000 rpm for 15 min at 4 °C.
7. The pellet was washed with one ml absolute ethanol by centrifugation at 12,000 rpm for 15 min at 4 °C.
8. The supernatant was discarded and the pellet dissolved in 25 µl autoclaved milli Q water

3.10.1.3 Checking purity of the RNA sample

The isolated RNA samples were run on a 0.8 per cent (w/v) agarose gel to check the quality of the sample as described in Section 3.2.

3.10.1.4 Quantification of RNA

Quantification of RNA was carried out using UV spectrophotometer (Spectronic Genesys 5). Fifteen µl of RNA sample was diluted to 1.5 ml with water. The absorbance was measured at 260 nm and 280 nm and the RNA concentration in pure sample was calculated using the following relationship.

OD_{260} = 1 is equivalent to 40 µg of single stranded RNA /ml sample.

Quantity of RNA present in the sample = OD_{260} X 40 µg/ ml.

3.10.2 Rapid Amplification of cDNA Ends (RACE)

3.10.2.1 First strand cDNA synthesis

Two 10-µl reactions were set as described below to convert the RNA into RACE-Ready first-strand cDNA.

1. The following reagents were combined in separate 0.5-ml microcentrifuge tubes:

For preparation of 5'-RACE-Ready cDNA	For preparation of 3'-RACE-Ready cDNA
1–3 µl RNA sample	1–3 µl RNA sample
1 µl 5'-CDS primer	1 µl 3'-CDS primer A
1 µl BD SMART II A oligo	

2. Sterile water was added to a final volume of 5 µl for each reaction.
3. The contents were mixed well and the tubes incubated at 70°C for 2 min.
4. The tubes were cooled on ice for 2 min.

5. After spinning the tubes, the following reagents were added to each reaction tube (already containing 5 µl):

2 µl 5X First-Strand Buffer
1 µl DTT (20 mM)
1 µl dNTP Mix (10 mM)
1 µl BD PowerScript Reverse Transcriptase
10 µl Total volume

6. The contents of the tubes were mixed by gentle pipetting.
7. The tubes were incubated at 42°C for 1.5 hr in a hot-lid thermal cycler.
8. The first-strand reaction product was diluted with 100 µl Tricine-EDTA Buffer:
9. Tubes were heated at 72°C for 7 min.
10. Samples were stored at –20°C.

3.10.2.2 Rapid Amplification of 5' and 3' cDNA

Two 25-µl reactions were set as described below to generate the 5' and 3' cDNA fragments. Gene specific primers used for the reaction are shown as GSP1 and GSP 2 and the universal primer mix is indicated as UPM.

3.10.2.2.1 Setting up 5'-RACE PCR reactions

Component	5'-RACE Sample	GSP 1 + 2 (+ Control)	UPM only (– Control)	GSP1 only (– Control)
5'-RACE-Ready cDNA	1.25µl	1.25µl	1.25µl	1.25µl
UPM (10X)	2.5µl	—	2.5µl	—
GSP1 (10 µM)	0.5µl	0.5µl	—	0.5µl
GSP2 (10 µM)	—	0.5µl	—	—
H_2O	—	2.0µl	0.5µl	2.5µl
Master Mix	20.75µl	20.75µl	20.75µl	20.75µl
Final volume	25.0 µl	25.0 µl	25.0 µl	25.0 µl

3.10.2.2.2 Setting up 3'-race PCR reactions

Component	3'-RACE Sample	GSP 1 + 2 (+ Control)	UPM only (– Control)	GSP2 only (– Control)
3'-RACE-Ready cDNA	1.25µl	1.25µl	1.25µl	1.25µl
UPM (10X)	2.5µl	—	2.5µl	—
GSP1 (10 µM)	—	0.5µl	—	—
GSP2 (10 µM)	0.5µl	0.5µl	—	0.5µl
H_2O	—	2.0µl	0.5µl	2.5µl
Master Mix	20.75µl	20.75µl	20.75µl	20.75µl
Final volume	25.0 µl	25.0 µl	25.0 µl	25.0 µl

3.10.2.2.3 Thermal cycler programme

The following programme was set to amplify the 5' and 3' ends of the gene.

- 5 cycles:
  - 94°C 30 sec
  - 72°C 3 min
- 5 cycles:
  - 94°C 30 sec
  - 70°C 30 sec
  - 72°C 3 min
- 25 cycles:
  - 94°C 30 sec
  - 68°C 30 sec
  - 72°C 3 min

3.10.3 Sequencing of RACE products

The reaction products were run on 0.8 per cent (w/v) agarose gel and the band obtained in the RACE reaction was eluted from the gel as described in Section 3.5.

The eluted product was cloned in pGEMT vector and the competent bacterial cells were transformed with this recombinant plasmid as described in Section 3.6. The recombinant clones were selected based on blue-white screening and confirmation of presence of insert was done as described in Section 3.7. The positive clones were sent for sequencing at University of Delhi South Campus (UDSC) using T_7 primer

3.11 Sequence analysis

Theoretical analysis of the sequence was carried out as described in Section 3.9

Results

4. RESULTS

The results of the study on molecular characterization of *hmgr* gene from solanaceous plants undertaken at the Centre for Plant Biotechnology and Molecular Biology are presented below.

4.1 PLANT DNA ISOLATION AND GEL SEPARATION

Genomic DNA was isolated from the leaf tissue of the plants and checked on 1 per cent (w/v) agarose gel to detect the purity (Plate 2). A single sharp band was obtained which indicated that DNA was intact. No RNA contamination was noticed.

4.1.2 Quantification of DNA

The quantity of DNA present in the sample was determined spectrophotometrically. The quantity of DNA in the sample varied from 233.5 µg/ml to 425.4 µg/ml. The OD_{260}/OD_{280} ratios ranged between 1.848 and 1.930 (Table 1).

4.2 PRIMER DESIGNING

The complete cDNA / mRNA sequences of the *hmgr* gene of potato, tobacco, and capsicum were downloaded and open reading frames were found out using the 'ORF Finder' tool (http://www.ncbi.nlm.nih.gov). Multiple sequence alignment of ORFs of potato, tobacco and capsicum sequences was done using ClustalW 1.83 available from European Bioinformatics Institute (www.ebi.ac.uk/clustal) (Fig.3). Two sets of specific primers were designed (Table 2) based on the conserved sequences obtained in multiple sequence alignment and they were synthesized from Bangalore Genei Pvt. Ltd. Details of different primer combinations are given in Table 3.

Table 1. Quality and quantity of DNA extracted from solanaceous plants

Sl No.	Plant species	Optical Density Values		Quantity of DNA (µg/ml)	Ratio OD_{260}/OD_{280}	Remarks on quality
		OD_{260}	OD_{280}			
1.	*Solanum xanthocarpum*	0.0851	0.0463	425.5	1.848	Good
2.	*Solanum nigrum*	0.0726	0.0375	363.0	1.930	Good
3	*Solanum torvum*	0.0467	0.0249	233.5	1.875	Good
4	*Physalis minima*	0.0494	0.0256	247.0	1.926	Good

Table 2. Details of gene specific primers designed

Primer	Type	Primer sequence	Length (bp)	Tm (°C)
HMF2	Forward	5' GGGAT(C/T)GGGTTTGTTCAG 3'	17	56°C
HMF3	Forward	5' ACAGAAGGATGTTTAGTGGCTAG 3'	23	57°C
HMR2	Reverse	5'AGATATGCCGATGAC(A/G)TCCATGTC 3'	23	60°C
HMR3	Reverse	5' CTTGCCATCATTTACAGCCTCC 3'	22	60°C

Fig. 3. Multiple sequence alignment of plant *hmgr* genes. The conserved sequences selected for designing forward and reverse primers are shown in boxes. HMF2 and HMF3 are the forward primers and HMR2 and HMR3 are the reverse primers.

```
capsicum      1 ATGGACGTTCGCCGGAGATCTGAAGAGGCTGTTTACTCATCAAAGGTCTTTGCCGCCGAT
nicotiana     1 ATGGACGTTCGCCGGAGATCTGACAAGCCTGCATATCCAACCAAAGAATTTACCGCCGGC
Potato        1 ATGGACGTTCGCCGGCGACCTGTTAAGCCTCTATACACATCTAAAGATGCTTCCGCCGGC

capsicum     61 GAAAAACCTCTCAAGCCTCACAAGCAACAACAAGAAGAAGACAATACCCTTCTCATTGAT
nicotiana    61 GAAAAACCTCTCAAACCCCACAAACAACAACAAGAACAGGACAACTCCCTTCTCATT---
Potato       61 G---AACCTCTGA------------AACAACAAGAAG--------TTTCTTCTCCTAAA-

capsicum    121 GCCTCTGATGCCCTTCCGCTCCCTTTGTATTTTACTAATGGGTTGTTTTTCACCATGTTT
nicotiana   118 GCCTCCGATGCCCTCCCACTCCCTTTGTACCTCACAAATGGGTTGTTTTTCACCATGTTT
Potato       97 GCATCTGATGCGCTTCCACTCCCATTGTACCTAACCAATGGGTTGTTTTTCACCATGTTT

capsicum    181 TTCTCTGTTATGTATTTTCTTCTTTCTAGGTGGCGTGAGAAAATCAGGAATTCTACTCCT
nicotiana   178 TTCTCCGTTATGTATTATCTTCTCAGTAGGTGGCGTGAGAAAATCAGGAACTCCACTCCT
Potato      157 TTCTCTGTTATGTATTTTCTTCTCGTAAGGTGGCGTGAGAAGATCCGTAATTCTATTCCT

capsicum    241 CTCCATGTTGTTACGCTTTCTGAATTGGGTGCTATTGTTTCGTTGATCGCTTCTGTTATT
nicotiana   238 CTCCACGTGGTTACCTTTTCTGAATTAGTTGCCATTATTTCGTTGATCGCTTCCGTGATT
Potato      217 CTTCATGTGGTTACCCTTTCTGAATTGTTAGCTATGGTGTCATTGATTGCTTCCGTTATA

                                          HMF2
capsicum    301 TATCTTCTTGGGTTCTTTGGGATTGGGTTTGTTCAGACATTTGTGGCTAGGGGGAATAAT
nicotiana   298 TATCTTCTGGGGTTCTTCGGGATCGGGTTTGTTCAGTCGTTCGTTTCCAGGGATAACAAT
Potato      277 TATCTTTTGGGTTTCTTTGGGATTGGGTTTGTTCAGTCGTTTGTGTCCAGGTCGAATAGT

capsicum    361 GAT---TCTTGGGATGAGGAGGATGAAAATGATGAGCAGTTTATCTTGGAGGAAGATAGT
nicotiana   358 GATGATTCTTGGGATGTTGAGGATGAAAACGATGAACAATTTCTCTTGGAAGAAGATAGT
Potato      337 GAT---TCATGGGATATTGAGGATGAGAATGCTGAGCAGCTTATTATTGAGGAAGATAGC

capsicum    418 CGTCGTGGACCTTGCGCCGCTGCGACTACTCTTGGTTG---TGCTGTCCCTACACCACCT
nicotiana   418 CGTCGTGGACCT----------GCCACTACTCTTGGCTGCACTGCTGTTCCACCACCACCT
Potato      394 CGCCGTGGACCATGTGCTGCTGCCACTACTCTTGGCTGC---GTTGTGCCTCCACCACCT

capsicum    475 GCTAAACATATTGCACCAATAGTACCACAGCAACCTGCT------GTATCCATTGCA---
nicotiana   469 GCTCGACAATTTGTCCCAATGGTACCACCGCAACCCGCCAAGATCGCAGCTATGTCT---
Potato      451 GTTCGAAAAATTGCCCCAATGGTTCCACAGCAACCTGCTAAG---GTAGCTTTGTCCCAA

capsicum    526 ---GAGAAACCTGCACCGTTGGTTACACCAGCAGCATCTGAGGAAGACGAAGAGATAATA
nicotiana   526 ---GAAAAACCTGCGCCGTTGGTTACACCAGCAGCCTCTGAGGAAGACGAGGAGATCATA
Potato      508 ACGGAGAAGCCTTCGCCAATAATTATGCCAGCATTATCGGAAGATGACGAGGAGATTATA
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capsicum    583 AAATCTGTGGTGCAAGGGAAAATTCCATCGTACTCTTTGGAATCCAAACTCGGTGATTGT
nicotiana   583 AAATCCGTGGTGCAGGGGAAAATGCCGTCGTACTCTTTGGAATCGAAACTCGGTGATTGT
Potato      568 CAATCTGTTGTTCAGGGTAAAACACCATCATATTCGTTGGAATCAAAGCTTGGTGATTGT

capsicum    643 AAGAGAGCTGCATCGATCAGGAAGGAGGTGTTGCAGAGGATTACAGGGAAGTCTCTAGAA
nicotiana   643 AAGAGAGCTGCTTCCATTCGTAAAGAGGCGTTGCAGAGGATTACGGGGAAGTCCCTAGAA
Potato      628 ATGAGAGCTGCTTCGATTCGAAAAGAGGCGTTACAGAGGATTACAGGGAAGTCATTGGAA

capsicum    703 GGGCTACCATTGGATGGATTTAACTATGAATCCATTCTCGGGCAGTGCTGTGAGATGACA
nicotiana   703 GGGCTCCCATTGGAGGGATTTGATTATGAATCCATTCTTGGGCAGTGCTGTGAGATGCCA
Potato      688 GGGCTCCCATTGGAGGGATTTGACTATTCGTCTATTCTTGGACAGTGCTGTGAGATGCCT

capsicum    763 ATTGGTTATGTGCAGATACCCGTGGGAATAGCAGGGCCATTGTTGCTTAATGGGAGAGAG
nicotiana   763 ATCGGCTACGTGCAGATACCCGTTGGAATAGCCGGGCCGTTGTTGCTCGACGGGAGAGAG
Potato      748 GTAGGATATGTGCAAATACCGGTGGGTATTGCTGGGCCTTTGTTGCTTGATGGGAGAGAG

                                                HMF3
capsicum    823 TATTCAGTGCCGATGGCAACCACAGAAGGATGTTTAGTGGCTAGCACCAATAGAGGTTGC
nicotiana   823 TATTCGGTGCCAATGGCAACCACTGAAGGATGTTTAGTGGCTAGCACCAACAGGGGTTGC
Potato      808 TACTCAGTGCCAATGGCAACTACAGAAGGATGTTTAGTGGCTAGCACCAACAGGGGTTGC

capsicum    883 AAGGCTATCTATGCTTCTGGTGGCGCCACCAGCATTTTGCTCCGTGATGGAATGACCAGA
nicotiana   883 AAGGCTATCTATGCTTCTGGCGGCGCCAATAGCGTGTTGCTCCGCGATGGGATGACCAGA
Potato      868 AAGGCTATCTTTGTCTCTGGTGGCGCCGACAGCGTTTTGCTCAGAGATGGGATGACAAGA

capsicum    943 GCACCCTGTGTCAGGTTCGGCACAGCCAAAAGGGCAGCAGAGTTGAAGTTCTTTGTTGAA
nicotiana   943 GCACCTTGTGTCAGGTTTGGCACTGCCAAAAGGGCCGCGGAGTTGAAGTTCTTTGTTGAA
Potato      928 GCTCCGGTTGTCCGGTTCACCACCGCCAAAAGAGCCGCTGAGTTGAAATTCTTCGTTGAG

capsicum   1003 GATCCTATCAACTTTGAGACACTTGCTAATGTTTTCAACCAATCAAGCAGATTTGCCAGA
nicotiana  1003 GATCCTGTGAAATTTGAGACACTTGCTGCTGTTTTCAACCAGTCAAGCAGATTTGCCAGA
Potato      988 GATCCCCTCAACTTTGAGACTCTTTCTCTTATGTTCAACAAATCAAGCAGATTTGCTCGA

capsicum   1063 TTACAAAGGATTCAGTGTGCAATTGCGGGAAAGAATCTGCACATGAGATTTGTATGTAGC
nicotiana  1063 TTACAAAGGATTCAATGCGCAATTGCGGGAAAGAATCTGTACATGCGATTTGTGTGTAGC
Potato     1048 TTACAGGGCATTCAATGTGCTATAGCTGGTAAAAATCTGTATATCACATTTAGCTGTAGC

capsicum   1123 ACCGGTGATGCAATGGGAATGAATATGGTGTCCAAAGGTGTACAAAATGTTCTTGATTAC
nicotiana  1123 ACTGGTGATGCAATGGGAATGAACATGGTGTCCAAAGGTGTACAAAATGTTCTTGATTAC
Potato     1108 ACTGGTGATGCAATGGGAATGAACATGGTATCCAAAGGTGTCCAGAACGTTCTGGATTAC

                                                HMR2
capsicum   1183 CTTCAGAATGAATACGCTGACATGGATGTCATCGGCATATCTGCGAACTTTTGCTCGGAT
nicotiana  1183 CTCCAGAATGAATATCCCGACATGGATGTCATCGGCATATCTGGGAACTTTTGCTCGGAC
Potato     1168 CTTCAGAGTGAATATCCAGACATGGACGTCATCGGCATATCTGGGAACTTTTGTTCGGAT

capsicum   1243 AAGAAGCCAGCAGCAGTTAATTGGATTGAGGGGAGAGGAAAGTCTGTAGTTTGTGAGGCA
nicotiana  1243 AAGAAGCCAGCAGCAGTTAACTGGATTGAGGGGAGAGGAAAGTCTGTAGTTTGTGAGGCA
Potato     1228 AAGAAGCCAGCAGCAGTTAACTGGATTGAAGGTAGAGGAAAATCAGTAGTTTGCGAGGCA
```

Fig. 3 Contd.

```
capsicum   1303 ATTATCACGGAAGAGGTGGTGAAGAAAGTTCTGAAAACTGAGGTTGCTGCTCTTGTGGAG
nicotiana  1303 ATTATCACGGAAGAGGTGGTGAAGAAAGTTCTGAAAACTGAGGTTGCTGCTCTTGTGGAG
Potato     1288 ATAATCAAGGAGGAGGTAGTGAAGAAAGTGTTGAAAACTGAGGT----------GTGGAG

capsicum   1363 CTGAACATGCTTAAAAATCTTACTGGCTCTGCATTGGCTGGTGCCCTTGGTGGTTTCAAT
nicotiana  1363 CTGAACATGCTTAAAAATCTTACTGGCTCTGCCATGGCTGGTGCGCTTGGTGGTTTCAAT
Potato     1338 CTGA----------------------------------------------------

capsicum   1423 GCCCATGCCAGCAATATTGTCTCAGCTGTGTATATAGCTACTGGTCAGGACCCAGCACAA
nicotiana  1423 GCCCACGCCAGCAATATCGTTTCAGCTGTGTTTATAGCAACTGGTCAGGACCCAGCTCAG
Potato          ----------------------------------------------------------

                                                        HMR3
capsicum   1483 AACATAGAGAGTTCACACTGCATCACTATGATGGAGGCTGTAAATGATGGCAAGGACCTC
nicotiana  1483 AACATAGAGAGCTCTCATTGTATCACTATGATGGAGGCTGTAAATGATGGCAAGGACCTC
Potato          ----------------------------------------------------------

capsicum   1543 CATATTTCTGTTACAATGCCTTCCATTGAGGTTGGTACTGTTGGTGGTGGAACTCAGCTT
nicotiana  1543 CATGTTTCTGTTACAATGCCTTCCATTGAGGTTGGTACTGTTGGAGGTGGAACTCAGCTT
Potato          ----------------------------------------------------------

capsicum   1603 GCATCTCAGTCAGCTTGCTTAAACTTATTGGGAGTGAAAGGTGCCAACAGAGAGGCACCA
nicotiana  1603 GCTTCTCAGTCAGCTTGCTTGAACTTATTGGGAGTGAAAGGTGCCAACAGAGAGGCACCA
Potato          ----------------------------------------------------------

capsicum   1663 GGGTCAAATGCAAGGCTTTTGGCCACAATAGTAGCTGGTTCTGTTCTTGCCGGGGAGCTA
nicotiana  1663 GGGTCAAATGCAAGACTCTTGGCCACAATAGTAGCTGGTTCTGTTCTTGCTGGGGAGCTA
Potato          ----------------------------------------------------------

capsicum   1723 TCCCTCATGTCAGCTATCTCAGCTGGGCAGCTGGTTAATAGCCACATGAAATACAATAGA
nicotiana  1723 TCCCTCATGTCAGCTATCTCAGCTGGGCAGCTGGTTAAGAGCCACATGAAATACAATAGA
Potato          ----------------------------------------------------------

capsicum   1783 TCTACCAAAGATGTCACCAAGGCATCCTCCTAA
nicotiana  1783 TCTACCAAAGATGTCACAAAGGCATCCTCCTAA
Potato          --------------------------------
```

Fig. 3 Contd.

4.3 AMPLIFICATION OF *hmgr* GENE FROM GENOMIC DNA

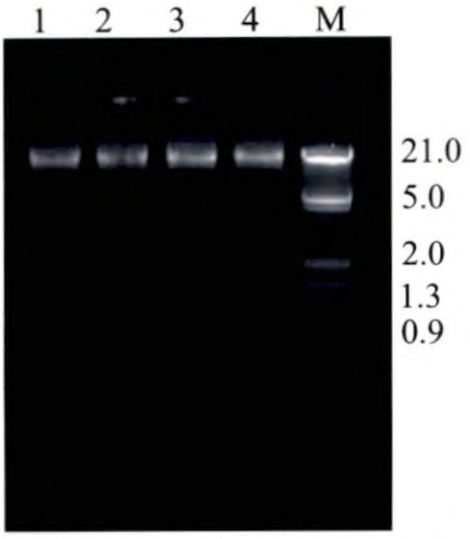
Three bands were obtained from *Solanum xanthocarpum* which were of approximate size 880 bp, 1325 bp and 1520 bp. There were two bands each in *S. nigrum* and *S. torvum* corresponding to 945 bp and 1835 bp. In *Physalis minima* , no amplification was observed (Plate 3). There were no bands in the negative control suggesting that there was no primer dimer formation. Two bands were eluted from each lane. The details are given in Table 4. Only one band each from *S. xanthocarpum* and *S. nigrum* was of good quality after elution, whereas two good quality bands were eluted from *S. torvum* (Plate 4).

4.4 TRANSFORMATION OF THE LIGATED PRODUCT INTO *E. coli.*

A large number of colonies were obtained when the competent cells were checked for competence by transformation using a plasmid containing ampicillin resistance marker (Plate 5). After the confirmation of competence, the ligated product (eluted band ligated into pGEMT vector) was transferred into competent *E. coli* (DH5α) cells using the heat shock method at 42 °C. A combination of blue and white colonies was obtained after overnight incubation confirming successful transformation (Plate 6).

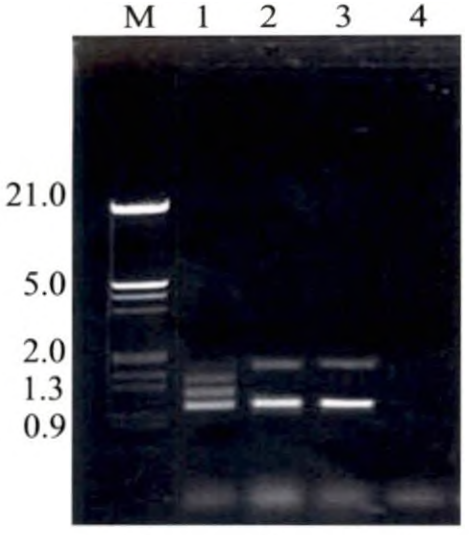
4.5 SCREENING OF THE TRANSFORMED COLONIES

The agar plates containing the transformed colonies were screened for recombinant plasmid. Four white colonies were picked up from each plate and were grown in LB broth. One blue colony was also inoculated in order to set the negative control. Plasmids were isolated and checked on 0.7 per cent (w/v) agarose gel (Plate 7). The plasmid isolated from the blue colony was of low molecular weight as it lacked the insert.



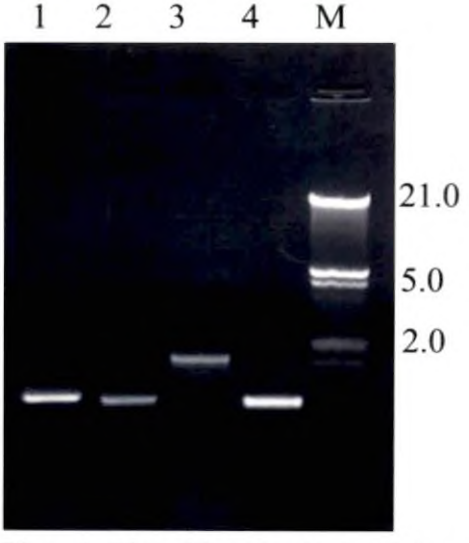
Lane 1- *Solanum xanthocarpum*
Lane 2- *S. nigrum*
Lane 3- *S. torvum*
Lane 4- *Physalis minima*
Lane M- Molecular weight marker

Plate 2. Genomic DNA isolated from the four solanaceous plants



Lane M- Molecular weight marker
Lane 1- *Solanum xanthocarpum*
Lane 2- *S. nigrum*
Lane 3- *S. torvum*
Lane 4- *Physalis minima*

Plate 3. Amplification of *hmgr* gene from the selected plants



Lane 1- *Solanum xanthocarpum*
Lane 2- *S. nigrum*
Lane 3- *S. torvum* upper band
Lane 4- *S. torvum* lower band
Lane M- Molecular weight marker

Plate 4. Eluted bands obtained from various *hmgr* amplicons

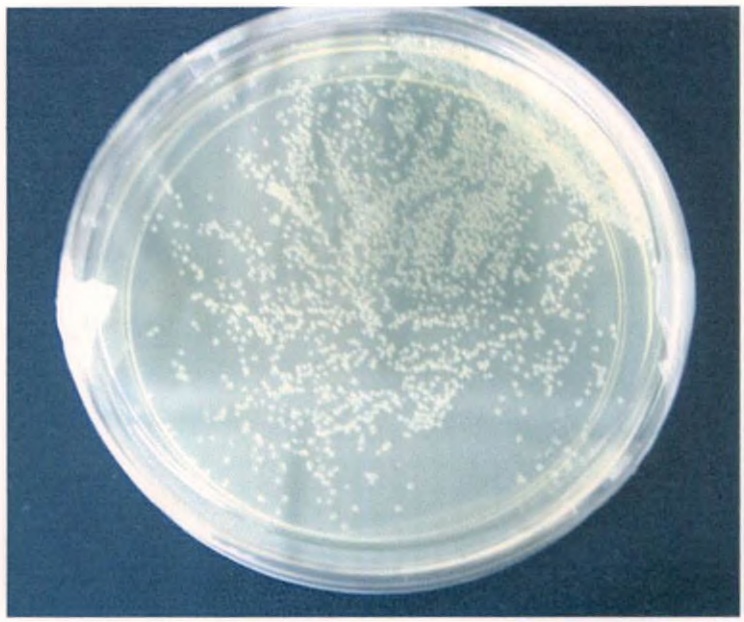


Plate 5. Checking the competence of the competent cells

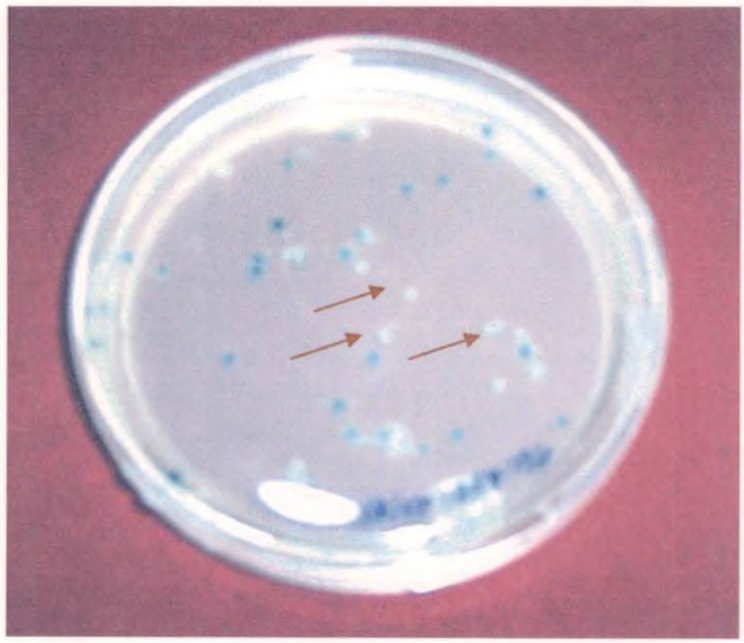


Plate 6. Blue white screening of transformed *E. coli* cells.
White colonies are transformed and blue colonies are non-transformed.
White colonies are indicated with arrow marks.

4.5.1 Confirmation of recombination by PCR

The plasmid was checked for the presence of insert by PCR confirmation. The plasmid DNA was used as template and the insert was amplified using gene specific primers. The PCR products were checked on 0.7 per cent (w/v) agarose gel (Plate 8). A positive control PCR reaction was also set up using the plant genomic DNA as template. Single amplified band exactly similar to the genomic DNA amplification product was obtained confirming the presence of plasmid.

4.5.2 Confirmation of recombination by restriction digestion

As a secondary confirmation test for the presence of insert, the plasmid was restricted using *Eco*RI enzyme. After digestion, it was checked on 0.7 per cent (w/v) agarose gel. Two bands, one small band of size exactly similar to the amplicon and another band of size equal to that of the plasmid were observed (Plate 9). The high molecular weight band corresponded to pGEMT vector and the low molecular weight band confirmed the presence of insert (*hmgr* gene) in the plasmid.

4.6 SEQUENCING OF THE CLONE

The screened colonies in which the presence of insert was confirmed were sent for automated sequencing using T7 universal primer. (Fig. 4). The *hmgr* sequences obtained in all the plants were submitted to GenBank through BankIt programme at NCBI site (http://www.ncbi.nlm.nih.gov/BankIt/). The sequence length and the corresponding accession numbers are given in Table 5. The sequences from *S. xanthocarpum, S. nigrum* and *S.torvum* were designated as *Sxhmgr, Snhmgr* and *Sthmgr* respectively. The two clones from *S. torvum* were indicated as *Sthmgr1 and Sthmgr*2. The sequences are given in Fig. 5.

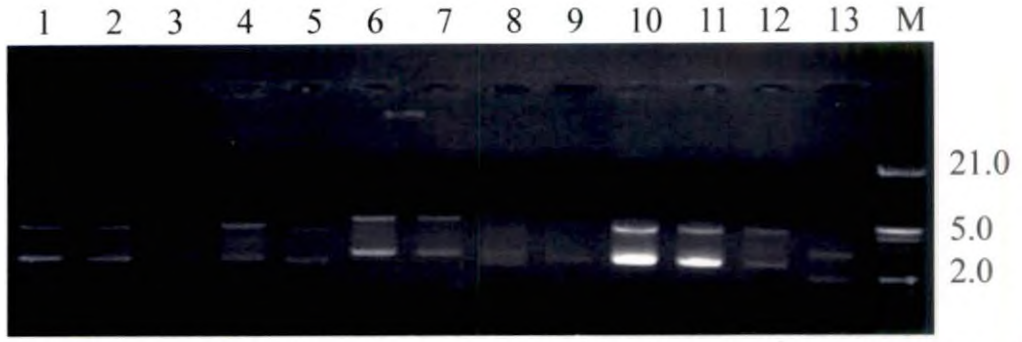


Plate 7. Plasmids isolated from the recombinant and non recombinant colonies

Lanes 1-3 : Plasmid from *hmgr* clone from *S. xanthocarpum*
Lanes 4-6 : Plasmid from *hmgr* clone from *S. nigrum*
Lanes 7-9 : Plasmid from *hmgr* clone from *S. torvum 1*
Lanes 10-12 : Plasmid from *hmgr* clone from *S. torvum 2*
Lane 13 : Plasmid from non recombinant blue colony
Lane M : Molecular weight marker

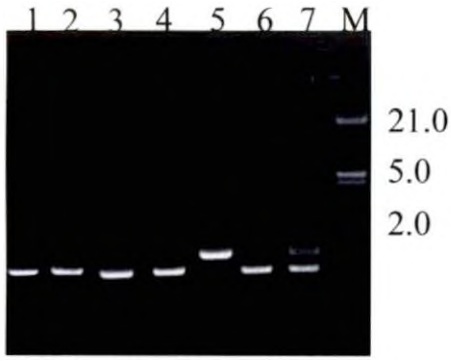


Plate 8. Reamplification of *hmgr* gene from the recombinant plasmids

Lane 1 : *Sxhmgr* plasmid
Lane 2 : *S. xanthocarpum* genomic DNA
Lane 3 : *Snhmgr* plasmid
Lane 4 : *S. nigrum* genomic DNA
Lane 5 : *Sthmgr 1* plasmid
Lane 6 : *Sthmgr 2* plasmid
Lane 7 : *S. torvum* genomic DNA
Lane M : Molecular weight marker

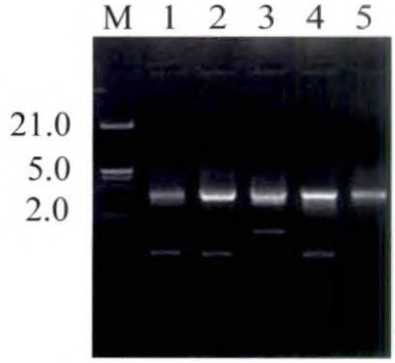


Plate 9. Restriction analysis of the cloned inserts

Lane 1 : *Sxhmgr* plasmid
Lane 2 : *Snhmgr* plasmid
Lane 3 : *Sthmgr 1* plasmid
Lane 4 : *Sthmgr 2* plasmid
Lane 5 : Plasmid from non recombinant blue colony
Lane M : Molecular weight marker

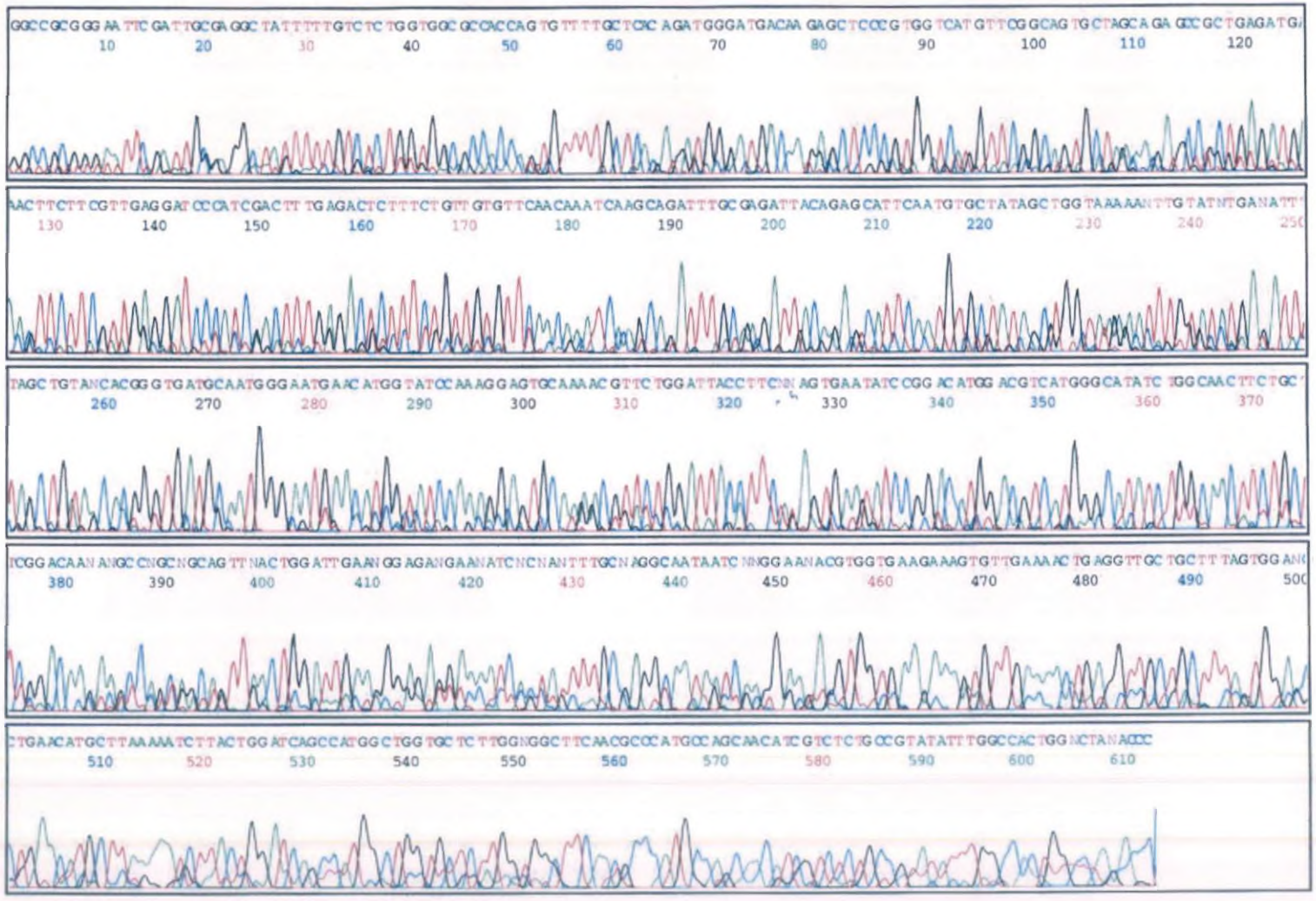


Fig . 4 A. Graphical output of *Sxhmgr* sequence data

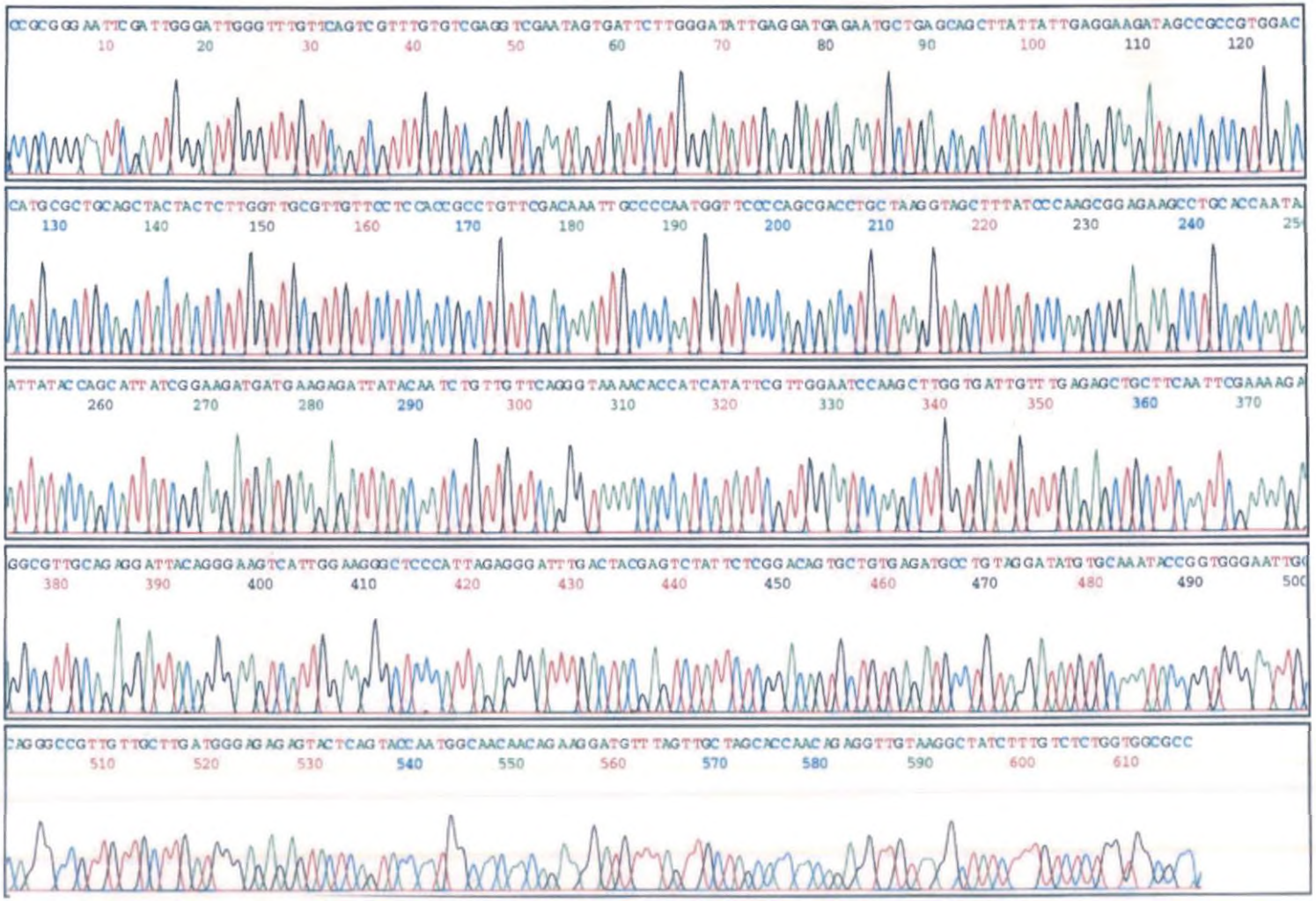


Fig . 4 B. Graphical output of *Snhmgr* sequence data

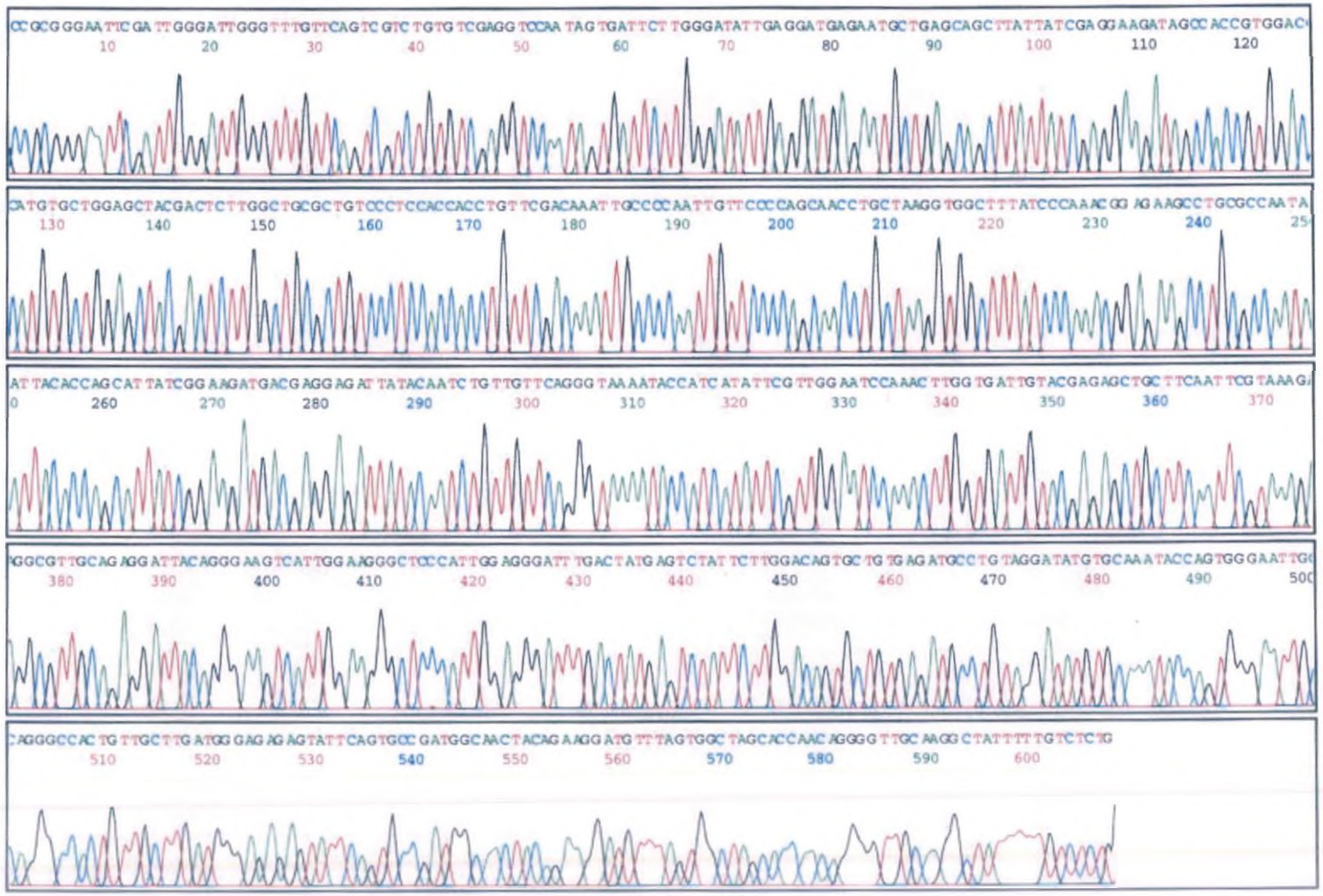


Fig . 4 C. Graphical output of *Sthmgr1* sequence data

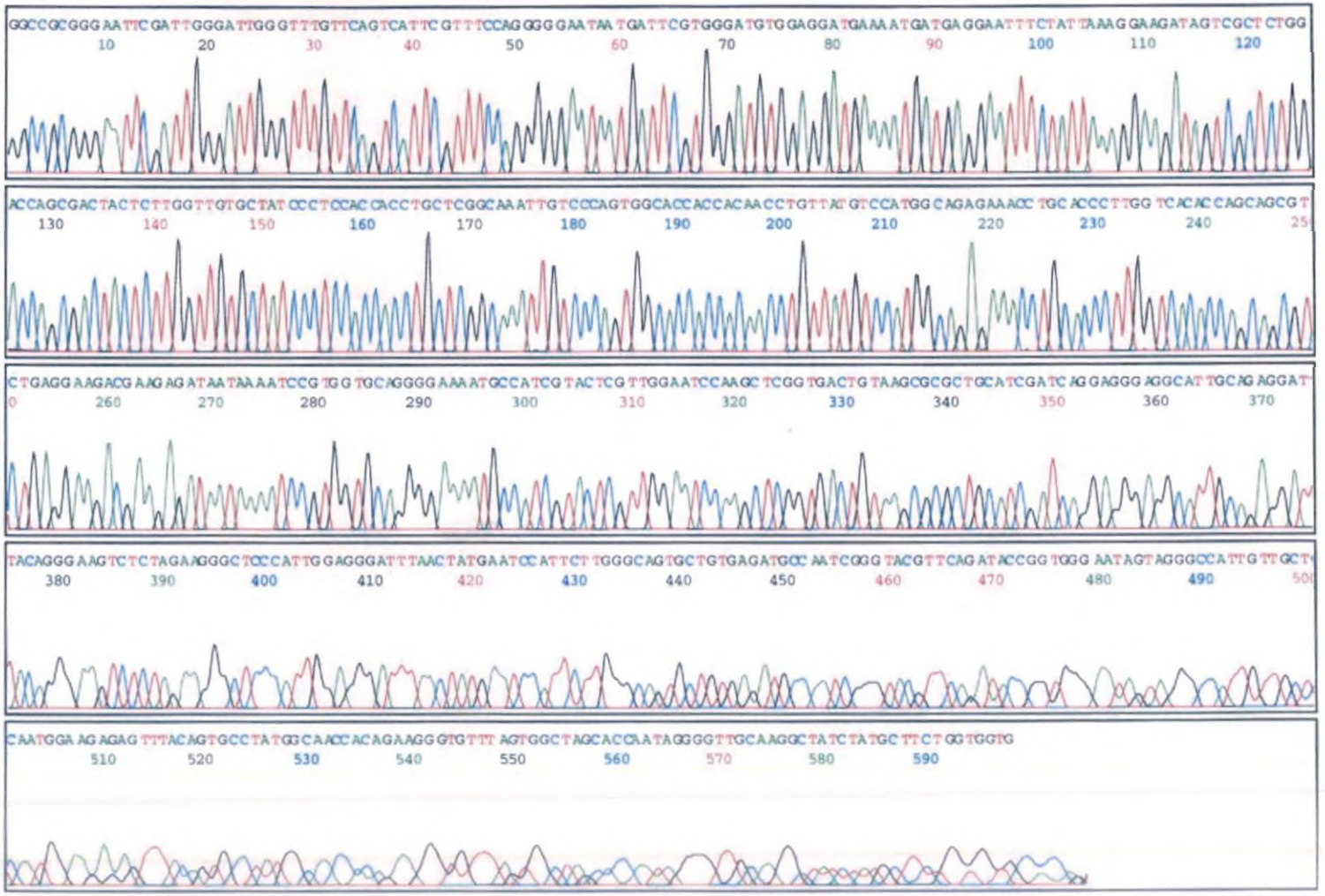


Fig . 4 D. Graphical output of *Sthmgr2* sequence data

Table 3. Details of different combinations of primers

Primer combination	Amplicon size (bp)	Annealing temperature (^{0}C)
HMF2-HMR2	896	53
HMF2-HMR3	1196	54
HMF3-HMR2	322	56
HMF3-HMR3	665	57

Table 4. Details of bands obtained in PCR reaction

Plant genomic DNA source	Positive primer combination	No. of bands eluted	Approximate size of eluted bands (bp)	Bands used for cloning (bp)
Solanum xanthocarpum	HMF3R3	2	880, 1325	880
Solanum nigrum	HMF2R2	2	945, 1835	945
Solanum torvum	HMF2R2	2	945, 1835	945, 1835
Physalis minima	-	-	-	-

Table 5. *Hmgr* gene sequences in different solanaceous plants.

Hmgr gene	Accession number	Sequence length	
		Nucleotide (bp)	Amino acid
Sxhmgr	AY 228453	535	178
Snhmgr	DQ 229901	582	194
Sthmgr 1	DQ 248943	574	191
Sthmgr 2	DQ 246549	941	313

```
      T  N  R  G  C  K  A  I  F  V  S  G  G  A  T  S  I  L  L  R
2     accaacaggggttgcaaggctatttttgtctctggtggcgccaccagtattttgctcaga  61
       D  G  M  T  R  A  P  V  V  R  F  A  T  A  K  R  A  A  E  M
62    gatgggatgacaagagctcccgtggtcagattcgccactgccaaaagagccgctgagatg 121
       K  F  F  V  E  D  P  I  N  F  E  T  L  S  L  V  F  N  K  S
122   aagttcttcgttgaggatcccatcaactttgagactctttctcttgtgttcaacaaatca 181
       S  R  F  A  R  L  Q  S  I  Q  C  A  I  A  G  K  N  L  Y  M
182   agcagatttgcaagattacagagcattcaatgtgctatagctggtaaaaatttgtatatg 241
       R  F  S  C  S  T  G  D  A  M  G  M  N  M  V  S  K  G  V  Q
242   agatttagctgtagcaccggtgatgcaatgggaatgaacatggtatccaaaggagtgcaa 301
       N  V  L  D  Y  L  Q  N  E  Y  P  D  M  D  V  M  G  I  S  G
302   aacgttctggattaccttcagaatgaatatccggacatggacgtcatgggcatatctggc 361
       N  F  C  S  D  K  K  P  A  A  V  N  W  I  E  G  R  G  K  S
362   aacttttgctcggacaagaagccagcagcagttaactggattgaagggagaggaaaatca 421
       V  V  C  E  A  I  I  K  E  E  V  V  K  K  V  L  K  T  E  V
422   gtagtttgcgaggcaataatcaaggaagaggtggtgaagaaagtgttgaagactgaggtt 481
       A  A  L  V  E  L  N  M  L  K  N  L  T  G  S  A  M  A
482   gctgctttagtggagttgaacatgcttaaaaatcttactggatcagccatggct  535
```

Fig. 5A. ***Sxhmgr*** **gene sequence**

```
      S  F  V  S  R  S  N  S  D  S  W  D  I  E  D  E  N  A  E  Q
1     tcgtttgtgtcgaggtcgaatagtgattcttgggatattgaggatgagaatgctgagcag  60
       L  I  I  E  E  D  S  R  R  G  P  C  A  A  A  T  T  L  G  C
61    cttattattgaggaagatagccgccgtggaccatgcgctgcagctactactcttggttgc 120
       V  V  P  P  P  P  P  V  R  Q  I  A  P  M  V  P  Q  R  P  A  K
121   gttgttcctccaccgcctgttcgacaaattgccccaatggttccccagcgacctgctaag 180
       V  A  L  S  Q  A  E  K  P  A  P  I  I  I  P  A  L  S  E  D
181   gtagctttatcccaagcggagaagcctgcaccaataattataccagcattatcggaagat 240
       D  E  E  I  I  Q  S  V  V  Q  G  K  T  P  S  Y  S  L  E  S
241   gatgaagagattatacaatctgttgttcagggtaaaacaccatcatattcgttggaatcc 300
       K  L  G  D  C  L  R  A  A  S  I  R  K  E  A  L  Q  R  I  T
301   aagcttggtgattgtttgagagctgcttcaattcgaaaagaggcgttgcagaggattaca 360
       G  K  S  L  E  G  L  P  L  E  G  F  D  Y  E  S  I  L  G  Q
361   gggaagtcattggaagggctcccattagagggatttgactacgagtctattctcggacag 420
       C  C  E  M  P  V  G  Y  V  Q  I  P  V  G  I  A  G  P  L  L
421   tgctgtgagatgcctgtaggatatgtgcaaataccggtgggaattgcagggccgttgttg 480
       L  D  G  R  E  Y  S  V  P  M  A  T  T  E  G  C  L  V  A  S
481   cttgatgggagagagtactcagtaccaatggcaacaacagaaggatgtttagttgctagc 540
       T  N  R  G  C  K  A  I  F  V  S  G  G  A
541   accaacagaggttgtaaggctatctttgtctctggtggcgcc  582
```

Fig. 5B. ***Snhmgr*** **gene sequence**

```
     R  M  L  S  S  L  L  S  R  K  I  A  T  V  D  H  V  L  E  L
1    agaatgctgagcagcttattatcgaggaagatagccaccgtggaccatgtgctggagcta  60
     R  L  L  A  A  L  S  L  H  H  L  F  D  K  L  P  Q  L  F  P
61   cgactcttggctgcgctgtccctccaccacctgttcgacaaattgccccaattgttcccc 120
     S  N  L  L  R  W  L  Y  P  K  R  R  S  L  R  Q  *  L  H  Q
121  agcaacctgctaaggtggctttatcccaaacggagaagcctgcgccaataattacaccag 180
     H  Y  R  K  M  T  R  R  L  Y  N  L  L  F  R  V  K  Y  H  H
181  cattatcggaagatgacgaggagattatacaatctgttgttcagggtaaaataccatcat 240
     I  R  W  N  P  N  L  V  I  V  R  E  L  L  Q  F  V  K  R  R
241  attcgttggaatccaaacttggtgattgtacgagagctgcttcaattcgtaaagaggcgt 300
     C  R  G  L  Q  G  S  H  W  K  G  S  H  W  R  D  L  T  M  S
301  tgcagaggattacagggaagtcattggaagggctcccattggagggatttgactatgagt 360
     L  F  L  D  S  A  V  R  C  L  *  D  M  C  K  Y  Q  W  E  L
361  ctattcttggacagtgctgtgagatgcctgtaggatatgtgcaaataccagtgggaattg 420
     Q  G  H  C  C  L  M  G  E  S  I  Q  C  R  W  Q  L  Q  K  D
421  cagggccactgttgcttgatgggagagagtattcagtgccgatggcaactacagaaggat 480
     V  *  W  L  A  P  T  G  V  A  R  L  F  L  S  L
481  gtttagtggctagcaccaacaggggttgcaaggctatttttgtctctg  528
```

Fig. 5C. ***Sthmgr1*** **gene sequence**

```
     S  F  V  S  R  G  N  N  D  S  W  D  V  E  D  E  N  D  E  E
1    tcattcgtttccaggggaataatgattcgtgggatgtggaggatgaaaatgatgaggaa  60
     F  L  L  K  E  D  S  R  S  G  P  A  T  T  L  G  C  A  I  P
61   tttctattaaaggaagatagtcgctctggaccagcgactactcttggttgtgctatccct 120
     P  P  P  A  R  Q  I  V  P  V  A  P  P  Q  P  V  M  S  M  A
121  ccaccacctgctcggcaaattgtcccagtggcaccaccacaacctgttatgtccatggca 180
     E  K  P  A  P  L  V  T  P  A  A  S  E  E  D  E  E  I  I  K
181  gagaaacctgcacccttggtcacaccagcagcgtctgaggaagacgaagagataataaaa 240
     S  V  V  Q  G  K  M  P  S  Y  S  L  E  S  K  L  G  D  C  K
241  tccgtggtgcagggaaaatgccatcgtactcgttggaatccaagctcggtgactgtaag 300
     R  A  A  S  I  R  R  E  A  L  Q  R  I  T  G  K  S  L  E  G
301  cgcgctgcatcgatcaggagggaggcattgcagaggattacagggaagtctctagaaggg 360
     L  P  L  E  G  F  N  Y  E  S  I  L  G  Q  C  C  E  M  P  I
361  ctcccattggagggatttaactatgaatccattcttgggcagtgctgtgagatgccaatc 420
     G  Y  V  Q  I  P  V  G  I  V  G  P  L  L  L  N  G  R  E  F
421  gggtacgttcagataccggtgggaatagtagggccattgttgctcaatggaagagagttt 480
     T  V  P  M  A  T  T  E  G  C  L  V  A  S  T  N  R  G  C  K
481  acagtgcctatggcaaccacagaagggtgtttagtggctagcaccaataggggttgcaag 540
     A  I  Y  A  S  G  G  A  T  S  I  L  L  R  D  G  M  T  R  A
541  gctatctatgcttctggtggtgccaccagtattttgcttcgtgatgggatgaccagagca 600
     P  C  V  R  F  G  T  A  K  R  A  A  E  L  K  L  F  V  E  D
601  ccctgtgtcaggtttggcacagccaaaagggcggcagagttgaagttatttgttgaagat 660
     P  T  K  F  E  T  L  A  N  V  F  N  Q  *  V  H  A  I  N  L
661  cccaccaaatttgagactcttgctaatgttttcaaccagtaagtgcatgccattaatcta 720
     F  V  I  L  C  F  Y  T  S  S  Y  L  I  V  V  F  E  W  D  V
721  ttcgttatattgtgtttttatactagtagttatctaattgttgtatttgaatgggatgtt 780
     A  D  Q  A  D  L  P  D  Y  K  G  F  S  V  Q  L  P  G  R  I
781  gcagatcaagcagatttgccagattacaaaggattcagtgtgcaattgccgggaagaatc 840
     C  T  *  D  S  Y  V  A  L  V  M  Q  W  E  *  I  W  C  P  K
841  tgtacatgagattcgtatgtagcactggtgatgcaatgggaatgaatatggtgtccaaag 900
     V  Y  K  T  F  L  L  T  F  R  M  N  I
901  gtgtacaaaacgttcttgcttaccttcagaatgaatatcct  941
```

Fig. 5D. ***Sthmgr2*** **gene sequence**

4.7 SEQUENCE ANALYSIS BY VARIOUS COMPUTER ALGORITHMS

4.7.1 Nucleotide sequence analysis

The nucleotide sequences were aligned against other *hmgr* sequences using BLAST program (http://www.ncbi.nlm.nih.gov/blast/). Nucleic acid statistics of the sequences obtained was done in the Biology Workbench (http://seqtool.sdsc.edu/). *Sxhmgr* was comparatively rich in A+T (55.3 %) and lowest A+T was noticed in *Sthmgr2* (50 %). In all cloned sequences, except *Sthmgr2,* G+C content was less than fifty per cent (Table 6). The sequences were translated in all six reading frames and longest open reading frame (ORF) was found out using NCBI 'ORF finder' (http://www.ncbi.nlm.nih.gov/ORFfinder/). There were seven ORFs in *Sthmgr2,* five in *Sxhmgr* and *Sthmgr1* and four in *Snhmgr* when the size threshold was hundred. Longest ORF was noticed in *Sxhmgr* (483 bases) and shortest ORF in *Sthmgr1* (282bases) (Fig.6). The location and length of the ORFs are specified in Table 7.

All the longest ORFs were subjected to BLAST and identity percent with other *hmgr* sequences was found out. The level of identity ranged between 80 per cent and 94 per cent. Maximum homology was shown when the sequences were compared with *hmgr* gene of other solanaceous plants (Table 8). Phylogenetic connection of the sequences with other plant *hmgr* genes was found out and the tree was built depicting the evolutionary relationship between each sequence. *Snhmgr* and *Sthmgr1* showed maximum homology with tomato *hmgr*, whereas, *Sthmgr2* was found to be similar to *Capsicum hmgr*. *Sxhmgr* formed part of another subcluster consisting of *Taxus* and *Ginkgo* (Fig. 7).

Ten different restriction enzymes were analyzed for the presence of cleavage sites in the cloned sequences (http://seqtool.sdsc.edu/). *Alu* I and *Hinf* I had maximum number of sites when compared to others. *Bam* HI, *Pst* I, *Hpa* I and *Hind* III could produce cleavage in single sequence only (Table 9). The diagrammatic representation showing the location of recognition sequences of different restriction enzymes is given

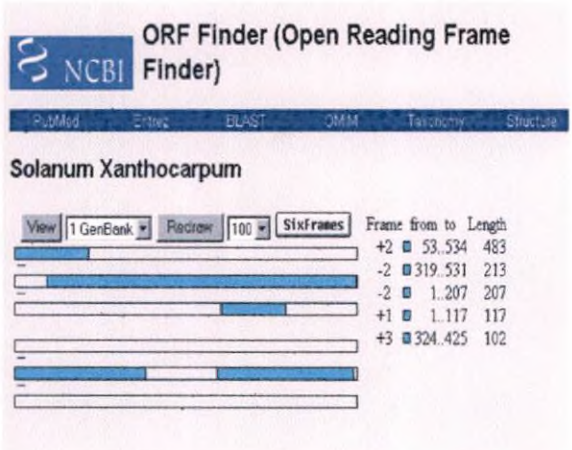
Table 6. Nitrogen base composition of *hmgr* clones

Gene sequence	Nitrogen base percentage (%)					
	A	T	G	C	A and T	G and C
Sxhmgr	29.2	26.2	26.5	18.1	55.3	44.7
Snhmgr	25.4	27.1	19.8	27.7	52.6	47.4
Sthmgr 1	26.1	25.9	27.1	20.8	52.1	47.9
Sthmgr 2	26.0	24.0	29.2	20.8	50.0	50.0

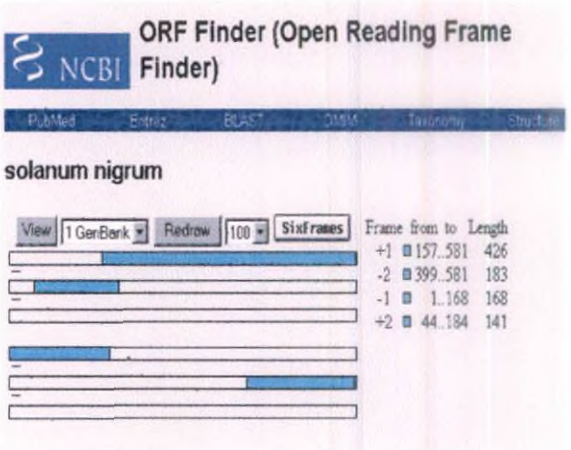
Table 7. Open reading frames (ORF) of *hmgr* gene in different plants

Sl No.	Gene	ORF location	ORF length	Reading frame
1	*Sxhmgr*	53-534	483	+2
2		319-531	213	- 2
3		1-207	207	- 2
4		1-117	117	+1
5		324-425	102	+3
6	*Snhmgr*	157-581	426	+1
7		399-581	183	+2
8		1-168	168	- 1
9		44-184	141	+2
10	*Sthmgr 1*	292-573	282	+1
11		239-439	201	+2
12		399-572	174	- 3
13		44-217	174	+2
14		1-171	171	- 2
15	*Sthmgr 2*	169-702	393	+1
16		3-317	315	- 1
17		754-939	186	-3
18		771-940	171	+3
19		200-310	111	- 1
20		546-713	168	-1
21		804-940	138	-1

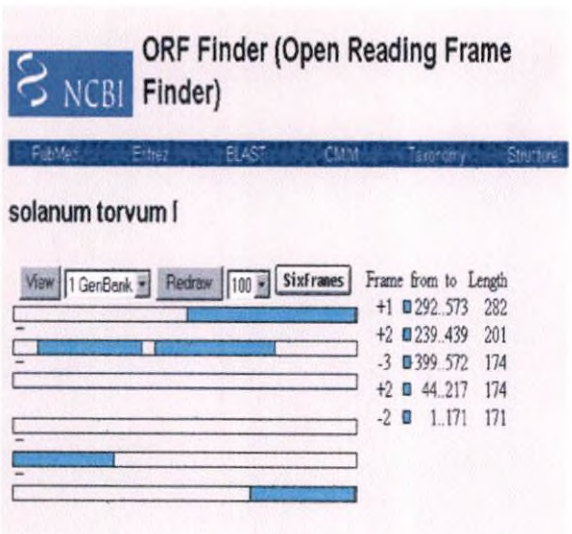
A) *Sxhmgr*



B) *Snhmgr*



C) *Sthmgr1*



D) *Sthmgr 2*

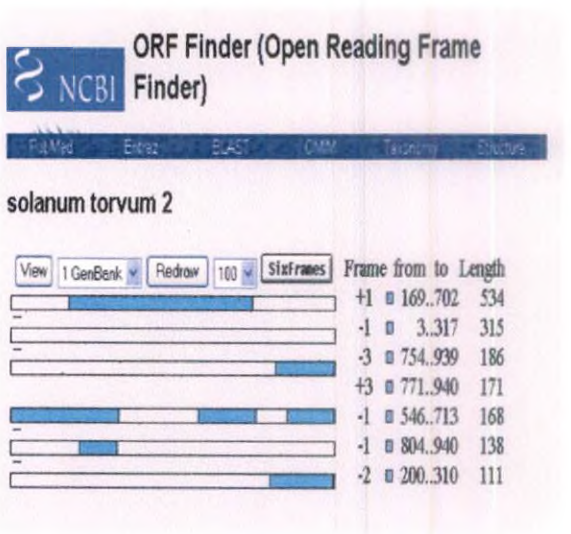


Fig. 6. Open reading frames of the sequenced clones.

The DNA sequence is displayed as six parallel horizontal bars, each one corresponding to one of the six possible translation frames.(+1, +2, +3 and –1, -2, -3 on the reverse strand). The ORFs verifying the size threshold (100 bp) are shown as small green shaded areas on each bar.

Table 8 A. Deduced amino acid sequence of *Sxhmgr* ORF and identity with other plant *hmgr* genes

ORF & Length(bp)	Aminoacid sequence	Details of sequences sharing homology		
		Accession No.	Plant species	% identity
+2 483	M T R A P V V R F A T A K R A A E M K F F V E D P I N F E T L S L V F N K S S R F A R L Q S I Q C A I A G K N L Y M R F S C S T G D A M G M N M V S K G V Q N V L D Y L Q N E Y P D M D V M G I S G N F C S D K K P A A V N W I E G R G K S V V C E A I I K E E V V K K V L K T E V A A L V E L N M L K N L T G S A M A	L01400 L40938 U60452 AF110383 AF542543 X63649 AF038046 AY623812 AY706757 AF303583 U72146	*Solanum tuberosum* *Lycopersicon esculentum* *Nicotiana tabacum* *Capsicum annuum* *Nicotiana attenuata* *Nicotiana sylvestris* *Gossypium hirsutum* *Catharanthus roseus* *Hevea braziliensis* *Pisum sativum* *Camptotheca acuminata*	86 91 91 86 87 86 84 82 82 79 80

Table 8 B. Deduced amino acid sequence of *Snhmgr* ORF and identity with other plant *hmgr* genes

ORF & Length(bp)	Aminoacid sequence	Details of sequences sharing homology		
		Accession No.	Plant species	% identity
+1 426	M V P Q R P A K V A L S Q A E K P A P I I I P A L S E D D E E I I Q S V V Q G K T P S Y S L E S K L G D C L R A A S I R K E A L Q R I T G K S L E G L P L E G F D Y E S I L G Q C C E M P V G Y V Q I P V G I A G P L L L D G R E Y S V P M A T T E G C L V A S T N R G C K A	L01400 L40938 U60452 AF110383 AF542543 X63649 AF038046 AY623812 AY706757 AF303583 U72146	*Solanum tuberosum* *Lycopersicon esculentum* *Nicotiana tabacum* *Capsicum annuum* *Nicotiana attenuata* *Nicotiana sylvestris* *Gossypium hirsutum* *Catharanthus roseus* *Hevea braziliensis* *Pisum sativum* *Camptotheca acuminata*	93 92 88 84 85 85 82 80 80 82 81

Table 8 C. Deduced amino acid sequence of *Sthmgr 1* ORF and identity with other plant *hmgr* genes

ORF & Length(bp)	Aminoacid sequence	Details of sequences sharing homology		
		Accession No.	Plant species	% identity
+1 282	L E S K L G D C T R A A S I R K E A L Q R I T G K S L E G L P L E G F D Y E S I L G Q C C E M P V G Y V Q I P V G I A G P L L L D G R E Y S V P M A T T E G C L V A S T N R G C K A I F V S	L01400 L40938 U60452 AF110383 AF542543 X63649 AF038046 AY623812 AY706757 AF303583 U72146	*Solanum tuberosum* *Lycopersicon esculentum* *Nicotiana tabacum* *Capsicum annuum* *Nicotiana attenuata* *Nicotiana sylvestris* *Gossypium hirsutum* *Catharanthus roseus* *Hevea braziliensis* *Pisum sativum* *Camptotheca acuminata*	94 93 91 88 88 87 83 81 88 80 80

Table 8 D. Deduced amino acid sequence of *Sthmgr2* ORF and identity with other plant *hmgr* genes

ORF & Length(bp)	Aminoacid sequence	Details of sequences sharing homology		
		Accession No.	Plant species	% identity
+1 393	M S M A E K P A P L V T P A A S E E D E E I I K S V V Q G K M P S Y S L E S K L G D C K R A A S I R R E A L Q R I T G K S L E G L P L E G F N Y E S I L G Q C C E M P I G Y V Q I P V G I V G P L L L N G R E F T V P M A T T E G C L V A S T N R G C K A I Y A S G G	L01400 L40938 U60452 AF110383 AF542543 X63649 AF038046 AY623812 AY706757 AF303583 U72146	*Solanum tuberosum* *Lycopersicon esculentum* *Nicotiana tabacum* *Capsicum annuum* *Nicotiana attenuata* *Nicotiana sylvestris* *Gossypium hirsutum* *Catharanthus roseus* *Hevea braziliensis* *Pisum sativum* *Camptotheca acuminata*	92 91 89 91 88 88 82 80 84 81 80

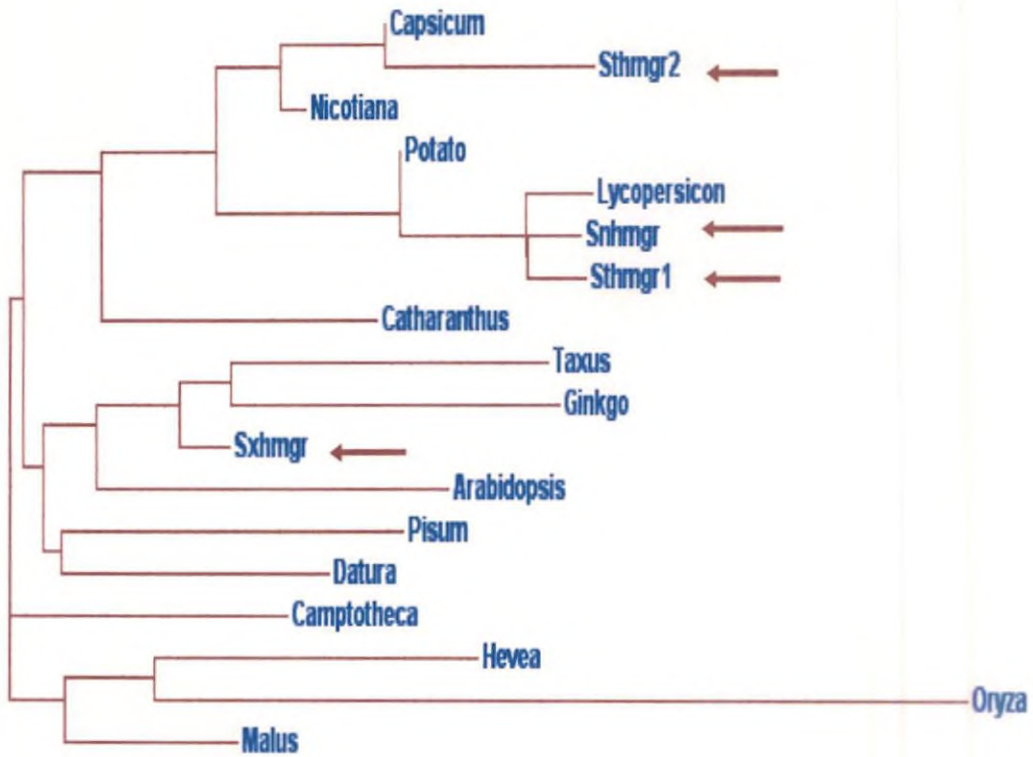


Fig. 7. Phylogenetic tree showing diversity among various plant *hmgr* genes.
The cloned sequences are indicated with arrows.

Table 9A. Restriction analysis of *Sxhmgr* sequence

Restriction enzyme	Recognition sequence	No. of cut(s)	Position of restriction sites	Fragment sizes (bp)
Alu I	AG'CT	3	78, 222, 250	58,78,144,285
Bam HI	G'GATC_C	1	137	137,398
Btg I	C'CryG_G	2	82,529	6,82,447
Dpn I	GA'TC	2	139,524	11,139,385
Hae II	r_GCGC'y	1	43	43,492
Hind III	A'AGCT_T	0	-	-
Hinf I	G'AnT_C	2	91,155	64,91,380
Hpa I	GTT'AAC	1	395	140,395
Mbo I	'GATC_	2	137,522	13,137,385
Pst I	C_TGCA'G	0	-	-

Table 9B. Restriction analysis of *Snhmgr* sequence

Restriction enzyme	Recognition sequence	No. of cut(s)	Position of restriction sites	Fragment sizes (bp)
Alu I	AG'CT	5	61,104,185, 304,323	19,43,61,81, 119,259
Bam HI	G'GATC_C	0	-	-
Btg I	C'CryG_G	1	85	85,497
Dpn I	GA'TC	0	-	-
Hae II	r_GCGC'y	1	582	0,582
Hind III	A'AGCT_T	1	302	280,302
Hinf I	G'AnT_C	3	26,296,404	26,108,178,270
Hpa I	GTT'AAC	0	-	-
Mbo I	'GATC_	0	-	-
Pst I	C_TGCA'G	1	103	103,479

Table 9C. Restriction analysis of *Sthmgr1* sequence

Restriction enzyme	Recognition sequence	No. of cut(s)	Position of restriction sites	Fragment sizes (bp)
Alu I	AG'CT	3	15,28,277	15,43,219,251
Bam HI	G'GATC_C	0	-	-
Btg I	C'CryG_G	1	39	39,489
Dpn I	GA'TC	0	-	-
Hae II	r_GCGC'y	0	-	-
Hind III	A'AGCT_T	0	-	-
Hinf I	G'AnT_C	3	63,250,358	63,108,170,187
Hpa I	GTT'AAC	0	-	-
Mbo I	'GATC_	0	-	-
Pst I	C_TGCA'G	0	-	-

Table 9D. Restriction analysis of *Sthmgr2* sequence

Restriction enzyme	Recognition sequence	No. of cut(s)	Position of restriction sites	Fragment sizes (bp)
Alu I	AG'CT	1	286	276,286
Bam HI	G'GATC_C	0	-	-
Btg I	C'CryG_G	2	174,243	69,174,319
Dpn I	GA'TC	3	314,660,786	126,155,314,346
Hae II	r_GCGC'y	0	-	-
Hind III	A'AGCT_T	0	-	-
Hinf I	G'AnT_C	7	26,278,386,676, 813,837,851	14,24,26,90,108, 137,252,290
Hpa I	GTT'AAC	0	-	-
Mbo I	'GATC_	3	312,658,784	126,157,312,346
Pst I	C_TGCA'G	0	-	-

Abbreviations :

r – Either of the two purines

y – Either of the two pyrimidines

n – Any of the four bases

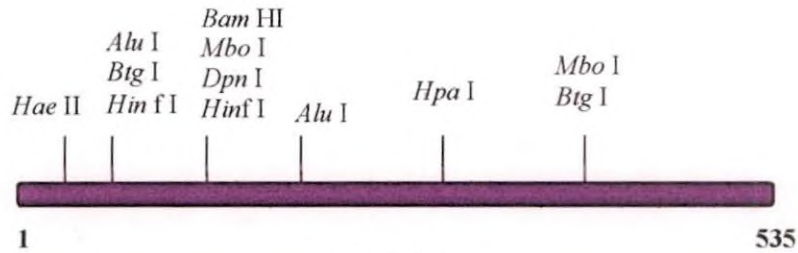
in Fig. 8. Gene prediction analysis of the sequences using the tool 'Genscan' (www.genes.mit.edu/genscan/) revealed that *Sxhmgr* and *Snhmgr* encode initial exon only, whereas, *Sthmgr* clones code for internal exons (Fig. 9).

4.7.2 Amino acid sequence analysis

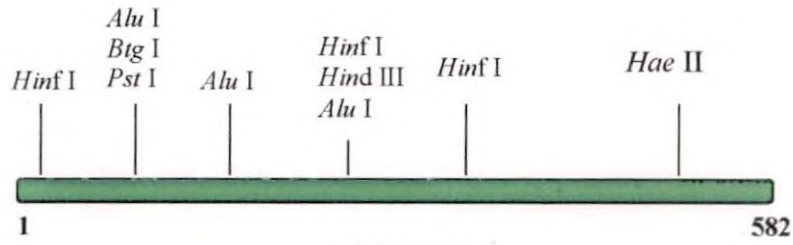
Deduced amino acid sequences of HMGR protein were subjected to multiple sequence alignment with other HMGR protein sequences (Fig. 10). The two genomic clones of *S. torvum* did not show any general trend of homology when compared with other *hmgr* genes. Hence these two clones were subjected to BLAST analysis and per cent identity with other solanaceous *hmgr* was plotted. *Sthmgr1* showed maximum homology with potato *hmg1* whereas, *Sthmgr2* showed maximum identity with potato *hmg2*, tomato *hmg2* and tobacco *hmg2* (Fig.11). The proportion of each amino acid was calculated using 'AASTAT' tool (http://seqtool.sdsc.edu/). Histidine residue was not found in any of the sequences. Tryptophan residue was absent in all the sequences except *Sxhmgr* (Table 10). The secondary structure of protein was predicted by 'GOR' algorithm offered by 'Biology Workbench' and proportion of each structure types were found out. *Sxhmgr* was comparatively richer in alpha helix and all other sequences (Fig. 12 & 13).

The sequences were compared with conserved domain database (CDD) to find out conserved regions present in them (Fig. 14). All the major conserved domains showed high-level homology to HMGR proteins belonging to class I and class II. Domains involved in mevalonate synthesis, CoA reduction and lipid metabolism were detected.

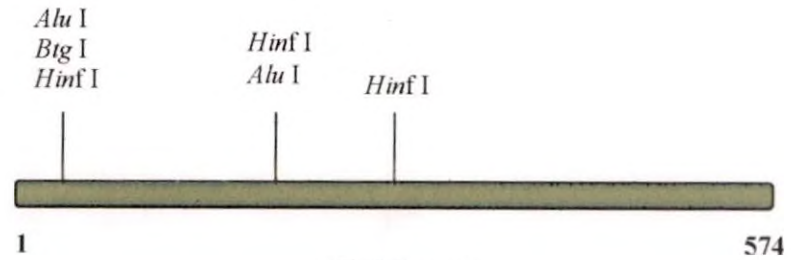
Important functional domains were located using 'InterProScan' (www.ebi.ac.uk/InterProScan/). All the sequences had hydroxyl methyl glutaryl CoA reductase domain. An NAD binding domain was present in *Sxhmgr* and substrate-binding domains were present in all the other sequences (Fig. 15).



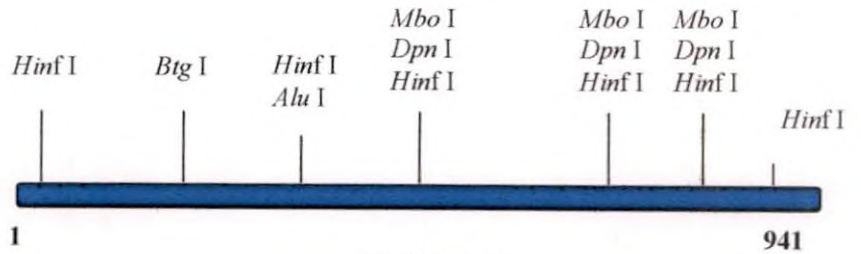
A) *Sxhmgr*



B) *Snhmgr*



C) *Sthmgr1*

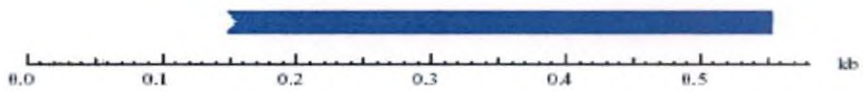


D) *Sthmgr2*

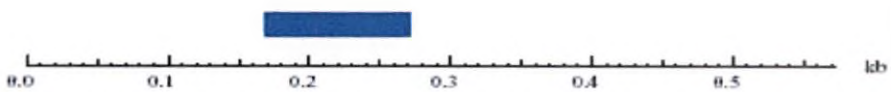
Fig. 8 Diagrammatic representation of restriction sites present in the *hmgr* Sequences. Lengths of the sequences are indicated as numbers below.

A) *Sxhmgr*

B) *Snhmgr*



C) *Sthmgr 1*



D) *Sthmgr 2*

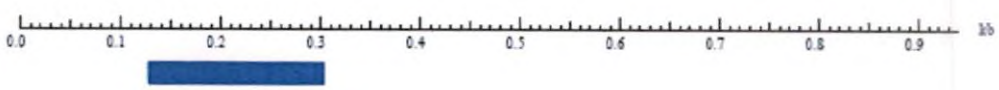


Fig. 9. Exon sequences present in *hmgr* genes. The length of each exon region is indicated in the scale.

Fig. 10 A. Multiple sequence alignment of deduced amino acid sequence of *Sxhmgr*

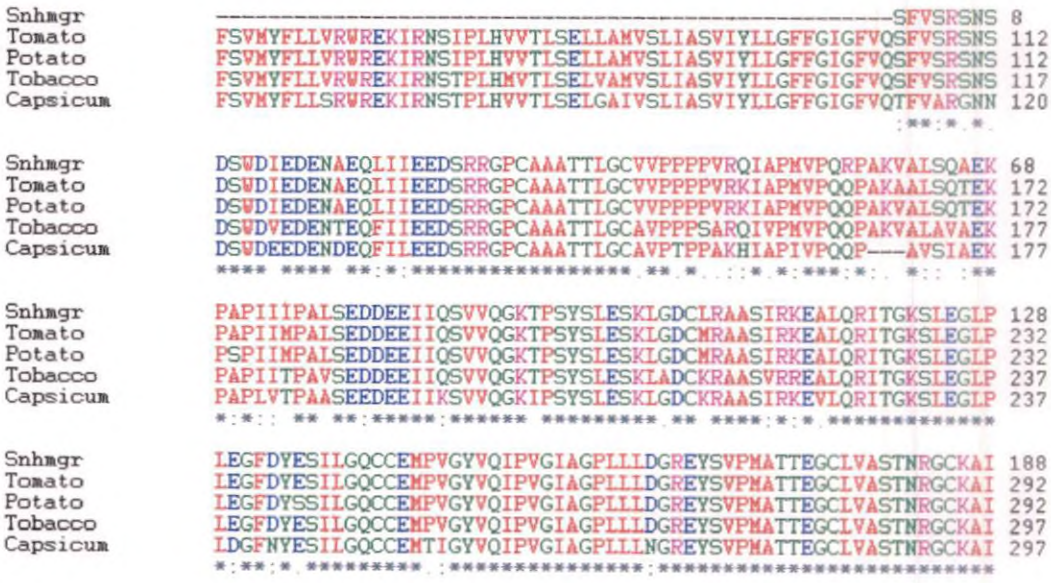


Fig. 10 B. Multiple sequence alignment of deduced amino acid sequence of *Snhmgr*

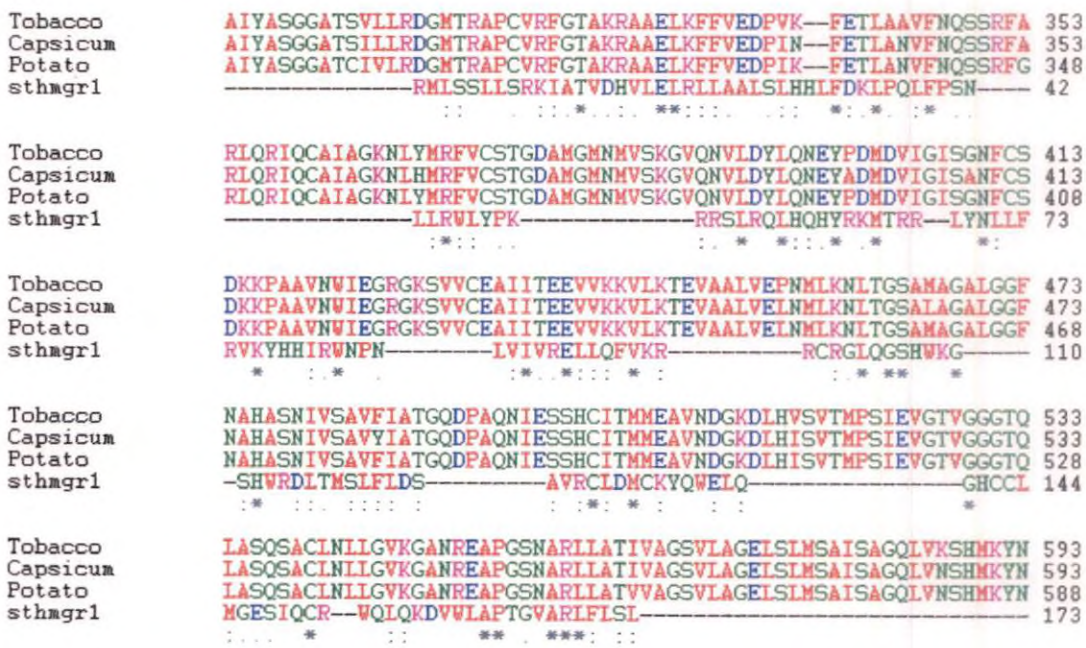


Fig. 10 C. Multiple sequence alignment of deduced amino acid sequence of *Sthmgr1*

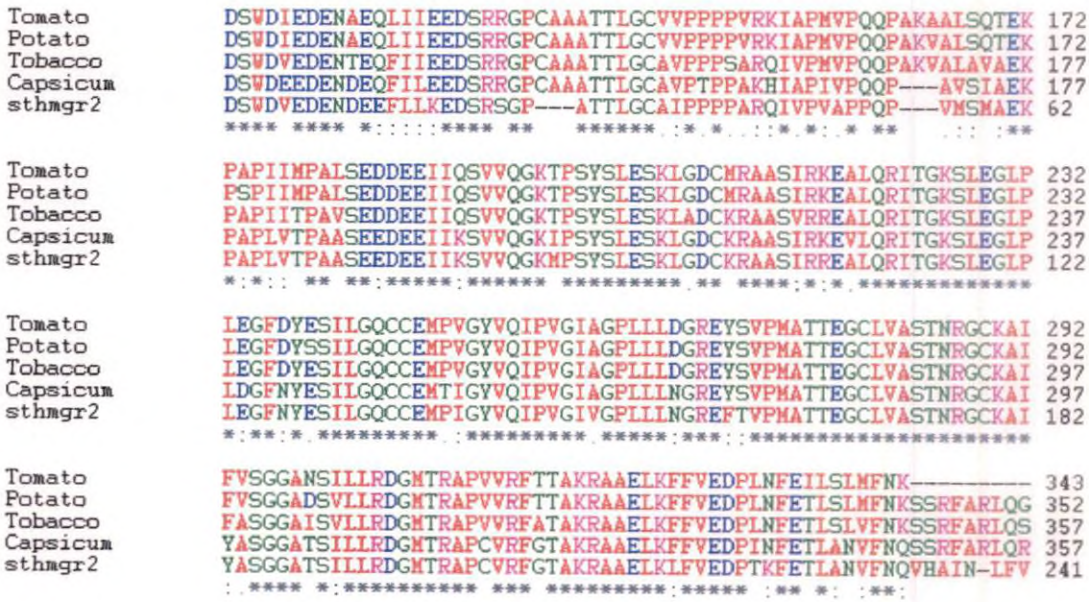


Fig. 10 D. Multiple sequence alignment of deduced amino acid sequence of *Sthmgr2*

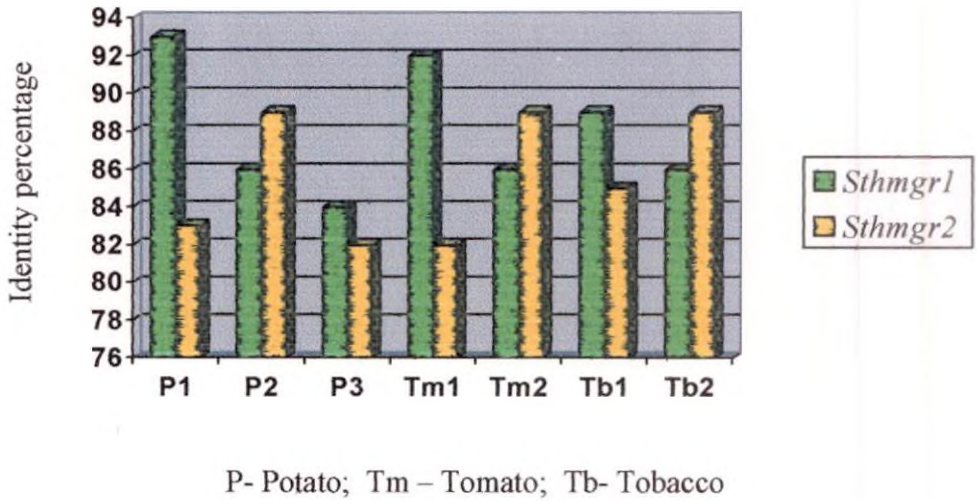


Fig. 11. The identity level difference in the two genomic clones of *S. torvum.*

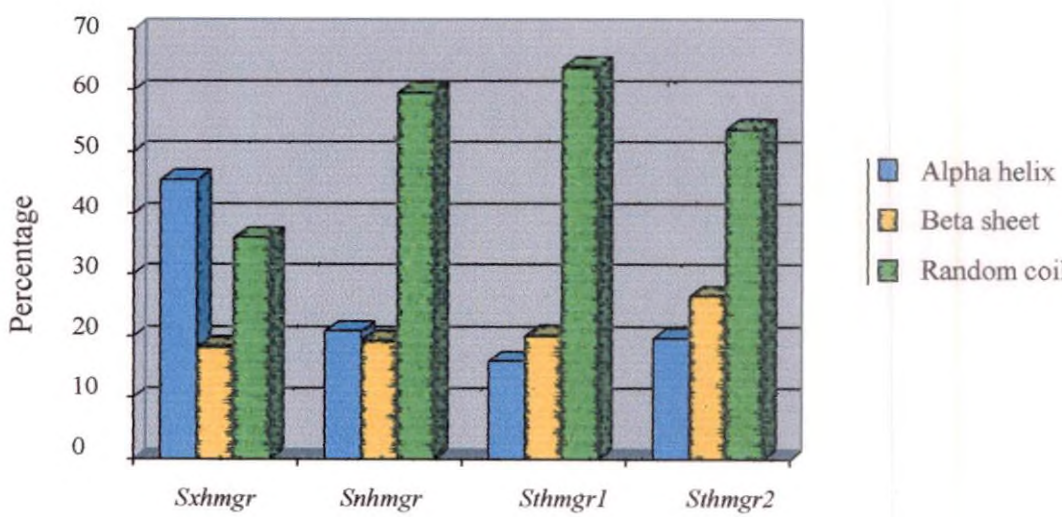


Fig. 12. Graphical representation of proportion of different secondary structure in the *hmgr* genes

```
Sequence    LLRDGMTRAP VVRFATAKRA AEMKFFVEDP INFETLSLVF NKSSRFARLQ
Structure   CCCCCCCCCH HHHHHHHHHH HHHHHHHCCC CCCCHHHHHH CCCCHHHHHH

Sequence    SIQCAIAGKN LYMRFSCSTG DAMGMNMVSK GVQNVLDYLQ NEYPDMDVMG
Structure   HHHHHHCCCC EEEEEEEEEE CCCEEEEEEC CCHHHHHHHC CCCCCCEEEE

Sequence    ISGNFCSDKK PAAVNWIEGR GKSVVCEAII KEEVVKKVLK TEVAALVELN
Structure   ECCCCCCCCC CCEEEEEECC CCCCHHHHHH HHHHHHHHHH HHHHHHHHHH

Sequence    MLKNLTGSAM A
Structure   HHHCCCCCEE C
```

Alpha Helix = H Beta Sheet = E Random Coil = C

Fig. 13 A. Predicted secondary structure of the amino acid sequence encoded by the longest open reading frame of *Sxhmgr*

```
Sequence    MVPQRPAKVA LSQAEKPAPI IIPALSEDDE EIIQSVVQGK TPSYSLESKL
Structure   CCCCCCCHHH HHHCCCCCCE EEECCCCCCH HHHHEEECCC CCCEEECCCC

Sequence    GDCLRAASIR KEALQRITGK SLEGLPLEGF DYESILGQCC EMPVGYVQIP
Structure   CHHHHHHHHH HHHHHHHHCC CCCCCCCCCC CEEEEECCCC CCCCCEEEEC

Sequence    VGIAGPLLLD GREYSVPMAT TEGCLVASTN RGCKA
Structure   CCCCCCCCCC CCCCEECCCC CCCCEEECCC CCEEC
```

Alpha Helix = H Beta Sheet = E Random Coil = C

Fig. 13 B. Predicted secondary structure of the amino acid sequence encoded by the longest open reading frame of *Snhmgr*

```
Sequence   LESKLGDCTR AASIRKEALQ RITGKSLEGL PLEGFDYESI LGQCCEMPVG
Structure  CCCCCCCCHH HHHHHHHHHH HHHCCCCCCC CCCCCCEEEE ECCCCCCCCC

Sequence   YVQIPVGIAG PLLLDGREYS VPMATTEGCL VASTNRGCKA IFVS
Structure  EEEECCCCCC CCCCCCCCCE ECCCCCCCCE EECCCCCCEE EEEC
```

Alpha Helix = H Beta Sheet = E Random Coil = C

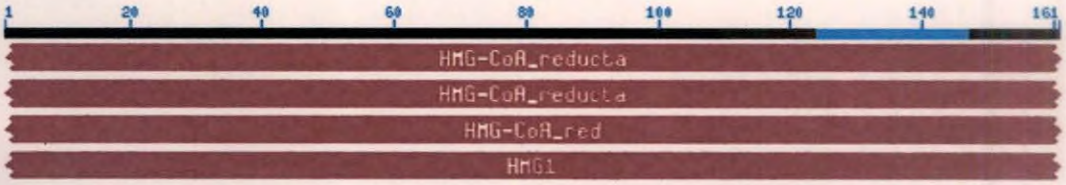
Fig. 13 C. Predicted secondary structure of the amino acid sequence encoded by the longest open reading frame of *Sthmgr1*

```
Sequence   SFVSRGNNDS WDVEDENDEE FLLKEDSRSG PATTLGCAIP PPPARQIVPV
Structure  CCCCCCCCCC CEECCHHHHH HHHHHCCCCC CCCEECCCCC CCCCCCEECC

Sequence   APPQPVMSMA EKPAPLVTPA ASEEDEEIIK SVVQGKMPSY SLESKLGDCK
Structure  CCCCCCCCCC CCCCCCCCCC CHHHHHHHHH HHHCCCCCCE EEECCCCCHH

Sequence   RAASIRREAL QRITGKSLEG LPLEGFNYES ILGQCCEMPI GYVQIPVGIV
Structure  HHHHHHHHHH HHHHCCCCCC CCCCCCCEEE ECCCCCCCCC CEECCCCCEE

Sequence   GPLLLNGREF TVPMATTEGC LVASTNRGCK AIYASGGATS ILLRDGMTRA
Structure  CCCCCCCCCC EEEECCCCCC EEECCCCCCE EEEECCCCCE EEEECCCCCC

Sequence   PCVRFGTAKR AAELKLFVED PTKFETLANV FNQVHAINLF VILCFYTSSY
Structure  CEEEECCHHH HHHHHHHCCC CCCHHHHHHH HHHHHEEEEE EEEECCCCEE

Sequence   LIVVPEWDVA DQADLPDYKG FSVQLPGRIC TDSYVALVMQ WEIWCPKVYK
Structure  EEEEEEECCC CCCCCCCCCC CEEEECCCCC CCEEEEEEEE EEEECCCCCE

Sequence   TFLLTFRMNI
Structure  EEEEEECEEC
```

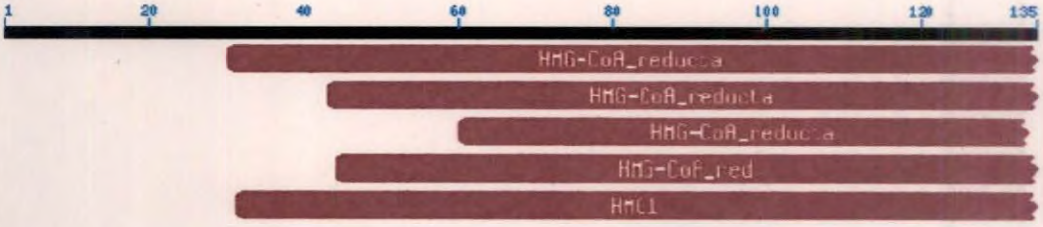
Alpha Helix = H Beta Sheet = E Random Coil = C

Fig. 13 D. Predicted secondary structure of the amino acid sequence encoded by the longest open reading frame of *Sthmgr 2*

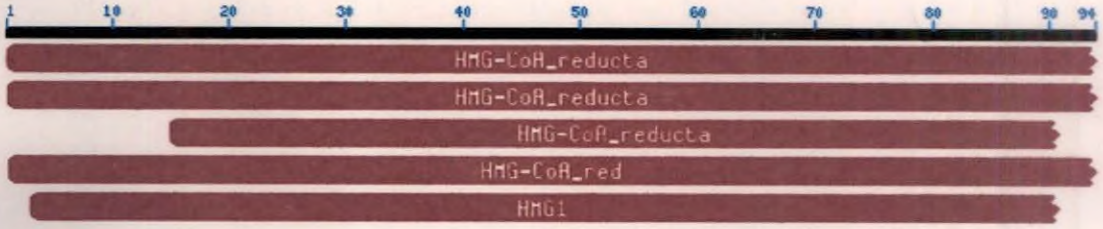
A) *Sxhmgr*



B) *Snhmgr*



C) *Sthmgr1*



D) *Sthmgr 2*

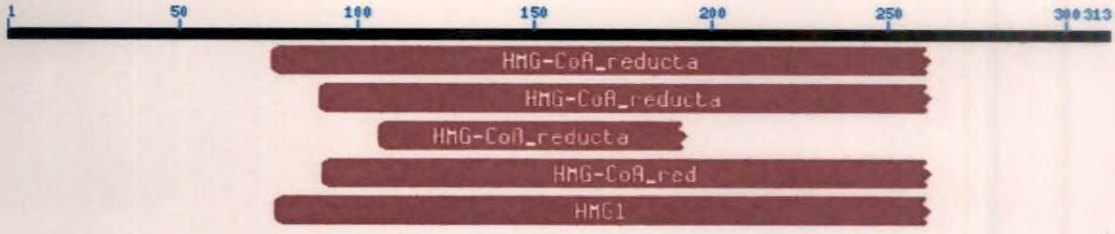


Fig. 14. Conserved domains of the cloned sequences, which show similarity to other *hmgr* coding sequences. The bars indicate the position of conserved regions.

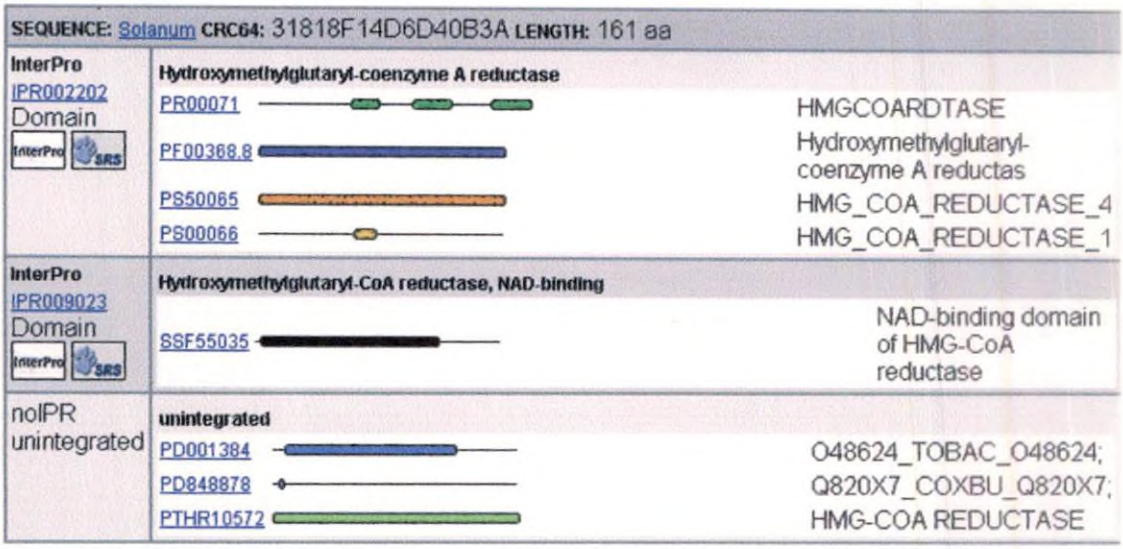


Fig. 15 A. The functional domains present in the cloned *Sxhmgr* sequence

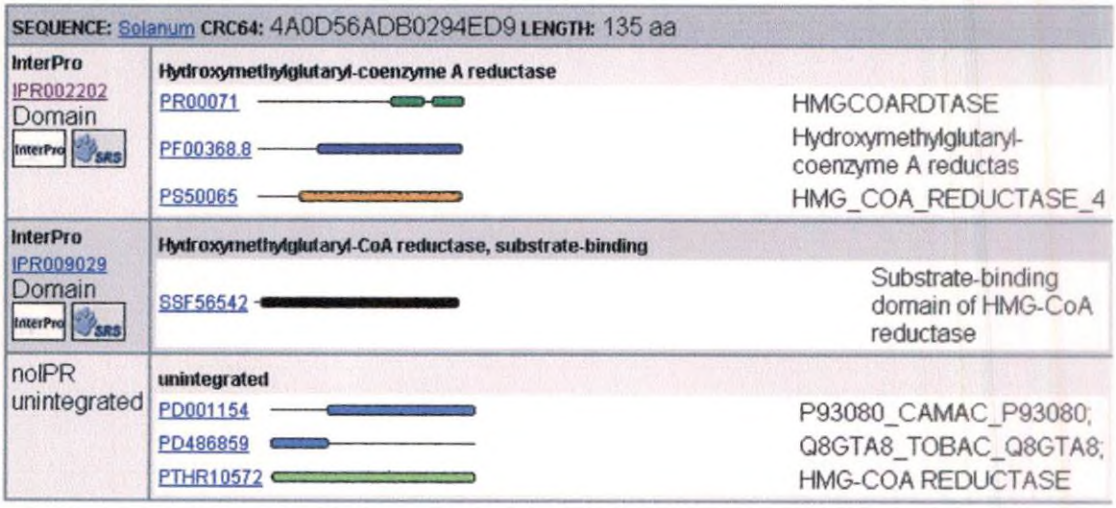


Fig. 15 B. The functional domains present in the cloned *Snhmgr* sequence

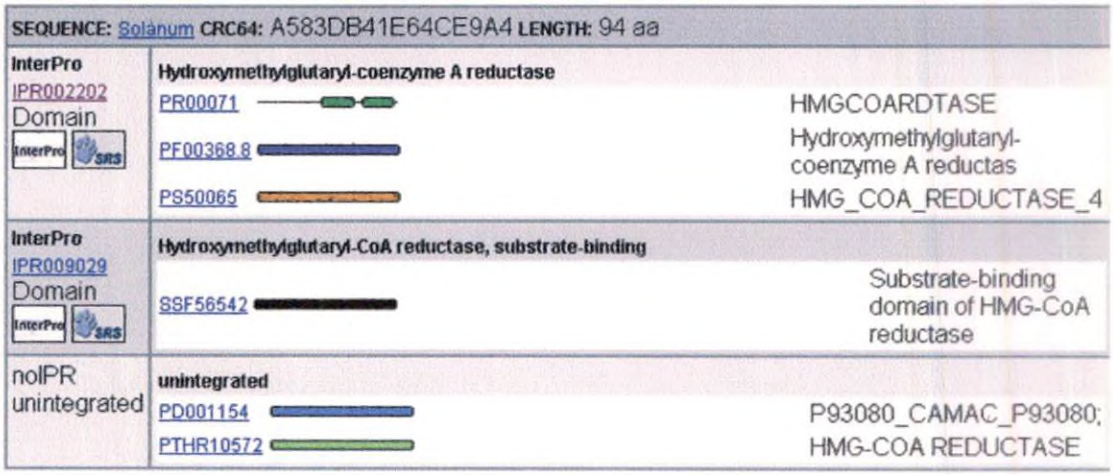


Fig. 15 C. The functional domains present in the cloned *Sthmgr1* sequence

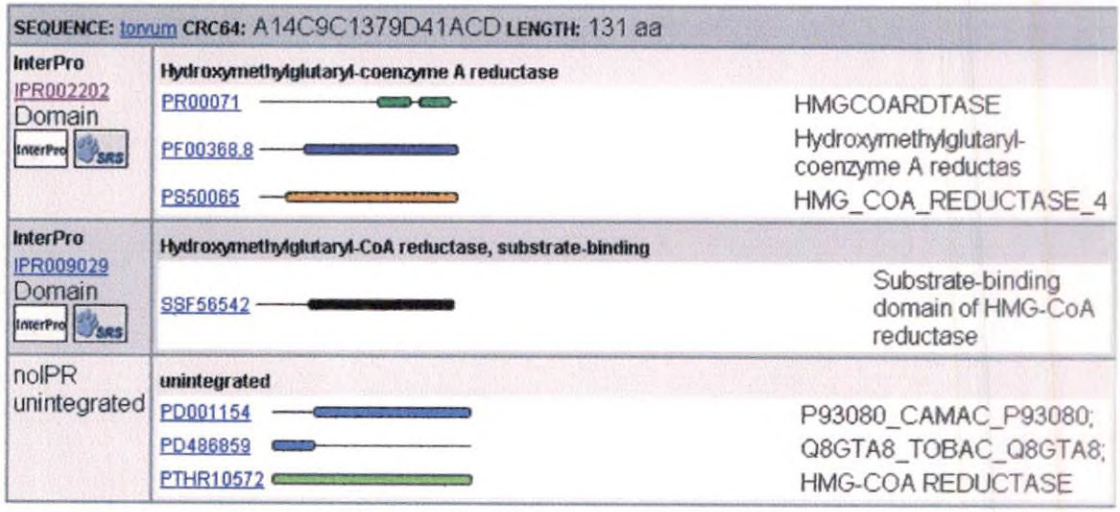


Fig. 15 D. The functional domains present in the cloned *Sthmgr2* sequence

Protein structures of major functional domains were compared with CATH protein structural database (www.biochem.ucl.ac.uk/bsm/cath/) and are depicted in Fig. 16. The analysis revealed the presence of oxidoreductase domain and domain 2 of chain A in the sequences.

Protein motifs, profiles and patterns were predicted using 'Mol biol online analysis' tools (http://motif.genome.jp/). Motif analysis revealed the presence of HMGR signature I and HMGR family profile in *Sxhmgr* sequence. All the other sequences showed presence of HMGR family profile only (Table 11). Hydropathy plot of each sequence was constructed by means of 'Kyte and Doolittle' analysis (http://occawlonline.pearsoned.com). No putative trans membranes were detected in any sequences (Fig. 17).

4.8 ISOLATION OF FULL-LENGTH GENE

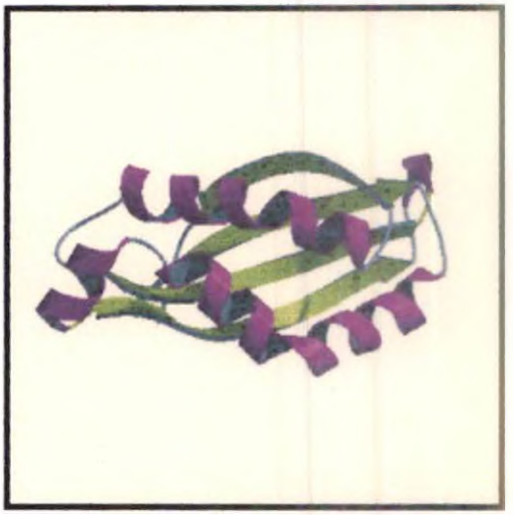
4.81 RNA isolation

Different concentrations of abscisic acid were used to induce HMGR mRNA in higher quantity. Wilting of twigs was first observed after four-hour treatment with 1000 mg l^{-1} solution. At lower concentrations wilting was observed after 8 to 12 hours. RNA was isolated using trizol reagent and run on 0.8 per cent agarose gel (Plate 10). Three distinct bands corresponding to 28s, 18s and 5s rRNA were obtained. RNA was quantified spectrophotometrically (Table 12). The second sample was found to be more suitable for cDNA synthesis due to increased quality and quantity.

4.8.2 Rapid Amplification of cDNA Ends (RACE)

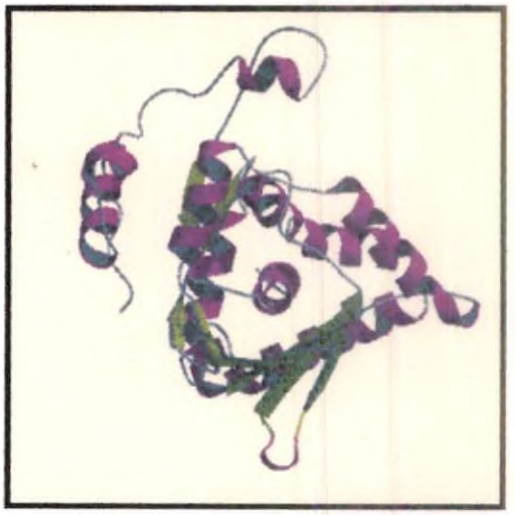
Reverse transcriptase enzyme was used to synthesize the 5' and 3' cDNA from the RNA preparation and the HMGR cDNA was amplified using gene specific primers (Table 13) in two separate reactions. The 5' and 3' RACE reactions were carried out with the suitable primer combination. GSP1 (gene specific primer 1) and UPM (universal primer mix) were used in 5' RACE and GSP2 and UPM were used in 3'

Oxido reductase domain
Sequence family 3.30.70.420.1



Oxido reductase domain
Sequence family 3.30.70.420.2

HMGR chain A, domain 2
Sequence family 3.90.770.10.1



HMGR chain A, domain 2
Sequence family 3.90.770.10.2

Fig. 16. Protein structure of various domains of HMGR. The alpha helix is shown in magenta (helix shaped) , β- sheets in yellow (wide ribbon shaped) and the random coils in gray (line shaped).

Table 10. Amino acid composition of different HMGR protein sequences

Aminoacid			Molar percentage of amino acid ((Mol %)			
			Sxhmgr	*Snhmgr*	*Sthmgr1*	*Sthmgr2*
Non polar		Gly	6.21	8.89	11.70	10.69
		Ala	9.94	8.89	7.45	8.40
		Val	9.94	6.67	6.38	6.11
		Leu	8.07	10.37	11.70	9.16
		Ile	4.35	7.41	6.38	6.87
		Met	6.21	2.22	2.13	3.82
		Pro	2.48	8.15	5.32	6.87
		Phe	4.97	0.74	2.13	1.53
		Trp	0.62	0.00	0.00	0.00
Polar	Uncharged	Ser	6.83	8.15	7.45	8.40
		Thr	3.73	3.70	5.32	4.58
		Cys	2.48	3.70	5.32	3.82
		Tyr	1.86	2.96	3.19	3.05
		Asn	6.21	0.74	1.06	2.29
		Gln	2.48	5.19	3.19	3.05
	Basic	Lys	8.07	5.19	4.26	5.34
		Arg	4.97	4.44	5.32	4.58
		His	0.00	0.00	0.00	0.00
	Acidic	Asp	4.35	3.70	3.19	1.53
		Glu	6.21	8.89	8.51	9.92

Table 11. Details of motifs found in different HMGR sequences

Gene	Motif	Position	Prosite ID	Description
Sxhmgr	HMGR 1	64-78	PS00066	HMGR signature 1
	HMGR4	1-161	PS50065	HMGR family profile
Snhmgr	HMGR4	29-135	PS50065	HMGR family profile
Sthmgr 1	HMGR4	1-94	PS50065	HMGR family profile
Sthmgr2	HMGR4	19-131	PS50065	HMGR family profile

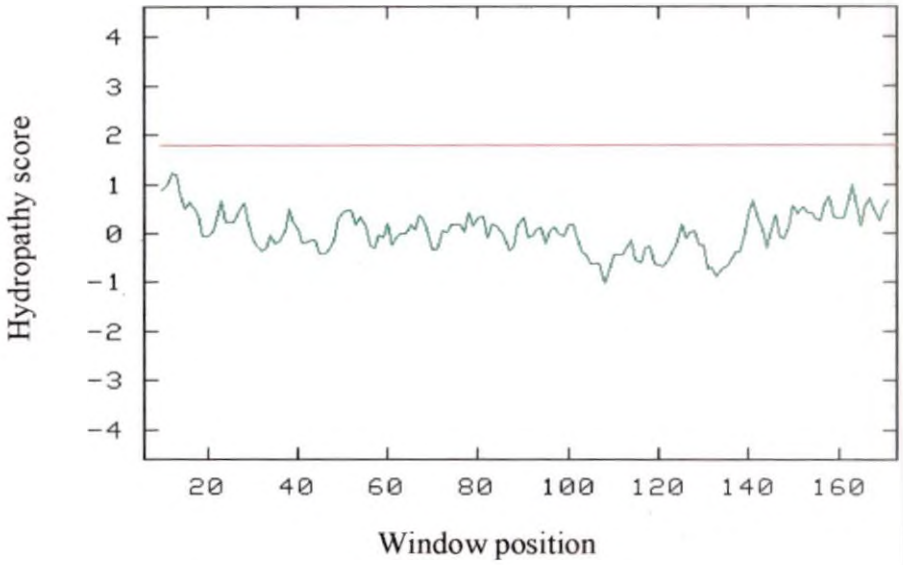


Fig. 17 A. Hydropathy plot of *Sxhmgr* sequence.

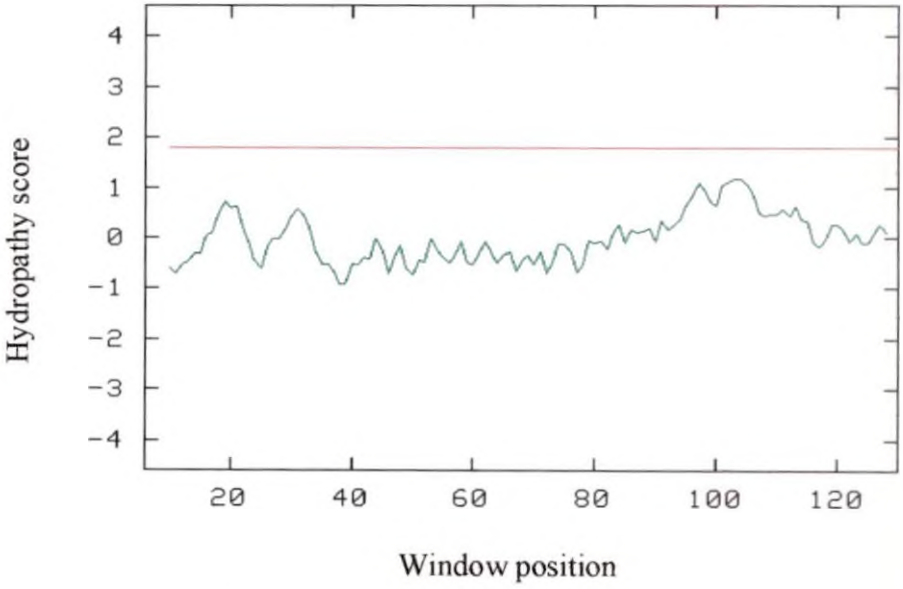


Fig. 17 B. Hydropathy plot of *Snhmgr* sequence.

The average hydrophobicity of each amino acid residue was calculated using the algorithm of Kyte and Doolittle over a window of 19 amino acids and was plotted as a function of window position

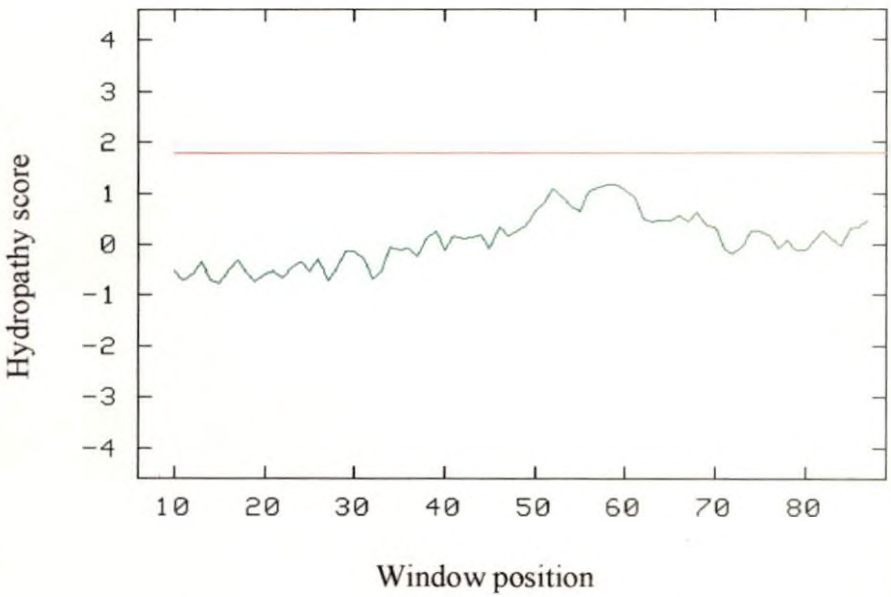


Fig. 17 C. Hydropathy plot of *Sthmgr1* sequence.

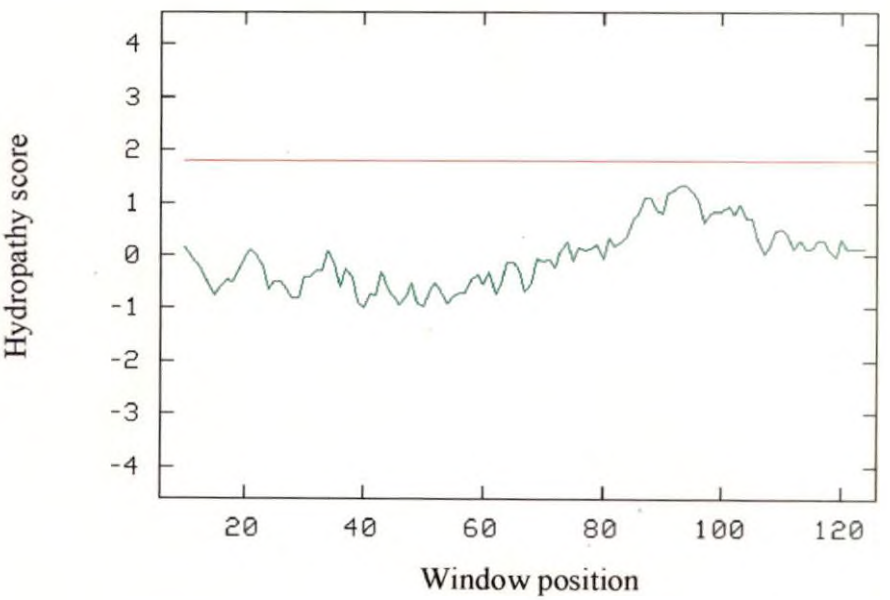


Fig. 17 D. Hydropathy plot of *Sthmgr2* sequence.

The average hydrophobicity of each amino acid residue was calculated using the algorithm of Kyte and Doolittle over a window of 19 amino acids and was plotted as a function of window position

Table 12. Quality and quantity of RNA extracted from *S. xanthocarpum*

RNA sample	Optical density		Ratio OD_{260} / OD_{280}	Quantity of RNA(μg/μl)
	OD_{260}	OD_{280}		
sample 1	0.023	0.017	1.35	0.46
sample 2	0.104	0.057	1.83	2.1

Table 13. Details of gene specific primers used in RACE reactions

Primer	Type	RACE	Primer sequence	Tm (^{0}C)
GSP1	Forward	5'	5' GCA AGG CTA TTT TTG TCT CTG GTG GCG 3'	82
GSP2	Reverse	3'	5' CAC CAC CTC TTC CTT GAT TAT TGC CTC GC 3'	88

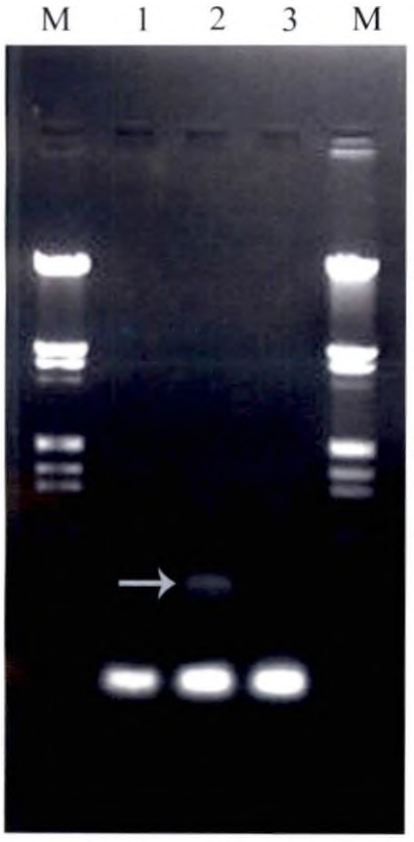
RACE. The products were run on 0.8 per cent agarose gel. No bands were obtained in the 5' reaction. An amplicon of size 540 bp was produced in the 3' RACE. The size was very low when compared with the expected size of one Kb. (Plate 11). This product was eluted and cloned in pGEMT vector and sequenced. The sequence analysis using BLAST confirmed the similarity of the cloned sequence with other *hmgr* genes. The multiple sequence alignment of the 3' sequence with some major *hmgr* genes is given in Fig. 18.

Lane 1 : *S. xanthocarpum* RNA 1

Lane 2 : *S. xanthocarpum* RNA 2

Lane M : Molecular weight marker

Plate 10. RNA isolated from *Solanum xanthocarpum*



Lane 1 : *S. xanthocarpum* 5' RACE

Lane 2 : *S. xanthocarpum* 3' RACE

Lane 3 : Negative control

Lane M : Molecular weight marker

Plate 11. Amplified band obtained in the 3' RACE reaction

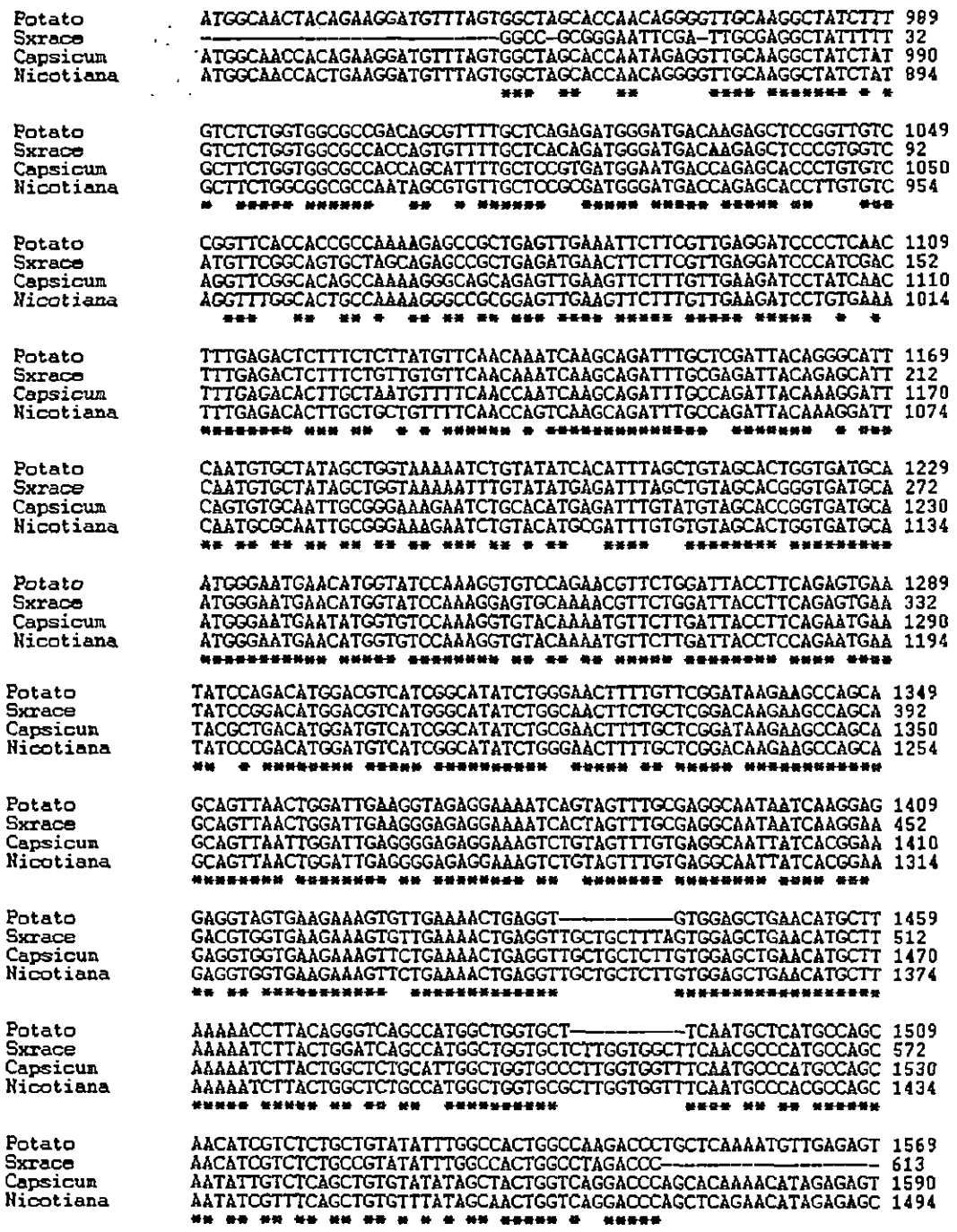


Fig. 18. The multiple sequence alignment of *S. xanthocarpum* 3'RACE with other solanaceous *hmgr* genes. The RACE product is indicated as ' Sxrace'

Discussion

5. DISCUSSION

Solanaceous plants are widely distributed throughout the world, especially in the tropics. Many of the weed plants belonging to the family possess a rich genetic base for resistance against pests and diseases. Most of them have various medicinal uses also. In the present study, four such plants were selected viz., *Solanum xanthocarpum, S. nigrum, Physalis minima* and *S. torvum*, based on their wide distribution, resistance to biotic stress and other medicinal properties.

Solanum xanthocarpum has insecticidal, molluscicidal and larvicidal activities (Singh and Bansal, 2003). It can also be considered as a medicinal plant based on the presence of saponins which can act as anti-allergic and anti-asthmatic (Gupta, 1994). *Solanum nigrum* is well known for it's nematicidal properties (Scholte, 2000). *Physalis minima* is highly resistant to insect pests and diseases (Parmar and Kaushal, 1982). The extracts of *S. torvum* can impart resistance to fungal pathogens and wilt causing bacteria (Rahman *et al.*, 2002; Thangavelu *et al.*, 2004). Moreover, *S. torvum* rootstocks are resistant to root-knot nematode and can be used in breeding of resistant brinjal varieties (Garibaldi *et al.*, 2005).

Many of the compounds involved in imparting such defence related properties to the plants are synthesized by isoprenoid pathway (Bach, 1995). Growth regulators, phytoalexins, carotenoids and terpenoids are some of the end products in this pathway. They carry out various cellular functions like photosynthesis, chemical signaling, growth and development and defence compound production (Arigoni, 1997; Newman and Chappel, 1999). The major rate-limiting enzyme in this pathway is 3-hydroxy 3-methyl glutaryl CoA reductase (HMGR) that is encoded by *hmgr* gene. The knowledge about the gene can reveal various functional aspects of different isoprenoid compounds expressed in various plants. The present study was aimed at molecular characterization of *hmgr* gene from the selected solanaceous plants. The whole work was based on a strategy to clone PCR amplified gene fragment from genomic DNA.

5.1 DNA ISOLATION

A modified Doyle and Doyle (1987) method including freezing the tissue with liquid nitrogen was used. Since most of the selected plants had thick hard leaves, liquid nitrogen was used to powder the leaves. Low temperature provided by liquid N_2 could reduce DNase activity (He *et al.*, 1992). Tender leaves were used because of their suitablility for DNA isolation and better performance in yielding DNA with good quality and quantity. Young leaves usually contain actively dividing cells with lesser concentration of extranuclear materials like protein, oil, carbohydrates and other metabolites that interfere with nucleic acid extraction (Babu, 1997). Extraction buffer contains EDTA, which could effectively chelate Mg^{2+} irons and mediate aggregation of nucleic acid. Beta-mercaptoethanol used during the grinding of leaves could disrupt protein disulfide bonds and was thus capable of initiating protein degradation.

The detergent used in lysis buffer was SDS and it coud act as a nuclease inhibitor and is often used to dissolve membranes. Proteins were removed by chloroform: isoamyl alcohol treatment. In addition to denaturing proteins, chloroform was also useful in removing lipids.

Isopropanol (0.6 volume) was used only for initial precipitation of DNA at low temperature (-20°C) and two volumes of ethanol were used for final precipitation. The pellet was dissolved in TE buffer for long-term storage. EDTA present in TE buffer could chelate and remove Mg^{2+} ions, which were required for nuclease activity. The DNA yielded a single sharp band when run on agarose gel. Since no RNA contamination was noticed along with the bands; RNase treatment was not given (Plate 2).

The optical density values of diluted preparation of DNA were found out using UV spectrophotometer (Table 1). All DNA preparations recorded OD_{260}/OD_{280} values in the range of 1.8- to 2.0, indicating that DNA was good without much RNA or

protein contamination. Quantity of DNA was found to be highest in *S. xanthocarpum* (425.5 µg/ml) and lowest in *S. torvum* (233.5 µg/ml). Since one PCR reaction requires only 25-50 ng template, the DNA recovered was found to be sufficient for further PCR reactions.

5.2 PRIMER DESIGNING

The strategy devised for cloning the plant *hmgr* genes was based upon the assumption that enzymes, which catalyze key reactions in intermediary metabolism, may be conserved during evolution. In the case of HMGR, this hypothesis was supported by the results of Basson *et al.* (1986), who reported that the enzymes from two distantly related organisms, such as hamster and yeast, were very similar in the region containing the catalytic site. Hence primers could be designed from the sequences of the same gene in other plants. When multiple sequence alignment of *hmgr* sequences from a number of plants was done, the homology in conserved boxes was very less. Hence only the sequences from solanaceous plants were taken for further alignment and it showed a good number of conserved regions (Fig. 3). Two pairs of gene specific primers, one pair having degeneracy, were designed from such conserved regions (Table 2). While designing primers, complementary sequences and stretch of single nucleotide were avoided as much as possible. All the primers had melting temperature above 56^0 C.

5.3 AMPLIFICATION OF *hmgr* GENE

Genomic DNA was used to amplify the gene. From genomic DNA all genes of the *hmgr* family could be amplified. On the other hand, if we used cDNA, only the constitutively expressed members of the gene family would be amplified. All other tissue specific or inducible gene expression would be avoided. Hence genomic clones were preferred so as to maximize the amplification pattern. The thermal cycler program was standardized because DNA amplification was very sensitive to PCR conditions. Similar observations were also reported by Park and Kohel (1994).

Therefore, various parameters like DNA concentration, Taq DNA polymerase concentration, annealing temperature and number of cycles were standardized for the specific amplification of *hmgr* gene. All the possible combinations were used at appropriate annealing temperature for PCR amplification. *S. xanthocarpum* gave good amplification for HMF3-HMR3 primer combination with annealing temperature at 58°C. *S. nigrum* and *S. torvum* gave amplification when HMF2-HMR2 was used with annealing temperature at 55°C.

No amplicon was obtained for *Physalis minima*, irrespective of the primer combinations and annealing temperatures tried. This could be due to genetic difference between *P. minima* and other plants. All others belonging to the genus *Solanum* and *P. minima* may not possess any conserved regions based on which the primers were designed. If the template and primers were mismatched, particularly at the 3' end of the primer, amplification would be reduced or eliminated. This was most common when the plants were distantly related to those from which the primer sequences had been derived. Among more closely related taxa, sometimes introns were inserted within the priming site. Introns or large inserts could also be inserted between priming sites making the region too large to be amplified efficiently.

All the plants included in the study belonging to genus *Solanum* yielded two or more bands upon amplification by PCR (Plate 3, Table 4). This was similar to the observation of earlier workers that HMGR was encoded by a multigene family (Stermer *et al.*, 1994). The amplicons might represent isoforms of the enzyme HMGR. There were reports about the presence of two genes in *Arabidopsis* (Caelles *et al.*, 1989), four genes in tomato (Park *et al.*, 1992), three genes in rubber (Chye *et al.*, 1992), three genes in potato (Choi *et al.*, 1992), one to two genes in tobacco (Genschik *et al.*, 1992), four genes in wheat (Aoyagi *et al.*, 1993) and two genes in rice (Nelson *et al.*, 1994) encoding HMGR. The high molecular weight band corresponded to a size of 1835 bp in *S. nigrum* and *S. torvum*. The low molecular weight band was obtained from *S. xanthocarpum* and had a size of 882 bp.

5.4 BACTERIAL TRANSFORMATION

Competence of DH5α cells was confirmed by transforming the cells with a plasmid containing ampicillin resistance marker. *E. coli* cells alone could not grow in ampicillin containing media, since they had no resistance encoding sequence. But all the competent cells harbouring the plasmid could grow in that media (Plate 5).

The cloning vehicle used was pGEMT Easy vector with a size of three kb. It contains T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α peptide-coding region of the enzyme ß-galactosidase. Insertional inactivation of the α peptide allows the recombinant clones to be directly identified by the colour screening of indicator plates. The multiple cloning region of the vector includes restriction sites conveniently arranged for use. The sites allow the release of the insert by digestion with a single restriction enzyme.

Ligated product containing *hmgr* sequence was used to transform the cells which could later be picked up from the media containing 5-bromo 4-chloro 3-indolyl ß-D galactoside (X-gal) and isopropyl thiogalactoside (IPTG) based on blue white screening. The pGEMT vector contained polycloning sites inside a ß-galactosidase encoding gene. Insertion of a new sequence would disrupt the reading frame of galactosidase encoding gene. The bacterial cell and vector together provided the complete protein as a result of α-complementation (Ullmann *et al.*, 1967). The colonies which have not taken up the plasmid can further utilize the substrate and appear as blue colonies on X-gal chromogenic substrate (Horwitz *et al.*, 1964). All transformed colonies harbouring the recombinant plasmid appeared in white colour due to disruption of α-complementation (Plate 6).

Plasmids isolated from white and blue colonies gave bands with different molecular weights. All the plasmids from white colonies had higher molecular weight than the pGEMT vector alone. Plasmid from blue colony was pGEMT itself, since no

recombination had occurred. It gave a band corresponding to 3.1 kb which is the actual size of the plasmid (Plate 7).

Restriction analysis using *Eco* RI enzyme having recognition sequences on either side of the polycloning site, could cut the insert from the vector. Two separate bands corresponding to the vector and the insert were obtained (Plate 8). When plasmid from blue colony was digested, it yielded a single band corresponding to the linearised plasmid. PCR confirmation revealed the amplification of fragment exactly similar to that obtained from genomic DNA (Plate 9). Both the tests confirmed the presence of insert in the plasmid.

5.5 THEORETICAL ANALYSIS OF THE SEQUENCES

When the sequences were subjected to BLAST homology search, it revealed the identity per cent with *hmgr* genes from *Solanum tuberosum* (Choi *et al.*, 1992), *Lycopersicon esculentum* (Narita and Gruissem, 1989), *Capsicum annuum* (Ha *et al.*, 2003), *Nicotiana tabacum* (Genschik *et al.*, 1992), *Cotharanthus roseus* (Caelles *et al.*, 1989), *Arabidopsis thaliana* (Bach, 1987) and *Hevea braziliensis* (Chye *et al.*, 1992). *Solanum xanthocarpum* showed maximum identity per cent with tomato (91%) and tobacco (91%) (Table 8). All other sequences shared more identity with potato and tomato. *Snhmgr* showed 93 per cent identity with potato and 92 per cent identity with tomato. The two clones from *S. torvum* differed in the level of other identity with other *hmgr* genes. Eventhough *Sthmgr1* and *Sthmgr2* showed maximum identity with potato (94% and 91% respectively) and tomato (93%and 90% respectively), the level of homology varied between the sequences. Such situation had been reported earlier also. In *Arabidopsis thaliana* there are two differentially expressed *hmgr* genes with very low homology between them. *Athmg1* accumulates at relatively high levels in all parts of the plant, whereas the *Athmg2* mRNA is restricted to seedlings, root and inflorescence. *Hmg1* encodes a house keeping form of HMGR, while *hmg2* is involed in synthesis of specific isoprenoids required in actively dividing cells (Enjuto *et al.*,

1994). Similarly in *Hevea,* three genes encode HMGR enzyme in which *hmg1* is involved in rubber biosynthesis and expressed in laticifers and *hmg3* is expressed constitutively (Chye *et al.*, 1992). According to their expression profile, and protein properties, the nucleotide sequences also vary.

The high level of identity of the sequences with other plant *hmgr* genes might be due to the conserved catalytic domains in the sequence. All HMGRs show high-level homology at their 'C' terminal, which contains the catalytic site. But at the N terminal, they have sequences with less conserved regions, except the hydrophobic domains (Chye *et al.*, 1991).

The analysis for discovering nitrogen base composition indicated that A+T base pair proportion was more when compared to C+G pairs (Table 6). Maximum AT was found in *Sxhmgr* (55.3 %) while the minimum was in *Sthmgr2* (50.0%). When the ORFs were examined, all *hmgr*s except *Sxhmgr* were found to encode the largest ORF in +1 reading frame. The length of largest ORF was 426 b, 282 b, and 534 b for *Snhmgr, Sthmgr1* and *Sthmgr2* respectively. The largest ORF in *Sxhmgr* was 483 b long and located in +2 reading frame.

The sequence diversity of the gene among various plant species was found out and phylogenetic tree was constructed. Phylogram is a branching diagram assumed to be an estimate of a phylogeny, branch lengths are proportional to the amount of inferred evolutionary change. All hmgr sequences except *Sxhmgr* fall into the same cluster of solanaceous plants. The evolutionary distance of *Sxhmgr* was much lower when compared to other sequences. *Snhmgr* and *Sthmgr1* forms the part of the same cluster consisting of all other solanaceous crops viz., *C. annuum, N. tabacum, S. tuberosum* and *L. esculentum* (Fig. 7). Both of them were closely related with tomato *hmgr*. But the second clone from *S. tovrum, Sthmgr2* showed maximum relatedness with capsicum and occupied another position in the same solanaceous cluster. This factor points towards the sequence divergence of the gene within the same species. *Sthmgr1* and *Sthmgr2* differ in their sequence similarly with other plant *hmgr*s and

probably form part of different *hmgr* genes in the same family as in tomato (Park *et al.*, 1992), potato (Choi *et al.*, 1992), wheat (Aoyagi *et al.*, 1993) and rice (Nelson *et al.*, 1994). They might have different functional performance and expression pattern.

Restriction analysis of the sequence revealed the cleavage sites of different enzymes (Table 9). The uniqueness of nucleotide sequence in each clone is reflected in the restriction analysis also. Recognition sequences of ten restriction enzymes were checked for their presence. In *Sxhmgr*, only two of them were found to have no cleavage site (*Hin*dIII and *Pst*I). *Alu*I had maximum number of cleavage sites (Three sites, four fragments). *Snhmgr* recorded four enzymes with no recognition sequence. They were *Bam* HI, *Dpn*I, *Hpa*I and *Mbo*I. Similar to *Sxhmgr*, *Snhmgr* also had maximum number of recognition sites for *Alu* I (Five sites). Another feature revealed in restriction analysis of *Snhmgr* was the presence of one cleavage site and one fragment formed by the enzyme *Hae* II. It had the restriction site at the end of the sequence (582nd base). Hence the number of restriction sites and number of fragments formed were found to be the same (Table 9b).

The sequence variation present in the two clones of *Sthmgr* was clearly evident from their restriction analysis. Only three enzymes were found to restrict the *Sthmgr*1 sequence (*Alu* I, *Btg* I *and Hin*fI). On the other hand, five enzymes could cleave *Sthmgr*2 (*Alu* I, *Btg* I, *Dpn* I, *Hin*f I and *Mbo* I). Even for the enzymes, which produced cleavages in both the sequence *Sthmgr1* and *Sthmgr2*, the number of fragments generated varied (*Alu* I, *Btg* I and *Hin*f I). The unique restriction pattern also suggested that they were parts of different *hmgr* genes. Maximum number of fragments was observed in *Sthmgr2* for *Hin*f I (seven cuts and eight fragments). *Pst* I could cleave only *Snhmgr* (1 cut only).

The exons present in the sequence were analysed by 'Genscan' tool. *Sxhmgr* and *Snhmgr* were found to encode an initial exon whereas *Sthmgr 1* and *Sthmgr2* had internal exons. No terminal exon or poly A tail could be detected. The length of exon

varied from sequence to sequence. The longest exon was found in *Snhmgr* (400 bp) and the shortest one in *Sthmgr1* (100 bp) (Fig.9). Amino acid analysis revealed composition of different amino acids in HMGR (Table 10). The molar percent of glycine was found to be high in all the sequences. The conserved glycine residues may be important in the maintenance of the correct structure of β-domains (Caelles *et al.*, 1989). *Sthmgr1* had maximum molar percentage of glycine (11.7%, 11 residues) and minimum number in *Sxhmgr* (6.21%, 10 residues). HMGR requires a high concentration of thiol-reducing agents for its activity (Roitelman and Shechter, 1984) and needs some conserved cysteine residues. *Sthmgr1* had maximum number of cystein residues (residues 5.32%) and *Snhmgr* had a molar percentage of 3.7per cent. The conserved cystein residues reflected their importance, not only for the appropriate conformation of the catalytic site of the enzyme, but also for its active role in the catalytic process. No histidine residues were reported in the sequence. There were only two conserved histidine residues in other plant HMGR proteins, in which the residue present in the b2 domain got protonated during the conversion of HMG CoA to mevalonate (Liscum *et al.*, 1985). Since the cloned *hmgr* sequence was not corresponding to the b2 domain, no histidine residues were detected in the deduced amino acid sequence. Glycosylation sites of the protein are usually associated with asparagine residues (Maldonado-Mendoza *et al.*, 1992). *Sxhmgr* has maximum number of residues for asparagine (10 residues, 6.21%). All other sequences have only one or two residues in their sequences with a low molar percentage.

The 'motifscan' of the deduced amino acid sequence revealed the presence of HMGR family profile in all the sequences. *Sxhmgr* revealed an extra motif encoding HMGR signature 1 (Table 11). The secondary structure prediction of the sequences showed the proportion of different structures viz., alphahelix, betasheet and random coil (Fig. 12). Penetrating through most parts of the *Sxhmgr*, alphahelix and random coils were the most abundant structural elements while beta sheets were intermittently distributed in the protein *Snhmgr* had 59.7 per cent random coils forming the structural backbone and helices and sheets form rest of the structure (20.89% and 19.4%

respectively). In *Sthmgr1* and *Sthmgr2* also, random coils forms the major structure with more or less equal proportion of helices and strands. Conserved domain search of the sequences recorded four domains in *Sxhmgr* and five in all others (Fig. 14). They were structurally similar to conserved regions in HMGR class I and class II enzymes. Class I enzymes, which are found pre-dominantly in eukaryotes and contain N-terminal membrane regions and class II enzymes, which are found primarily in prokaryotes and are soluble as they lack the membrane region (Brown and Goldstein, 1980). Yeast and human HMGR were divergent in their N-terminal region, but were conserved in their active site. In contrast, human and bacterial HMGR differ in their active site architecture, while the prokaryotic enzyme is a homodimer, the eukaryotic enzyme is a homotetramer (Basson *et al.*, 1988). Another domain of HMGR associated with lipid metabolism was also detected in all the sequences. The analysis revealed that all the conserved domains associated with HMGR were conserved in the cloned sequences also.

Functional aspects of conserved domains were discovered through 'Inter Pro Scan'. CoA reductase and NAD binding domains were detected in *Sxhmgr* NAD binding domains were very well established in bacteria such as *Pseudomonas mevalonii*, which could use mevalonate as the sole carbon source. These bacteria use an NAD-dependent HMGR to deacetylate mevalonate into HMG CoA. All other *hmgr* had substrate-binding domain instead of NAD binding domain (Fig. 15). Structural representation of the domains was available from CATH structural database describing the architecture of major domains (Fig.16). The alpha helix, beta sheets and random coils were very well depicted in the structure.

Sequences were analyzed for presence of trans membrane domain using Kyte and Doolittle (1982) hydropathy plot analysis. All the cloned sequences were devoid of transmembrane regions. This was due to the fact that primer combinations used were designed for amplifying a region more near to 'C'-terminal. Since both the transmembranes were associated with N-terminal no putative transmembranes could

be detected. Normally all plant HMGR had two potential transmembranes associated with their N-terminal. They are involved in anchoring the enzyme to endoplasmic reticulum (Chin *et al.*, 1984; Basson *et al.*, 1988). Positive hydropathy scores indicate increased hydrophobicity and negative values show an increase in hydrophilic amino acids (Fig.17).

5.6 ISOLATION OF FULL LENGTH GENE

Rapid amplification of cDNA ends (RACE) was carried out in two separate reactions for amplifying the flanking regions of *Sxhmgr* upto 5' and 3' ends. The 5' RACE could not produce any amplification pattern. But 3'RACE resulted in a band of 540 bp, which was much below the expected size of 1.2 kb.

The homology search of the sequence revealed that the insert corresponded to *hmgr* gene. Multiple sequence alignment of the sequence along with *Sxhmgr* and other plant *hmgr* showed a mere increase of 60 bases in the 3'RACE sequence; compared to *Sxhmgr*. All other analyses also showed that changes made due to the extra 60 bases are negligible and the results were almost consistent with the *Sxhmgr* results. Hence the 3'RACE sequence analysis data has not been included here.

In conclusion, partial *hmgr* genes have been isolated and characterized from three solanaceous plants viz., *S. xanthocarpum, S. nigrum* and *S. torvum*. Two different genomic clones were obtained from *S. torvum*, which corresponds to two different *hmgr* genes of the same gene family. The cloned and sequenced fragments can be used to design primers for amplifying full-length gene from the plants through RACE. Moreover, these sequences will aid in the construction of probes, and isolation of complete gene from the genomic or cDNA library using those probes. The different patterns of expression of *hmgr* gene can be studied in different tissues under various environmental conditions. The *hmgr* genes are well known for their tissue specific expressions and regulation by environmental factors such as light. Hence mRNA

expression study will be useful in understanding the regulational aspects. Further investigations are required for the future applications of the gene in resistance breeding. This will be useful in pyramiding of different resistant genes in improving the effectiveness of protection and durability of resistance.


Summary

6. SUMMARY

A large number of isoprenoid compounds including monoterpenes, diterpenes, sesquiterpenes and sterols are formed via mevalonate pathway. HMG CoA reductase is the first key enzyme in this pathway. This enzyme is encoded by more than one copy of the gene, which is evidenced by the presence of isoforms. Usually one of the isoforms has a constitutive expression irrespective of tissue specificity and the other forms are related to production of a particular metabolite. The isoforms, which come under the second category, usually have tissue specific expression, which is regulated by external stimuli such as light. In solanaceous plants, end products of isoprenoid pathway are usually involved in different functional aspects of development. As an initial step to understand the molecular basis of isoprenoid pathway, *hmgr* gene fragments were cloned and characterized from *Solanum xanthocarpum, S. nigrum* and *S. torvum.* To date, this is the first report on the isolation and characterization of the genomic sequence of *hmgr* gene from the solanaceous plants selected for the study. The work undertaken and the result obtained in this study are summarized as follows:

1. The genomic DNA isolated from all the plants were of good quality and quantity. The quantity ranged from 233.5 µg/ml to 425.5 µg/ml. The ratio of optical density values of diluted DNA preparations at 260nm and 280nm were in the range of 1.8484 to 1.93, indicating the good quality.

2. Two pairs of gene specific primers were designed based on the homology within the conserved regions of other solanaceous *hmgr* genes. One pair was degenerate in nature due to less conserved regions present within the sequence. All the primers had a melting temperature of more than 56 ^{0} C and their sequence length varied from 17 to 23 bp.

3. The sequences encoding *hmgr* was amplified from the genomic DNA of *Solanum xanthocarpum, S. nigrum,* and *S. torvum. S. xanthocarpum* yielded

three bnads of sizes 882bp, 1188bp and 1325 bp. *S. nigrum* and *S. torvum* produced two bands of sizes 945 bp and 1835 bp. No amplicons were observed for *Physalis minima.*

4. The amplified gene fragments were eluted, cloned into plasmid vector and competent *E. coli* cells were transformed with the ligated product. A combination of blue and white colonies was obtained after overnight incubation confirming successful transformation.

5. Presence of insert was checked by PCR amplification of the cloned insert. Single amplified bands exactly similar to the genomic DNA amplification were obtained in plasmids of white colonies. Plasmid isolated from blue colony could not produce any amplification.

6. Presence of insert was confirmed by restriction analysis of plasmids. The plasmids isolated from white colonies yielded two bands upon restriction with *Eco* RI. One band was of size 3 kb (Size of plasmid) and the other one corresponding to insert size. Plasmid isolated from blue colony yielded only one band corresponding to 3 kb.

7. The cloned inserts of *hmgr* genes were sequenced using T7 universal primer. Theoretical analysis of the sequence showed about 85% identity with *hmgr* sequences from other solanaceous plants like tomato, potato, tobacco and capsicum.

8. Restriction analysis revealed distribution pattern of cleavage sites of different restriction enzymes. The two clones from *S. torvum* varied widely in the presence and distribution of cleavage sites suggesting the basic variation existing at nucleotide level.

9. Phylogenetic tree constructed for the sequences revealed high-level evolutionary relation with other *hmgr* sequences. Plants, which belong to families other than solanaceae, produced different clusters in the phylogram.

10. Gene prediction analysis indicated the presence and location of exons in the sequences. Both *Sxhmgr* and *Snhmgr* had initial exons encoded by the nucleotide sequence. But *Sthmgr1* and *Sthmgr2* showed the presence of internal exons. *Snhmgr* had the longest exon (400bp) and *Sthmgr1*had the shortest one (100 bp).

11. Secondary structure of the deduced amino acid sequence predicted using 'GOR' algorithm showed that random coils are the major structural components. Alpha helices and beta sheets are more or less equal in their proportion.

12. Conserved domain search of the sequences recorded four domains in *sxhmgr* and five in all others. They are structurally similar to conserved regions in HMGR class I and class II enzymes. Functional analysis of conserved domains showed CoA reductase, NAD binding and substrate binding activities.

13. Kyte and Doolittle hydropathy plot analysis revealed the absence of transmembrane regions in the deduced amino acid sequence of all the *hmgr* clones.

14. RNA isolated from *S. xanthocarpum* for cDNA synthesis and full-length gene amplification was good in quality and quantity. 5' RACE could not produce any amplification. But the 3' RACE reaction resulted in an amplicon of size 540 bp. The cloned and sequenced RACE product showed high homology with other *hmgr* genes when analysed using multiple sequence alignment.

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* Originals not seen

The reagents used for DNA isolation

1) Extraction buffer (4X)

Sorbitol - 2.5 g

Tris- HCl - 4.8 g

EDTA - 0.74g

The chemicals were dissolved in 60 ml sterile distilled water. The pH was adjusted to 7.5 and final volume was made up to 100 ml with distilled water and then autoclaved.

2) Lysis buffer

1M Tris-HCl (pH-8.0)	- 20 ml
0.25 M EDTA	- 20 ml
5 M NaCl	- 40 ml
CTAB	- 2 g
Distilled water	- 20 ml

Cetyl Trimethyl Ammonium Bromide (CTAB) was dissolved in 20 ml sterile distilled water. To this solution the required volumes of other stock solutions are added.

3) Tris-HCl 1M (pH-8.0)

Tris-HCl 15.76g was dissolved in 60 ml sterile distilled water. The pH was adjusted to 8.0 and final volume was made up to 100 ml with distilled water and then autoclaved.

4) EDTA 0.25 M

Ethylene Diamine Tetra Acetic acid (EDTA) 9.305 g was dissolved in 100 ml sterile distilled water and autoclaved.

5) NaCl 5M

Sodium chloride 29.22 g was dissolved in 100 ml sterile distilled water and autoclaved.

6) Sarcosine 5 %

Sarcosine 5 g was dissolved in 100 ml sterile distilled water and autoclaved.

7) TE buffer

(Tris HCl -10.0 mM; EDTA -1.0 mM)

Tris-HCl 1.0 M (pH 8.0) - 1.0 ml

EDTA 0.25 M (pH 8.0) - 0.4 ml

Distilled water - 98.6 ml

Autoclaved and stored at room temperature.

8) Ice-cold Isopropanol

9) Chloroform-Isoamyl alcohol (24:1 v/v)

To 24 parts of chloroform, 1 part of isoamyl alcohol was added and mixed properly. The mixture was stored in refrigerator before use.

10) Ethanol 70 per cent.

To 70 parts of absolute ethanol, 30 parts of double distilled water was added.

ANNEXURE II

Buffer and dyes used in gel electrophoresis

1) 6x Loading/Tracking dye

Bromophenol blue	- 0.25 %
Xylene cyanol	- 0.25 %
Glycerol	- 30 %

The dye was prepared and kept in fridge at 4°C

2) Ethidium Bromide (intercalating dye)

The dye was prepared as a stock solution of 10 mg /ml in water and was stored at room temperature in a dark bottle.

3) 50x TAE buffer (pH 8.0)

Tris base	- 242.0 g
Glacial acetic acid	- 57.1 ml
0.5 M EDTA (pH 8.0)	- 100 ml
Distilled water	- 1000 ml

The solution was prepared and stored at room temperature.

ANNEXURE III

Reagents used for plasmid isolation

1) Solution I (Resuspension buffer)

Glucose	- 50 mM
Tris	- 25 mM
EDTA	- 10mM
pH	- 8.0

2) Solution II (Lysis buffer)

NaOH	- 0.2 M
SDS	- 1 %

3) Solution III (Neutralization buffer)

CH_3 COOK	- 5 M
pH	- 5.5

Annexures

MOLECULAR CHARACTERIZATION OF 3-HYDROXY-3-METHYL GLUTARYL COA REDUCTASE *(hmgr)* GENE FROM SOLANACEOUS PLANTS

By

SMITHA JOSE

ABSTRACT OF THE THESIS

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ABSTRACT

3-Hydroxy-3-methyl coenzyme A reductase (HMGR) is a key enzyme in the synthesis of mevalonate, which is the precursor of terpenoid compounds, that are vital in biological processes like respiration, photosynthesis, growth, reproduction and defense mechanisms in plants. It is also the rate-limiting enzyme in tri- and sesqui-terpene biosynthesis.

The gene encoding HMGR protein has been cloned and sequenced in several plants like *Arabidopsis*, rubber, potato, tobacco, tomato, wheat, rice, apple, periwinkle, chilli and black pepper. In the present study, an attempt was made to isolate and characterize *hmgr* gene from four plants belonging to family Solanaceae: *Solanum xanthocarpum*, *S. nigrum*, *S. torvum* and *Physalis minima*, having medicinal/ insecticidal properties. Gene was amplified through polymerase chain reaction with two pairs of gene-specific primers, designed on the basis of conserved boxes in HMGR from other solanaceous plant species. The amplified products were cloned in the plasmid vector pGEMT and sequenced. A total of four sequences were characterized: two from *S. torvum* (945 and 1835bp) and one each from *S. xanthocarpum* (882bp) and *S. nigrum* (945bp).

Theoretical analysis using various tools revealed conserved domains corresponding to HMGR in all the four sequences. The sequences were found to be rich in glycine, which is important in maintaining the correct structure of β-domain. These sequences exhibited a high degree of identity with *hmgr* genes in tomato, tobacco and potato, all belonging to family Solanaceae. Generally, HMGRs exhibit a high level of homology at the catalytic domain, present at the C-terminal region. Two functional domains (CoA reductase and NAD binding) could be located on *Sxhmgr*, the sequence from *S. xanthocarpum*. However, no putative transmembrane region could be located in any of the sequences, probably because the N-terminal region has not been represented in the cloned sequence. Normally, two

transmembrane regions are associated with plant HMGRs, involved in anchoring the enzyme to endoplasmic reticulum. Attempts to obtain 5' and 3' ends of *Sxhmgr* did not prove successful.

Sequence diversity of *hmgr* gene among various plat species was determined. The evolutionary distance of *Sxhmgr* was much lower, compared to other sequences. The two sequences cloned from *S. torvum* showed divergence, indicating that *hmgr* genes could be divergent, within the species.

For further exploitation of the sequences in genetic improvement programmes, the full-length genes will have to be cloned from the mRNA population and expressed in *Escherichia coli.* The expression profile of mRNA in different parts and at different developmental stages of the plant will also throw light on the role of these genes in various metabolic processes taking place.