A DISSERTATION REPORT ON

"Sequence analysis, cloning and expression studies of few root expressed transcripts encoding *Cytochrome P450* and *BAG-domain* proteins from *Withania somnifera*"

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IN

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By

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DECLARATION

I, **SHAGUFA ARIF**, a student of the **"M.Sc. Microbiology" session 2020-2022, Department of Bioscience,** Integral University Lucknow, declare that I am solely responsible for all of the work presented in this thesis entitled **"Sequence analysis, cloning and expression studies of few root expressed transcripts encoding *Cytochrome P450* and *BAG-domain* proteins from *Withania somnifera*"** Which is being submitted to Integral University, Lucknow, Uttar Pradesh, India for partial fulfillment for the award of the degree of Master of Science in Microbiology (2022), has been carried out by me under the supervision of **Dr. Pradipto Mukhopadhyay,** principal Scientist, Biotechnology Division, CSIR-CIMAP, Lucknow, U.P., India. I further declare that I take responsibility for the accuracy of this dissertation report.

Shagufa Arif

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To whom it may concern

This is to certify that **Ms. Shagufa Arif,** a student of **M.Sc. Microbiology** (2nd year/4th semester), Integral University Lucknow, has completed her four-month dissertation work entitled **"**Sequence analysis, cloning and expression studies of few root expressed transcripts encoding Cytochrome P450 and BAG-domain proteins from *Withania somnifera*" successfully She has completed this work at CSIR-CIMAP (Central Institute of Medicinal and Aromatic Plants), Lucknow, under the guidance of **Dr. Pradipto Mukhopadhyay.** The dissertation was a compulsory part of her M.Sc. Microbiology degree.

I wish her good luck and a bright future.

Dr. Snober S. Mir
Head of department of Bioscience

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Shagufa Arif

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Abbreviation	Description
bp	Base Pair
DEPC	Diethylpyrocarbonate
DAS	Day after sowing
EDTA	Ethylenediaminetetraacetic Acid
EtBr	Ethidium Bromide
Kb	Kilobase
LB	Luria-Bertani
MOPs	Morpholino-pyropanesulfonic acid
MCT	Microcentrifuge tube
PCR	Polymerase Chain Reaction
Pol	Polymerase
qRT	Quantitative Real Time
TAE	Tris Acetate EDTA
TRIS	Tris (hydroxymethyl) Aminomethane
TF	Transcription Factor
V/V	Volume/volume
W/V	Weight/Volume
µg	Micro gram
µL	Micro litre
UTR	Untranslated region
ORF	Open reading frame
RPM	Rotation per minute
cDNA	Complementary DNA

1. INTRODUCTION

Since ancient times, people have been looking towards nature to treat their diseases. The usage of medicinal plants began instinctively, as in the case of animals (Stojanoski*et al.*, 1999). Plants are the foundation of sophisticated traditional medicine systems such as Ayurvedic, Unani, and Chinese medicine, to name a few. These medical systems gave birth to several key medications that are still in use today (Gurib *et al*., 2006). The objectives of using plants as sources of therapeutic agents are to isolate bioactive compounds for direct use as drugs, such as digoxin, digitoxin, morphine, reserpine, taxol, vinblastine, and vincristine; and produce bioactive compounds of novel or known structures as lead compounds for semi synthesis to produce patentable entities with higher activity and/or lower toxicity.

Plants are living chemical factories for the manufacture of a wide range of secondary metabolites (SMs), which are the building blocks for many commercial pharmaceutical medications and herbal therapies produced from medicinal plants. The various chemical compounds in medicinal plants have biological activity that can benefit human health through the pharmaceutical and food businesses, but they also have significant value in the perfume, agrochemical, and cosmetic industries (Hassan, 2012). Many SMs are being studied for therapeutic development, including alkaloids, terpenoids, and phenylpropanoids (Sharma, 2018).

One of the medicinal plants is *Withania somnifera*, also known as 'Ashwagandha', it belongs to Solanaceae family. Solanaceae family is comprised of 84 genera that include about 3,000 species, scattered throughout the world. Members of this family are generally annual shrubs. The genera *Withania* play an important role in the indigenous medicine of South East Asia, represented by Unani and Ayurvedic systems. The twenty-three known *Withania* species are widely distributed in the drier parts of tropical and subtropical zones, ranging from the Canary Islands, the Mediterranean region, and northern Africa to Southwest Asia.

Only two species, *W. somnifera* and *W. coagulans* are economically and medicinally important, both being cultivated in various parts of the world. In India, Ashwagandha being cultivated in different state like Utter Pradesh, Bihar, Chhattisgarh, Rajashthan etc. Over 200 formulations in Ayurveda, Siddha, and Unani medicine contain Ashwagandha roots, which are used to treat a variety of physiological ailments (Winters *et al.,* 2006). *W. somnifera* produces different secondary metabolites (withanolids, withaferin, withanone etc) to use as medicine to cure different kinds of disease like anti-arthritic, anti-aging, anti-tumor, neuroprotetive, anti-inflammatory property, immune enhancer, rejuvenating property, stress releasing, reproductive potential enhancer. (Choudhary *et al.*, 2005; Singh *et al*., 2011). There are different morphotypes/ varieties of *W. somnifera* available, and a couple have already been released by CSIR-CIMAP like Poshita, (Nagori) NMITLI-101, NIMTLI-118 and NMITLY-135, etc. Poshita is an extensively cultivated morphotype with a larger root and shoot production than Nagori. This morphotype has the potential to meet industrial market need; however it is limited by its root high fiber texture due to strong secondary xylary fibre development, which is an undesired characteristic in the industrial market. The Nagori morphotype has a finer texture. The root quality of Nagori is considered exceptional, but its root biomass production is likewise low, making it unsuitable for meeting industrial market needs. Although much effort has been put into the breeding sector of *W. somnifera*, the created varieties or mophotypes are still limited by their root yield or fibrous texture to have good quality and high root biomass production to meet industrial market demand. *Withania somnifera* root is source of important alkaloid and lactones which are pharmaceutically important, however, the content of metabolite is low ranging from of dry weight (Singh and Kumar 1998). To meet the demand, there is a need for biotechnological intervention to increase biomass of the pharmaceutically important components.

In the lab, transcriptome data of different growth root growth stages of Nagori and Poshita was generated following a 150 bp pair-end protocol on the

Illumine novaseq 6000 platform. The RNAseq data of five growth stages of Nagori (45 DAS, 9 (days after sowing) pots grown and 45 DAS, 85 DAS, 110 DAS, and 125 DAS field grown) and three growth stages of Poshita (85 DAS, 110 DAS, and 125 DAS field grown)

We have taken a few sets of selected transcripts from DEGs (Differentially expressed genes) of different growth stages of the Nagori morphotype. The following are *CYP734A1,CYP72A15, and BAG-domain*. As per literature, CYP734A1 (previously CYP72B1) is a C-26 hydroxylase that converts brassinolide and castasterone to 26-hydroxybrassinolide and 26-hydroxycastasterone, respectively, to inactivate brassionsteroids(Fujioka et al., 2003). Recently, many members of Cytochrome P450 have been characterised to play a role in Withanolide biosynthesis. Of these, the CYP72A family has been hypothesised to play a role in oxidation of sesquiterpene backbones as well as hydroxylation of bioactive gibberellins (GAs), which directly or indirectly play a role in increasing metabolite content through cortex cell proliferation. BAG (Bcl-2-associated anthogene) proteins are an evolutionarily conserved multifunctional group of chaperone regulators. They regulate apoptosis-like processes like pathogen attack and plant development into abiotic stress. Roots undergo morphological changes and are subjected to various programmed cell death activities to form different cell layers regulating withanolide content. The role of BAG in root anatomical development is not completely understood to date. As a result, molecular or biotechnological intervention is required to solve itThis would necessitate molecular characterization of variables involved in plant growth and development within and between varieties/morphotypes. Keeping this in mind, the following goals were designed.

Major objectives of this project:

1) Analysis of RNA seq data for the association of Bcl-2-associated athanogene(BAG) domain and Cytochrome P450in the tuberous root development of *W.somnifera*

2) Sequence and phylogenic analysis of the BAG-domain and Cytochrome P450 transcripts and the encoded proteins
3) qRT-PCR validation of the BAG-domain and Cytochrome P450and their cloning.

2. Review of Literature

2.1: Withania somnifera

Ashwagandha is also known as "Indian Ginseng" or "Indian Winter Cherry" (*Withania somnifera*, fam. Solanaceae). It is one of the most significant herbs in Ayurveda (India's traditional medical system), and it has been used as a Rasayana for millennia for its wide-ranging health effects. Rasayana is herbal or metallic concoction that promotes a youthful physical and mental state of health as well as happiness. This plant's leaf and root extracts were found to have a variety of secondary metabolites that have been utilised as medicines for a variety of ailments, including cancer, neurological disorders, and immunological disorders, with few side effects. Different types of withanolides are found in different regions of the plant among the secondary metabolites (Lele *et al*., 2010). These medicines are given to young children as tonics, and they are also used by the middle-aged and elderly to extend their lives. Ashwagandha is the most well-known of the Rasayana herbs in Ayurveda. The herb is known as "Sattvic Kapha Rasayana" (Changhadi, 1938).

Ashwagandha is sold as a churna, a finely sieved powder that can be combined with water, ghee (clarified butter), or honey. It boosts memory and improves the function of the brain and neurological system. It promotes a healthy sexual and reproductive balance by improving the function of the reproductive system. It boosts the body's stress resistance because it's a powerful adaptogen. By increasing cell-mediated immunity, Ashwagandha boosts the body's resistance against disease. It also has powerful antioxidant qualities, which help to protect cells from free radical damage (Singh *et al.,* 2010).

2.1.1. Taxonomical classification of *W. somnifera*

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Angiosperma
Class	Dicotyledons
Order	Tubiflorae
Family	Solanaceae
Genus	*Withania*
Species	*Somnifera*

Figure2.1. *Withania somnifera* plant at 110 days after sowing (DAS)

2.1.2. Morphological characteristics

Ashwagandha is a shrub that grows 50-70 cm tall and it can grow twice in a year (winter season and in summer season), it is erect, , and branched, and has a strong foul odor similar to horse urine. *Withania* stems are brownish dark in color and upright, with few or no leaves on the lowest section of the stem. The leaves are alternate (opposite on flowering stems), simple, with slightly wavy margins, and tapered into 5–20 mm long petioles, which are generally broadly ovate or oblong, 29–80 mm long and 21–50 mm broad. It's a stellate tomentose, grey, under shrub that grows 30-40 cm tall and has long woody tuberous root. The 5-lobed, constrictly campanulate corolla measures 5–8 mm in length and is light yellow to yellow-green in colour. The Ashwagandha fruit is a spherical hairless berry that is 5–8 mm in diameter, orange-red to scarlet in ripe state, and is encased by the expanded calyx. The majority of the seeds are pale brown in colour, 2.5 mm in diameter, kidney-shaped, and compressed with a rough and netting surface. The flowering season for *Withania* is from October to June, and the fruiting season is from October to July. The scarlet fruit of *Withania somnifera* is surrounded by a brownish, papery, turgid calyx. Collectors have characterized it as a foul-smelling bush with generally strong-smelling roots, as well as having a strong green tomato odor in the leaves (Gaurav et al., 2015).

2.1.3. Distribution and habitat

Withania somnifera natural distribution stretches from the Mediterranean to the tropical regions of Africa, including South Africa, and also from the Cape Verde Islands and Canary Islands to the Arabian and Middle East regions, including India, southern China, and Sri Lanka. Ashwagandha is a native herb in New South Wales and South Australia, where it is propagated and cultivated in gardens in the warmer and drier regions of Europe. It is commonly grown as a medicinal crop in India and other parts of the world, most likely for its fleshy roots (Govindaraju *et al*., 2003).

Ashwagandha grows in dry areas in India, on the Himalayas under 1600m, Beluchistan, Sri Lanka and in the Mediterranean area: spontaneous in Sicily and Sardinia (Kapoor *et al*., 2018).

2.1.4. Climatic conditions for growth

Withania somnifera is grown in late rainy-season (kharif). It can be grown as a rainfed crop in semitropical locations receiving 500 to 750 mm of rainfall. Root development improves if one or two winter rains are experienced. During the growth season, the crop demands a relatively dry climate. It can withstand temperatures ranging from 20 to 38°C, as well as temperatures as low as 10°C. The plant grows from sea level to 1500 meters above sea level (Umadevi *et al.,* 2012).

2.1.5. Chemical constituents

Several chemical compounds, including steroidal lactones, alkaloids, flavonoids, tannin, and others, have been discovered, extracted, and isolated from *Withania* sps. From aerial parts, roots, and berries of *Withania* Sps., more than 12 alkaloids, 40 withanolides, and many sitoindosides (a withanolide containing a glucose molecule at carbon 27) have been identified and published. Withanolides, the principal chemical elements of these plants, are mostly found in the leaves, where their concentrations typically vary from 0.001

to 0.5 percent by dry weight (DW). The withanolides are a class of naturally occurring C28-steroidal lactones that are formed by oxidising C-22 and C-26 to generate a six-membered lactone ring on an undamaged or rearranged ergostane framework (Mirjalili*et al.*, 2009).

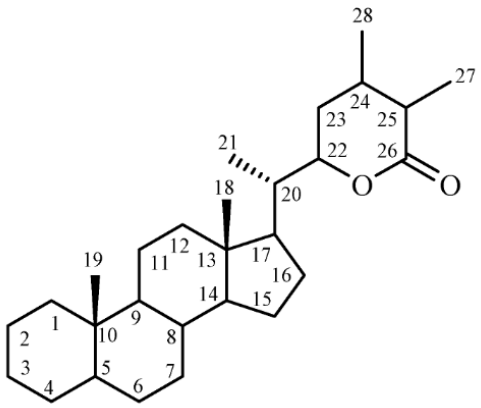

Figure.2.2. Backbone structure of Withanolides

2.1.6. Biochemical-properties

The biochemical composition of *Withania somnifera* has been widely studied and researched. Some 35 compounds have been analysed in the laboratory.

- Alkaloids: About 13 alkaloids are known as Isopelletierine, Anaferine, Cuseohygrine, Anahygrine, Tropine etc.
- Somniferin is a bitter alkaloid with some hypnotic activity.
- Steroidal Lactones: Withanolides, Withanoferins
- Saponins with an additional acyl group (Sitoindoside VII and VIII)
- Withanolides With Carbon At 27th Position: Sitoindoside IX and X
- Iron
- Others: resin, fat, coloring matters, a reducing sugar, phytosterol, Ipuranol, and a mixture of saturated and unsaturated acids.

2.1.7. W. somnifera in Ayurveda*l* traditional medicine

Only *W. somnifera* and *W. coagulans* (Rishyagandha) are regarded to have therapeutic properties among the 23 species of *Withania* (Mirjalili MH.et al 2009;).. Although *W. somnifera*is more often employed in traditional medicine,

W. coagulans is also used in some preparations. *W. coagulans* has been linked to a number of researches that suggest it could be useful as a type 2 diabetes treatment.

In Ayurveda, Siddha, and Unani medicine, *W. somnifera* roots are utilised in approximately 200 compositions. The powdered root of the plant, Ashwagandha churna, is commonly used to cure a number of diseases. Only the root of the plant is used in traditional medicine preparations of Ashwagandha dhilehyam, which is primarily used as a rejuvenation supplement, a therapy for male impotence, and an energy enhancer (Palliyaguru*et al*., 2016).

2.1.8. W.somnifera effects against COVID-19

Multiple pharmacological activity of Ashwagandha have been described, including immunological booster, hepatoprotective, anxiolytic, antidepressant, nootropic, anti-inflammatory, antioxidant, anti-stress, anticonvulsant, anticancer, anti-genotoxic, and immunomodulatory effects. Furthermore, a prior study reveals that *W. somnifera* has antiviral properties. Similarly, one recent study verified *W.somnifera* potential as a SARS-CoV-2 entrance and replication inhibitor in the treatment of COVID-19 (Chikhale andGurav*et al.,* 2020). *W. somnifera* also produces withanolides, which are beneficial to the immune system (Chengappa*et al.,* 2018), antiviral (Varshney *et al.,* 2020), and anti-inflammatory (Varshney *et al*., 2020). (Sun. *et al.,* 2016). As a result, withanolides' triplex effects may aid in the management of COVID-19 infection, as determined by gene ontology (GO) analysis and network pharmacology techniques.

By targeting 3 C-like protease (3CLpro), papain-like protease (PLpro), and spike, the current study studied the network pharmacology of withanolides from *W. somnifera* as an immunity booster and an anti-viral drug against COVID-19 (Khanal*et al.,* 2020).

2.2. Tuberose root

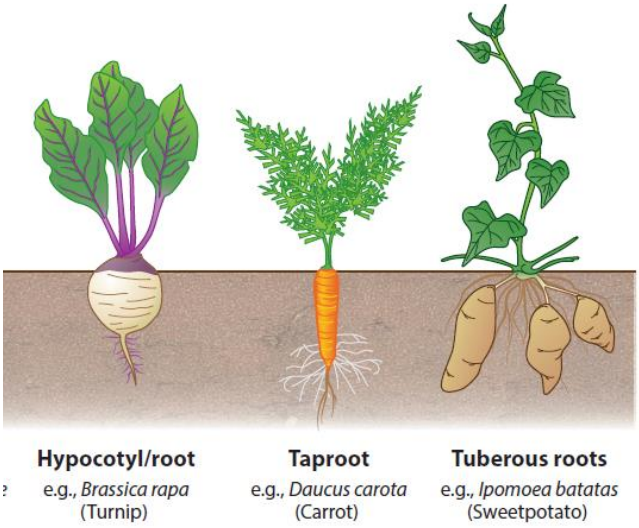

Figure2.3. tuberous root (Ziereret *al., 2021)*

2.3. Root development

Plant organogenesis can result in a wide range of interesting shapes and sizes. Tubers (e.g., potato), taproots (e.g., yam, carrot), tuberous roots (e.g., cassava, sweetpotato), hypocotyl/root transition structures (e.g., turnip), rhizomes (e.g., ginger, topinambur), corms (e.g., taro), and many more subterranean storage structures can be produced by root and tuber crops. Despite their structural differences, root and tuber crops have one thing is common, they contain a lot of water and carbohydrates, as well as varying levels of protein and minerals. Root and tuber crops are therefore crucial for human nutrition. Potato (*Solanum tuberosum*), sweetpotato (*Ipomoea batatas*), and cassava (*Manihot esculenta*) are three of the world's most important food crops. The plant's roots have a simple radial design, with the vascular cylinder or stele surrounded by concentric ground tissue layers (cortex, endodermis) that are separated from the surrounding environment by a root epidermis. Furthermore, the root has three longitudinal zones, meristematic, elongation, and differentiation, each of which is driven by a

different chemical component. Members of the PLETHORA (PLT) transcription factor family, such as the WUSCHEL-LIKE HOMEOBOX (WOX), are involved in controlling the early stages of root organogenesis and root development, which is regulated by specific ROOT GROWTH FACTOR (RGF)/GOLVEN (GLV) proteins and CLE peptides (CLAVATA3/EMBRYO SURROUNDING REGION-RELATED). WOXs are primarily regulated by two hormones one is auxin in the root apical meristem and cambium region and second is cytokinins in the shoot apical meristem region(Wu *et al.,* 2019; Baesso*et al.,* 2020). Medicinal plant roots are the main organs responsible for the accumulation of active components with important medicinal value. In root, the accumulation of active components is mainly affected by growth period, growth season and growth year. Some medicinal plants accumulate abundant of Secondary metabolites mainly during their reproductive growth period. The most essential environmental cues that trigger signals in leaves and transit to stolon for initiation of tuberous root are photoperiod and temperature.

2.4. Cytochrome P450 (CYP)

Cytochrome P450 (P450) is a class of enzymes with a wide range of functions that catalyze a wide range of biological processes. P450 genes regulate growth, development, and the manufacture of secondary metabolites. It has been recognized in all domains of organisms, such as plants, animals, fungi, protists, archaea, bacteria, and even viruses (Lamb et al. 2009). Members of the cytochrome P450 gene family in plants were categorized into ten clans (Nelson *et al.,* 2004). Four clans consisting of multifamily members are named CYP71, CYP72, CYP85, and CYP86, respectively, depending on their lowest-numbered family members. The remaining six are known by their family numbers, such as CYP51, CYP74, CYP97, CYP710, CYP711, and CYP727. Studies for structural organization and classification of cytochrome P450 genes has been done in plants, such as tobacco (Nebert and Russell

2002), *Arabidopsis*, rice (Nelson *et al*. 2004), soybean, flax, mulberry (Ma et al. 2013), poplar (Babu *et al.* 2013), and so on.

The P450 gene family is the third largest in *Arabidopsis*. The endoplasmic reticul -um, chloroplasts, mitochondria, and other secretory pathways are home to the majority of P450s identified in plants(Morant M *et al*., 2003). P450 monooxygenases are heme-thiolate enzymes that use oxygen and NADPH to catalyse a wide range of chemical processes such as epoxidation, sulfoxidation, dehalogenation, dealkylation, cleavage, ring extension, and reduction. CYP450s play a role in a variety of biological functions, including cell growth and development, secondary metabolite production, and xenobiotic detoxification.

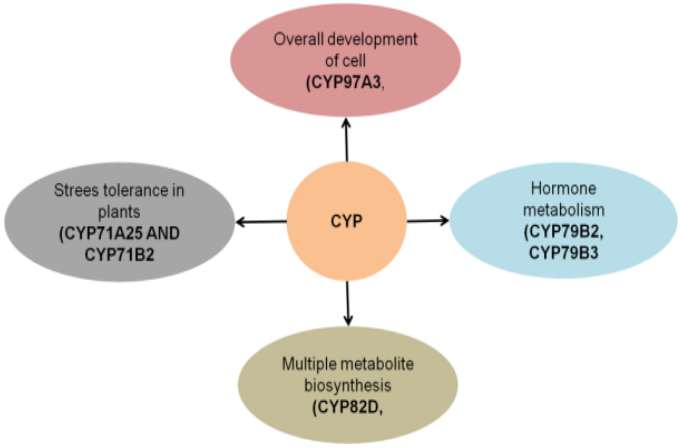

Figure.2.4. Role of different Cytochrome P450 Enzymes in Plant

2.4.1. CYP734A1

CYP734A1 (previously CYP72B1) is a C-26 hydroxylase that converts brassinolide and castasterone to 26-hydroxybrassinolide and 26-hydroxycastasterone, respectively, to inactivate brassionsteroids. CYP72C1 is a homolog of CYP734A1. Brassinolide, castasterone, 6-deoxocastasterone, 3-dehydro-6-deoxoteasterone, 6-deoxotyphasterol, and typhasterol were all identified as potential CYP72C1 substrates, but no corresponding C-26 hydroxylation products were identified. It suggests that CYP72C1, unlike

CYP734A1, uses a different mechanism to inactivate the brassinosteroids. It has been observed thatbrassinosteroids (BRs) production occurs in every plant organ and that its amount is developmentally regulated. Nevertheless, unlike other plant hormones, BRs do not undergo long-distance transport (Symons et al., 2004; Symons et al., 2006). BRs have been detected in very high concentrations in pollen grains and immature seeds but relatively low concentrations in mature organs. Defects in BR metabolism frequently result in varying degrees of plant height reduction. BRs regulate a wide range of physiological processes, including seed development and germination; cell division and elongation, which have a highly stimulating effect on plant growth; differentiation of tracheary elements; polarisation of the cell membrane; proton pumping to the apoplast and into a vacuole via transmembrane ATPases; and increasing photosynthesis efficiency by increasing CO_2 assimilation and Rubisco activity. BRs regulate cortical microtubule reorientation, which affects the organisation of cellulose microfibrills and increases the expression of alfa and beta-tubulin genes. This set of hormones was also found to stimulate leaf senescence. Exogenously given at high doses, BRs decrease root extension while promoting the development of lateral roots. Furthermore, BRs regulate photo-and skotomorphogenesis (etiolation) processes and are known to have a positive influence on reproductive development and flowering time regulation. BRs have gained popularity in recent years because of several reports indicating that BRs modulate plant metabolic responses to environmental biotic and abiotic stresses, including salt and drought stress tolerance, thermotolerance, oxidative stress tolerance, pathogen resistance, and herbicide and pesticide tolerance(Vriet *et al*., 2012). An earlier study found that CYP72B1 plays a role in the brassinosteroid signalling pathway. It converts the active brassinosteroids that have a role in plant development to an inactive form (Figure 2.5).

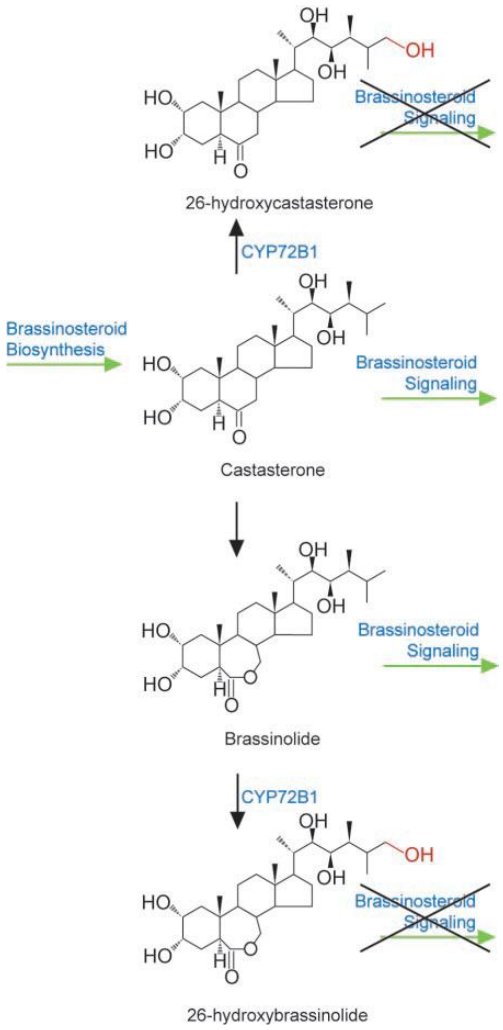

Figure.2.5. Active brassinosteroids have a catabolic route. The hydroxylation of carbon 26 by CYP72B1 is thought to inactivate growth-promoting brassinosteroids like BL and CS. (Neff et al., 1999).

2.4.2. CYP72A

The CYP72A gene subfamily, which is part of the CYP multiple-family clan, catalyses a number of key events in the biosynthesis route of a variety of specialised metabolites. Gibberellins (GAs), particularly low-bioactivity GAs, have a favourable effect on seed dormancy. In Arabidopsis thaliana, AtCYP72A9 has been proven to encode bioactive GA13-hydroxylase, which leads to the buildup of such low-activity GAs (Hou *et al*., 2021).

Furthermore, a large number of CYP72A genes are required for the manufacture of therapeutic metabolites. MtCYP72A67 is a crucial enzyme in the *Medicago* genus that catalyses hydroxylation at the C-2 site in the hemolytic sapogenin biosynthesis (Biazzi, E. et al. 2015). In *Gleditsia sinensis*, GsCYP72As have been found to be involved in the production of triterpenoid saponins (Kuwahara, *et al*. 2018). Furthermore, biotic and abiotic stressors can have a significant impact on the expression of several CYP72A genes. In ryegrass, the methylation level of LpCYP72A161 is tightly linked to the response to temperature stress (Dai, *et al.* 2017). It was postulated in fenugreek that the CYP72A gene family played a key role in diosgenin production and might also be involved in biosynthesis (Pral,*et al*., 2016).

2.5.BAG-domain

Programmed cell death (PCD) is a genetically controlled process that allows damaged and undesirable cells to be removed selectively. PCD is critical in plant development and response to both abiotic and biotic stress. Despite the fact that plants and animals share many of the same molecular and biochemical hallmarks of PCD, plants lack some crucial stages (phagocytosis) and important regulators (Caspases). In mammals, the B cell lymphoma 2 (Bcl-2) family of proteins plays a critical role in determining cell fate by modulating proapoptotic and antiapoptotic signals (Tanthrige, N. *et al*.2020). Plants, on the other hand, lack Bcl-2 proteins. ProteinslikeBcl-2-associated athanogene (BAG1) was discovered to initiate the antiapoptotic action of Bcl-2 proteins in mice in 1995 (Das *et al.*,2022).

The BAG family proteins are highly conserved throughout the kingdoms of yeast, animals, and plants. A protein from a mouse embryo complementary DNA (cDNA) library that interacted with human Bcl-2 was the first member of the BAG family to be identified.

BAG proteins have a conserved BAG-domain (BD) that interacts with the ATPase domain of heat-shock protein 70 (Hsc70/Hsp70) in the C-terminal area

but differs in the N-terminal region, giving selectivity to certain proteins and pathways(Jiang *et al*.,2022)

Plant BAG proteins are involved in biological processes such as plant programmed cell death (PCD) and autophagy, as well as co-chaperones that act as molecular switches, regulating the function of molecular chaperones, primarily HSP70/HSC70 (Brive *et al*., 2001). They also play a role in plant response to abiotic stresses such as salt, drought, temperature, and pathogen infection. BAG family genes have been studied in other plants, including rice, tomato, and wheatetc (Rana *et a*l., 2012; Ge *et al.,* 2016; He *et al.*, 2021; Yan et al., 2003).*Arabidopsis* was the first plant to uncover seven BAG family genes. The plant BAG protein family can be classified into two groups. The first category includes proteins with an N-terminal ubiquitin-like (UBL) domain and a BAG-domain, such as AtBAG1–4. They could be direct homologues of animal BAG1, with great structural and functional similarities. The second kind (BAG5-7 proteins) has a unique to plants isoleucine glutamine (IQ) motif that binds to Ca2+-free calmodulin (CaM) near the BAG-domain (Doukhanina*et al*., 2006; Nawkar*et al*., 2017).

AtBAG5 predominantly have four-helices (H1, H2, Ha and H3). H1, H2, and H3 form an extended three-helix bundle that resembles its human homologs structurally (Sondermann *et al.,* 2001). The IQ motif for CaM binding and the BAG-domain for Hsc70 binding are found in AtBAG5. The IQ motif is a plant-specific CaM-binding motif with the consensus sequence IQXXXRGXXXR that preferentially binds to apo-CaM19. The interaction between the IQ motif and the semi-open C-lobe predominantly involves an intense network of hydrophobic contacts, hydrogen bonds, and salt bridges, and this unique property implicates BAG proteins in the Ca2+/CaMsignalling pathway in plants (Putkey*et al.,* 2003).

3.MATERIALS AND METHODS

3.1. *In silico*sequence analysis:

3.1.1. BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

Using specialised web servers (NCBI, Phytozome v2, etc.) as online tools, BLAST (Basic Local Alignment Search Tool) is run with default parameters for protein (Blastp) or nucleic acid sequences (Blastn). Sequences that have an E value of $e^{-0.5}$or less are not taken into consideration for further analysis. The local BLAST tool uses a locally constructed Contig database and its translated 6frame database to perform a BLAST analysis of transcripts related to Withania somnifera transcripts. Below is a list of the commands used for these analyses.

Table3.1. Command used for BLAST

S. No.	Program file	Command
1.	Blast database preparation	Makeblastdb.exe –in “input file name’ –dbtype “prot (for protein) or nucl (for nucleotide)”
2.	Blastp (protein).	blastp.exe -query ‘input query file name’ -db ‘database name’ -out ‘output file name’.
3.	Blastn (nucleotide).	blastn.exe-query “input query file name” -db “database name” -out “output file name”
4.	Blastn for short sequence (for primer).	blastn.exe- query” input query file name”. –evalue 1000 –word_ size 5 –task blastn-short -db “database name “–out “output file name”

3.1.2. HMMsearch of nucleotide and protein

Hmmsearch was used to identify homologous protein or nucleotide sequence. For this, ClustalW alignment was used to create a multiple alignment of recognised sequences from the TAIR database. The alignment was converted to Stockholm format, and a build profile for a hidden Markov model was created. Using HMMsearch commands, this profile was used to find related proteins in the local Withania protein dataset. We chose the sequences with e-values less than $e^{-0.5}$. The chosen sequences were fetched from the database and Blast searched in the TAIR database; only those showing the best hits for the chosen class of proteins were kept for further analysis.

Table3.2. Command used for Hmm

S.NO.	Program File	Command
1.	Hmmbuild	Hmmbuild.exe --amino "output file name.hmm" "output file name.stockhom"
2.	Hmmsearch	Hmmsearch.exe -o "output file name" "input file name.hmm" db"database"

3.1.3. Multiple sequence alignment

All sequence alignments were done using Clustal W algorithm for Windows OS (ClustalX2) standalone or Clustal-omega algorithm as an online tool (https://www.ebi.ac.uk/Tools/msa/clustalo/) for multiple sequence alignment (protein and nucleic acid). The protein sequences in clustalX2 are aligned using default parameters. For nucleic acid alignment, the pore size and gap range are set to 10 and 0.2, respectively.

3.1.4. Motif analysis

All sequences of CYP and BAG of*Withania somnifera* and *Arabidopsis thaliana* are taken and motif analysis done by using MEME Suit online tool (https://meme-suite.org/meme/).

In BAG-domain, CYP734A1 and CYP72A15 3 conserved motif was selected and analysis was done.

3.1.5. Other in silicoanalysis tools

Table3.3. Various type of in silico analysis tools are as follows:

S.No.	Program/software Name	Online/standalone	Purpose
1.	CAP3 Sequence Assembly Program	Online	Contig Assembly Program is an online software program. This format allows us to assemble a set of contigs. (http://doua.prabi.fr/software/cap3)
2.	mEMBOSS	Standalone	Offline software used for reverse complementary of contig (https://memboss.software.informer.com/6.5/)
3.	MegaX	Standalone	statistical analysis of molecular evolution and for constructing phylogenetic trees (https://www.megasoftware.net/)
4.	GeneRunner.exe	Standalone	DNA sequence analyser program (https://gene-runner.software.informer.com/6.0/)

5.	Oligo Analyzer	Standalone	primer analysis tool for oligonucleotides (https://sg.idtdna.com/pages/tools/oligoanalyzer)
6.	Clustal Omega	Online	multiple sequence alignment program (https://www.ebi.ac.uk/Tools/msa/clustalo/)
7.	Clustal X2	Standalone	multiple sequence alignment (http://www.clustal.org/clustal2/)
8.	MeV4.9.0 Software	Standalone	NGS and Microarray analysis (https://sourceforge.net/projects/mev-tm4/files/mev-tm4/MeV%204.9.0/)
9.	Oligo Explorer	Standalone	Use to search PCR primers and primer pairs, analyses all important primer properties like Tm, GC%, primer loops, primer dimers and etc. (http://www.genelink.com/tools/gl-oe.asp)
10.	MEME Suit	Online	(https://meme-suite.org/meme/).

3.1.6. Primer designing for Real time PCR and cloning

The 3'UTR and 5'UTR regions of the chosen genes' nucleotide sequences were analyzed for primer design using the Gene Runner programme. To

design primers for all three gene families, the present more distinctive region was used to design the 3'UTR region for *CYP72A15* and the ORF region for *CYP734A1* and BAG-domain genes. To create the primers, we employ "PRIMEREXPRESS 2.0" software. I opened the software, then navigated to the file, reopened it, and then selected batch mode. The input file added to design the family of primers selected the type of primer required (e.g. qRT-PCR). Then go to the options to set the parameters (primer length 18 to 24 and qRT-PCR) and click Finish to save the primer list.For amplification of specific ORF or DNA fragment, nucleotide sequences of the selected genes were analyzed with oligoexplorer, we used nested PCR thus we designed two sets of primers. For each gene the first set of primer which was designed from the UTR region was named as F1R1 (forward and reverse) and the second set of primer which is dedicated to ORF region amplification was named as F2R2.

3.2. Isolation of RNA

3.2.1. Preparations of Diethylpyrocarbonate (DEPC) treated water

In order to prepare it, DEPC was diluted with MilliQ/HPLC water to a final concentration of 0.1 percent (v/v), shaken erratically, and agitated with a magnetic stirrer for 6 to 8 hours. Prior to usage, it was boiled for at least 10 minutes and twice autoclaved after being stored at 60 °C overnight.

3.2.2.Preparation of DEPC-treated glassware and plastic ware

DEPC at a concentration of 0.02 percent (v/v) was introduced to a sizable, clean plastic container full of water, and the mixture was agitated for 30 to 60 minutes. Then, plastic and glasses were dipped and kept in an agitated condition overnight. After being taken out, the glass and plastic items were dried in a hot air oven at 60 °C and autoclaved twice before being used.Table 3.4 lists many reagents made with autoclaved DEPC water.

Table3.4. Preparation of stock buffer for RNA isolation

S.No.	Component	Molarity/Normality	pH
1.	Tri-sodium citrate dehydrate	1M	6
2.	Guanidine thiocyanate	4M	-
3.	Sodium acetate	3M	5
4.	Ammonium thiocyanate	4M	-
5.	NaCl	1.2M	-

Table.3.5. Preparations of homemade Trizol reagent in 100 ml.

S.No.	Component	Composition(for 100 ml)
1.	Guanidine isothiocynate(4M)	20 ml
2.	Ammonium thiocynate(4 M)	10 ml
3.	Sodium acetate(3 M)	3.34 ml
4.	Glycerol (100%)	5 ml
5.	Phenol red	0.10g.

All chemicals were placed in a DEPC-treated brown bottle to a volume of 50 ml, then 38 ml of water saturated phenol was added, and then an appropriate amount of DEPC water was added to a final volume of 100 ml.

3.2.3. RNA isolation from *W.somnifera* root

Weighed root samples of W. somnifera were frozen in liquid nitrogen, labelled, and kept at -80°C. The samples were then taken out of the deep freeze, put in liquid nitrogen storage, ground into a fine powder with liquid nitrogen present, and homogenised using homemade Trizol reagent (1 ml / 100 mg fresh tissue). After that, sarcosine (50 µl per ml of trizol used in a % solution) and chloroform (200 µl per ml of trizol used) were applied to the homogenate. The resultant homogenate was centrifuged at 12000 g for 10 minutes at 4°C after being aggressively shook by hand for 15 to 30 seconds. Without disrupting the intermediate phase, the supernatant was collected after centrifugation, and 0.7 times the amount of isopropanol was added and gently mixed.After being incubated at room temperature for 15 to 30 min., samples were centrifuged at 12000 g for 15 min at 4 °C to pellet the RNA. The pellet was washed twice with 75 % ethanol, suspended, and centrifuged at 12000 X g for 10 minutes at 4 °C after the supernatant was removed. RNA samples were kept at 80°C for further usage. When the necessary samples were extracted, centrifuged, and the ethanol was eliminated. Following a 10- to 15-minute period of drying, the pellets were dissolved in DEPC-treated autoclaved HPLC grade water. For later usage, the remaining RNA samples were once again kept below 80°C. Plastic, glass, and ceramic items were treated with DEPC water, which was also used to prepare all of the reagents.

3.2.4.Spectrophotometric quantification and purity analysis of DNA & RNA

DNA and RNA samples were recorded (on a suitable quartz cuvette) at wavelengths of 230 nm, 260 nm, and 280 nm using a UV-Visible dual-beam spectrophotometer (Specord 200 Plus, Analytic Gena). For measurement, aliquot DNA or RNA is appropriately diluted with autoclaved nuclease-free water. Sample purity is estimated by calculating A260 / A230 (expected ratio> 2 for high quality RNA / DNA) and A260 / A280 (expected ratio ≥1.8 for DNA, expected ratio ≥2).The following formula was used to measure the

concentration of DNA or RNA. DNA concentration (ng/µL) = (50 x OD260 x dilution factor) / 1000

RNA concentration (ng /µL) = (40 x OD260 x dilution factor) / 1000.

3.2.5.Denaturing agarose gel electrophoresis for RNA analysis

For RNA gel electrophoresis, the 10X MOP buffer (200 mM MOP, 50 mM NaOAC, 10 mM EDTA, pH 7.0) was initially made in the dark. Prepare a 1% agarose gel (0.55 g agarose, 43.2 ml DEPC water, 5 ml MOP), microwave the agarose to dissolve it, add 0.9 ml 37 % formaldehyde, 0.9 ml DEPC H 2 O, and 1µl EtBr, and let the mixture cool before adding the RNA and 1X loading buffer (diluted from 10X stock; Table 3.6). The RNA in the loading samples was denaturated in 1X loading buffer at 80°C for 5 minutes and immediately placed it on ice. The samples wereloaded in the gel after a short spin, and electrophoresis on a constant 50 volt power source until the tracking dye covered approximately 70% of the runway. RNA bands in the gel were recorded with the gel documentation system "Gel.Pro CCD516, brand Biozen Zenith".

Table3.6. Loading buffer used in nucleic acid gel Electrophoresis

Name of the dye	**Component**
6X DNA loading buffer	0.05% (w\v) Bromophenol blue and/or xylene cyanol FF 0.05% (w\v), and 30% Glycerol
5X RNA loading dye	400 µl MOPs (10 x stock) ,72 µl formaldehyde (37%),308.4 µl formamide (100%), 8 µl EDTA (0.5 M stock, pH 8.0),200 µl Glycerol volume made up of 1 ml with Autoclaved DEPC treated water

3.2.6. Preparation of cDNA for gene cloning

For cloning, we prepared cDNA from diluted total RNA sample (1µl) in DEPC water upto 10 µl in a DEPC-treated PCR tube. Master Mix was prepared as mentioned Table 3.7.

Table3.7. Preparation of master mix for cDNA preparation

Components	Volume (in µl)
10X RT buffer	2.0
25X dNTPs (100mM)	0.8
10XRandom primer	2.0
Reverse transcriptase	1.0
DEPC water	4.2
Total volume	10

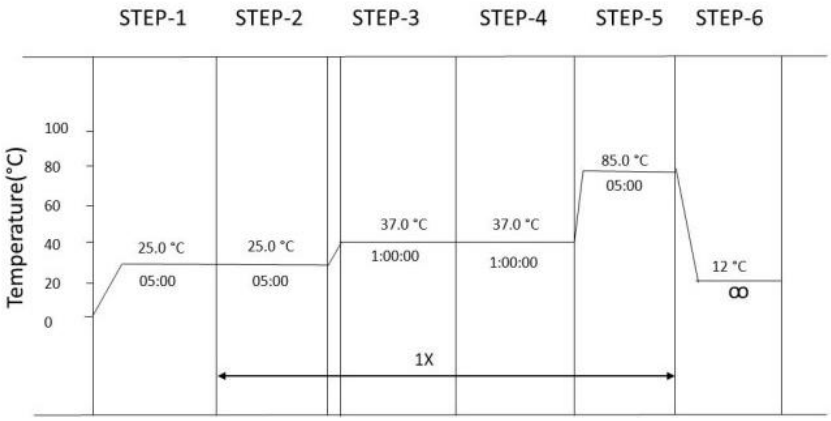

Figure3.1. cDNA amplification cycle

After preparing the master mix, add 10 µl to each tube and centrifuge for a short time, then tap and centrifuge again for a short time. Finally, set up the PCR reaction on the PCR machine. Perform PCR amplification to generate cDNA for cloning. After PCR is complete, the cDNA is stored at 20°C for future use in cloning of various types of genes.

Table.3.8. preparation of master mix for *qrt*-PCR preparation

S.No.	Components	Volume (µl)
1	SYBR green master mix	5
2	10mM gene specific primer (F&R)	1
3	HPLC water	2

cDNA (4x) of different stages was added to aliquots of master mix and total reaction mixture was loaded on real time plate. Real time PCR was performed in central facility of the institute. Ct value of experimental replicates was averaged for each stage and normalized to actin (endogenous control), after that ΔΔct value was calculated.

3.3. Cloning

3.3.1. Cloning of ORFs from *W. somnifera* cDNA

For the PCR reaction of F1R1 amplification, 10 times diluted cDNA of different stages of root was taken and used as a template for PCR amplification. For the second PCR reaction we used 2-3µl product of the first PCR reaction as a template for F2R2 amplification in the 20µl reaction.

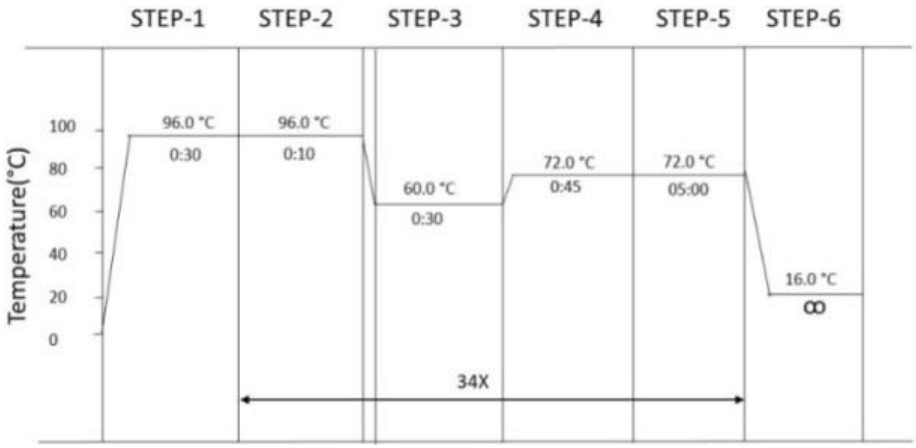

Figure3.2. amplification condition for PCR

Table3.9. Preparation of PCR reaction mix for cloning *WsN_CYP734A1_C18* and *WsN_CYP72A15_C4*_C4

Components	**Volume (µl)**
Water	13.4
10X dNTPs	0.4
5X Phusion Buffer (colourless)	4.0
10mM Primer (F1R1)	1.0
Phusion polymerase	0.2
cDNA	1.0

Preparation of PCR reaction mix for cloning *WsN_BAG5_SS3* genes

Components	**Volume (**µl)
Water	14.4
10X dNTPs	0.4
10X p*fu* Buffer (colourless)	2.0
10mM Primer (F1R1)	1.0
pfu+Taq mix enzyme	0.2
cDNA	2.0

All the chemicals were taken in 1.5 ml of MCT tube and mixed properly.

Gene name	**Annealing temperature**
WsN_CYP72A15_C4	F1R1 58°C
WsN_CYP734A1_C18	F1R1 62°C
WsN_BAG5_SS3	F1R3 56°C

Table3.10. Preparation of Master Mix during Nested PCR for *WsN_CYP734A1_C18* and *WsN_CYP72A15_C4*

Components	**Volume (µl)**
Water (HPLC)	13.4
10X dNTPs (10mM)	0.4

5X Phusion Buffer (colourless)	4.0
Phusion Polymerase	0.2
10mM Primer (F2R2)	1.0
PCR Product	1.0

For *WsN_BAG5_SS3*

Components	**Volume (**µl)
Water	14.4
10X dNTPs	0.4
10X Pfu Buffer (colourless)	2.0
10mM Primer (F2R2)	1.0
Pfu+Taq mix enzyme	0.2
PCR product	2.0

Mixture was prepared in 1.5 ml of MCT tube.

Gene name	**Annealing Temperature**
WsN_CYP734A1_C18	F2R2 64°C
WsN_CYP72A15_C4	F2R2 61.8°C
WsN_BAG5_SS3	F2R3 65°C

After performing nested PCR, the amplified products were ranon agarose gel electrophoresis, and the DNA bands were visualised. After confirming the size of the amplified product of the target sequence, we have proceeded with gel elution and purification.

3.3.2.Agarose gel electrophoresis for DNA analysis

By dissolving 0.4 g of agarose powder in 1X TAE buffer, 0.8 % agarose gel was prepared (diluted from 50X). The mixture was allowed to cool to below 50°C (until no fumes were visible), at this point 2.5 µl of ethidium bromide (10 mg/ml stock solution) was added and poured to a gel casting tray with a comb(for well

formation).After the gel has solidified, transfer the tray containing the gel into the working tank, filled with 1X TAE buffer just enough to submerge the gel. Then remove comb to form well. The DNA sample was mixed with a 6X DNA loading dye (final concentration 1X) (Table 3.5).Electrophoresis was performed at 70 to 80 volts. DNA was visualized by EtBr fluorescence and images were recorded with a gel documentation system.

Table.3.11. Preparationof 50X TAE buffer for DNA gel electrophoresis

S.No.	Component	Molarity	Composition
1.	Tris	-	242g/L
2.	Glacial Acetic Acid	-	57.1ml/L
3.	EDTA	0.5M(pH 8.0)	100ml/L

3.3.3: Elution of DNA bands from agarose gel

After gel electrophoresis, the band of interest was excised with a blade, and then transferred to a pre-weighed micro centrifuge tube under UV light with little exposure. A kit (Nucleospin Cat no. 740609.50) for extracting agarose gel was used for elution and purification.

3.3.4.Ligation of PCR products into plasmid vectors

The PCR-amplified DNA product after purificationwas ligated to a cloning vector, pJET1.2, according to the manufacturer's protocol with a 1: 3 vector: insert molar ratio and HPLC grade autoclave water (10µl) were added to a 2X reaction buffer to a total volume of 17 µl. 1 µl Blunting enzyme mixture was added to the reaction mixture, vortex briefly, centrifuged for 3-5 seconds, incubated at 70° C for 5 minutes, and immediately placed on ice. Next, 1 µl cloning vector pJET 1.2 (50 ng / µl) and 1µl T4 DNA ligase (5U / µl) were added to the final ligation mixture and incubated at 22°C for 5-20 minutes.*E.coli* DH5α competent cells were transformed with 10 µl ligation mix and then selected on LB agar medium containing ampicillin (final concentration 100 µg / ml).

Table3.12. Preparation of media and antibiotics

S.NO.	Component	Volume
1.	LB (Luria Bertani)	25g/L
2.	Agar-Agar	17.5g/L
3.	Ampicillin	10mg/ml
4.	Sodium acetate (3M)	24.612g/100ml

3.3.5. Plasmid DNA transformation of E. coli

For transformation, add 10 µL ligation mix or cells of 5-50 ng of plasmid DNA (up to 20 µL) to 100 µL thawed competent cells, place on ice, and remove from the refrigerator at 80 °C. The cells and DNA were then incubated on ice for 45 minutes, heat shocked at 42 °C (water / dry bath) for 90 seconds, and immediately returned to ice. After incubating on ice for 12 to 20 minutes, 900 µl LB broth was added to the transformation mixture and the cells were allowed to grow at 37°C and 200 rpm for 1 hour. Cells were pelleted by centrifugation at 6000 rpm for 5 minutes. All supernatants were then discarded except 100 µl, cells were resuspended, inoculated into LB agar medium (containing appropriate antibiotics) using a spreader, and incubated overnight at 37°C.

3.3.6. Colony PCR

Single colonies obtained after transformation were streaked and each streak cell was inoculate in 20µl HPLCwater anddenatured for 95 to 98°C for 3-7 minutes.

Use each 3 µl lysate as a DNA template and set up PCR with cDNA amplification primers and conditions. Alternatively, vector-specific primers can be used under appropriate annealing temperature conditions. Then perform

agarose gel electrophoresis to check for the presence of PCR amplification products. All components were kept on ice and the reaction was immediately transferred to the thermal cycler.

Table 3.13: Preparation of reaction mixture for colony PCR

Components	**Volume (in µl)**	**Final Volume (in µl)**
10X PCR buffer	2 X20	40
10 mM dNTPs	0.4 X20	8
F2R2 primer	1 X20	20
*Taq*DNA polymerase	0.2 X20	4
HPLC water	16.4 X20	328
Total volume	20 X20	400

Then gently mix the reaction mixture and collect all the liquid at the bottom of the tube by high speed centrifugation. The PCR tube was transferred from the ice to the PCR machine, the block was preheated to 95°C, and thermal cycling was started.

3.3.7.Plasmid Mini-preparation

A single colony of isolated E. coli cells was inoculated in 5 ml of LB media in culture tubes containing the correct antibiotic. The bacteria were then incubated overnight on a shaker at 37°C and 200 rpm. The plasmid was then isolated the following day using a plasmid miniprep kit (GENETIX BRAND cat no. #NP-37107), in accordance with the manufacturer's instructions.

4. RESULT AND DISCUSSION

4.1. Identification and sequence analysis of *CYP734A1, CYP72A15* and *BAG-domain* from *W. somnifera* transcriptome data

In the present lab large scale RNAseq data have been generated for multiple growth stages of Nagori and Poshita morphotype of *W. somnifera*. In total, five growth stages of Nagori morphotype *viz*. 45 days after sowing (DAS) in the pots inside the greenhouse, 45 DAS in field condition, 85 DAS in field condition, 110 DAS in field condition, and 125 DAS in the field condition were analyzed at RNA level. At 45 days, roots of Nagori plant in greenhouse were highly branched with significantly less tuberous-type initiation in root width and length compared to plant of similar age in field. The tuberous nature of the roots becomes more prominent from 85 days onward in field. From 110 DAS onward, xylem fibre formation initiated and become more apparent at 125 DAS.

Cytochrome P450 (P450) is a family of enzymes that catalyze a variety of biological activities. CYP734A1 is a C-26 hydroxylase that is known to inactivate brassionsteroids by converting brassinolide and castasterone to 26-hydroxybrassinolide and 26-hydroxycastasterone, respectively. CYP72A15 encode active GA 13-hydroxylases in Brassicaceae. Plants over-expressing CYP72A15 exhibited semi-dwarfism, which was caused by significant reduction in GA4 levels. Biochemical assays revealed that recombinant CYP72A15 protein catalyze the conversion of 13-H GAs to the corresponding 13-OH GAs. BAG-domain proteins are known to have apoptotsis-related activities and are involved in regulation of programmed cell death pathways.

To identify the members from *W. somnifera*, HMM (Hidden Markov Model) profiling of BAG-domain (PF02179) were obtainedfrom Pfam database in Stockholm format. The stockholm file was used to prepare the HMM matrix files which was used as a query in HMM search (windows-based HMMER 3.0) algorithm to identify similar sequence *from W. somnifera* Nagori using 6-frame

translated in-house transcript database, the sequences above less than $e^{-0.5}$ were selected for further analysis and BLASTp searched in *Arabidopsis d*atabase (TAIR10) using local Blast program. The final putative BAG-domain sequences were aligned using Clustal-omega algorithm(https://www.ebi.ac.uk/Tools/msa/clustalo/) and guide tree suggested that the nucleotide sequences were grouped into four subclades.

In case of *CYP72A15* and *CYP734A1,Arabidopsis thaliana* peptide sequence was downloaded from the TAIR Database (https://www.arabidopsis.org/). BLASTp was then performed using our in-house transcriptome database and hits above $e^{-0.5}$ were retained. Nucleotide sequences of obtained IDs were fetched using in-house transcriptome database. Orientation of the transcripts were verified by BLASTx analysis. The sequences of 4, 5 and 6 frame were reverse complimented using revseq program of the mEmboss (https://memboss.software.informer.com/6.5/) and were put into CAP3 (Contig Assembly Program-http://doua.prabi.fr/software/cap3) to remove degeneracy and form larger contigs. The contigs and the remaining single sequencesare shown in Table.4.1 and 4.2

Its multiple sequence alignment was done using Clustal-Omega online tool (https://www.ebi.ac.uk/Tools/msa/clustalo/) and converted to msf file (for Genedoc analysis) using ClustalX2 program. Phlogenetic tree (maximum likelihood, 1000 bootstrap) was generated forCYP72A15 and CYP734A1 by taking 10 members of CYP families which are already reported to belong to the same family. These two ORFs were found to group together in the same clade with Arabidopsis CYP734A1 and CYP72A8 (Figure. 4.4)

For phygenetic analysis of BAG-domain proteins of all known 7*Arabidopsis thaliana* BAG proteins sequences were taken from TAIR databaase and by using MegaX software (https://www.megasoftware.net/), with maximum likelihood method and 1000 bootstrap phylogenetic tree was generated (fig. 4.5). Final assembled contigs (C) and unassembled single sequences (SS) of CYP734A1and BAG-domain were taken for further analysis; their details and provided nomenclature are given in Table 4.1, 4.2 and 4.3. The BAG-domain

proteins were found to group with corresponding Arabidopsis proteins in two clades or groups as per earlier report. The position of the BAG-domain protein was determined from multiple alignment with corresponding *Arabidopsis* protein (Fig.4.2 and 4.3). Further, a MEME analysis (https://meme-suite.org/meme/) for conserved motifs in the CYPs and BAG-domain showed three conserved motif in each case as were found in Arabidopsis proteins (Fig. 4.6 and 4.7).

Table.4.1. Schematic representation of Blastx analysis of identified contigs of CYP734A1

S.No.	CYP450 (Blast hits)	*Withania CYP450* (Contigs)	Sequence IDs	*Arabidopsis* IDs	e.value
1	CYP72A9	WsN_CYP734A1_C1	TR_GS1_N_DN25323_c1_g1_i1_len=1029 TR_GS1_N_DN25323_c0_g1_i1_len=714	AT3G14630.1	7.00E-76
2	CYP72A15	WsN_CYP734A1_C2	TR_GS2_N_DN17182_c0_g4_i1_len=865 TR_GS2_N_DN17182_c0_g5_i2_len=932	AT3G14690.1	0
3	CYP72A7	WsN_CYP734A1_C3	TR_GS5_N_DN25840_c0_g1_i1_len=669 TR_GS5_N_DN18917_c0_g3_i1_len=1245 TR_GS4_N_DN6674_c1_g1_i1_len=862 TR_GS3_N_DN8919_c1_g3_i1_len=1100	AT3G14610.1	5.00E-160
4	CYP721A1	WsN_CYP734A1_C4	TR_GS2_N_DN7310_c0_g1_i1_len=1917 TR_GS1_N_DN1325_c0_g1_i1_len=539 TR_GS3_N_DN476_c0_g1_i1_len=765 TR_GS1_N_DN9474_c0_g1_i1_len=1320	AT1G75130.1	2.00E-177
5	CYP72A9	WsN_CYP734A1_C5	TR_GS3_N_DN19879_c0_g1_i1_len=1658 TR_GS5_N_DN25033_c0_g1_i1_len=1053	AT3G14630.1	3.00E-174
6	CYP735A1	WsN_CYP734A1_C6	TR_GS1_N_DN20421_c0_g1_i1_len=1744 TR_GS2_N_DN11787_c1_g1_i2_len=946	AT5G38450.1	0
7	CYP72A15	WsN_CYP734A1_C7	TR_GS2_N_DN16107_c1_g2_i1_len=1906 TR_GS5_N_DN17015_c0_g1_i4_len=1396 TR_GS2_N_DN16026_c0_g1_i2_len=715	AT3G14690.1	0
8	CYP72A15	WsN_CYP734A1_C8	TR_GS2_N_DN16026_c1_g1_i2_len=1759 TR_GS1_N_DN22419_c0_g5_i2_len=971 TR_GS5_N_DN19992_c1_g1_i1_len=1245	AT3G14690.1	0
9	CYP714A1	WsN_CYP734A1_C9	TR_GS2_N_DN18202_c4_g11_i1_len=1785 TR_GS5_N_DN22372_c0_g1_i1_len=1372 TR_GS3_N_DN8513_c0_g1_i1_len=1076	AT5G24910.1	0
6	CYP735A1	WsN_CYP734A1_C6	TR_GS1_N_DN20421_c0_g1_i1_len=1744 TR_GS2_N_DN11787_c1_g1_i2_len=946	AT5G38450.1	0
7	CYP72A15	WsN_CYP734A1_C7	TR_GS2_N_DN16107_c1_g2_i1_len=1906 TR_GS5_N_DN17015_c0_g1_i4_len=1396 TR_GS2_N_DN16026_c0_g1_i2_len=715	AT3G14690.1	0
8	CYP72A15	WsN_CYP734A1_C8	TR_GS2_N_DN16026_c1_g1_i2_len=1759 TR_GS1_N_DN22419_c0_g5_i2_len=971 TR_GS5_N_DN19992_c1_g1_i1_len=1245	AT3G14690.1	0
9	CYP714A1	WsN_CYP734A1_C9	TR_GS2_N_DN18202_c4_g11_i1_len=1785 TR_GS5_N_DN22372_c0_g1_i1_len=1372 TR_GS3_N_DN8513_c0_g1_i1_len=1076	AT5G24910.1	0
10	CYP72A15	WsN_CYP734A1_C10	TR_GS5_N_DN18917_c0_g2_i4_len=973 TR_GS2_N_DN16026_c1_g4_i1_len=794 TR_GS2_N_DN15795_c2_g1_i1_len=741	AT3G14690.1	0
11	CYP72A15	WsN_CYP734A1_C11	TR_GS4_N_DN9813_c1_g1_i1_len=864 TR_GS3_N_DN7994_c1_g1_i1_len=950 TR_GS4_N_DN9813_c0_g1_i1_len=709	AT3G14690.1	6.00E-173
12	CYP72A15	WsN_CYP734A1_C12	TR_GS2_N_DN16026_c1_g7_i1_len=1791 TR_GS4_N_DN1418_c0_g1_i1_len=678 TR_GS2_N_DN16026_c1_g5_i1_len=1093 TR_GS3_N_DN10848_c1_g3_i1_len=640	AT3G14690.1	0
13	BAS1	WsN_CYP734A1_C13	TR_GS5_N_DN18759_c0_g2_i2_len=1875 TR_GS1_N_DN25323_c2_g1_i2_len=1493 TR_GS2_N_DN16026_c1_g1_i2_len=1759 TR_GS1_N_DN22419_c0_g5_i2_len=971	AT2G26710.1	7.00E-110
14	BAS1	WsN_CYP734A1_C14	TR_GS3_N_DN15238_c0_g5_i1_len=1675 TR_GS2_N_DN7310_c0_g1_i1_len=1917	AT2G26710.1	0

Table continues on next page…

15	CYP721A1		TR_GS2_N_DN7310_c0_g1_i1_len=1917 TR_GS1_N_DN1325_c0_g1_i1_len=539	AT1G75130.1	4.00E-178
16	CYP72A15	WsN_CYP734A1_C15 WsN_CYP734A1_C16	TR_GS3_N_DN476_c0_g1_i1_len=765 TR_GS2_N_DN16026_c1_g7_i1_len=1791 TR_GS4_N_DN1418_c0_g1_i1_len=678 TR_GS5_N_DN18917_c0_g1_i3_len=808	AT3G14690.1	0
17	CYP72A15	WsN_CYP734A1_C17	TR_GS2_N_DN17182_c0_g5_i1_len=1827 TR_GS5_N_DN18917_c1_g1_i1_len=879	AT3G14690.1	0
18	BAS1	WsN_CYP734A1_C18	TR_GS5_N_DN18759_c0_g2_i2_len=1875 TR_GS1_N_DN25323_c2_g1_i2_len=1493	AT2G26710.1	2.00E-108
19	CYP72A7	WsN_CYP734A1_C19	TR_GS5_N_DN18917_c0_g3_i1_len=1245 TR_GS4_N_DN6674_c1_g1_i1_len=862	AT3G14610.1	4.00E-160
20	CYP721A1	WsN_CYP734A1_C20	TR_GS4_N_DN19354_c1_g2_i2_len=1271 TR_GS1_N_DN21131_c0_g2_i1_len=834	AT1G75130.1	6.00E-100
21	CYP72A7	WsN_CYP734A1_C21	TR_GS2_N_DN18368_c0_g2_i3_len=1132 TR_GS1_N_DN25323_c2_g4_i1_len=831	AT3G14610.1	5.00E-93
22	CYP72A15	WsN_CYP734A1_C22	TR_GS3_N_DN15700_c1_g1_i1_len=884 TR_GS3_N_DN14881_c2_g1_i3_len=942	AT3G14690.1	0
23	BAS1	WsN_CYP734A1_C23	TR_GS3_N_DN21967_c0_g1_i1_len=1252 TR_GS1_N_DN28390_c0_g1_i1_len=478 TR_GS4_N_DN19857_c1_g4_i1_len=497	AT2G26710.1	0
24	CYP721A1	WsN_CYP734A1_C24	TR_GS1_N_DN21131_c0_g1_i1_len=1806 TR_GS3_N_DN10112_c0_g1_i1_len=1041	AT1G75130.1	2.00E-165
25	CYP72A15	WsN_CYP734A1_C25	TR_GS2_N_DN16107_c1_g2_i1_len=1906 TR_GS5_N_DN17015_c0_g1_i4_len=1396	AT3G14690.1	0
26	CYP72A15	WsN_CYP734A1_C26	TR_GS2_N_DN16026_c1_g1_i2_len=1759 TR_GS1_N_DN22419_c0_g5_i2_len=971	AT3G14690.1	0
27	CYP72A15	WsN_CYP734A1_C27	TR_GS1_N_DN24085_c1_g2_i1_len=1414 TR_GS3_N_DN15700_c1_g2_i1_len=2416	AT3G14690.1	0
28	CYP714A1	WsN_CYP734A1_C28	TR_GS2_N_DN18202_c4_g11_i1_len=1785 TR_GS5_N_DN22372_c0_g1_i1_len=1372	AT5G24910.1	0
29	CYP72A15	WsN_CYP734A1_C29	TR_GS2_N_DN16026_c1_g5_i1_len=1093 TR_GS3_N_DN10848_c1_g3_i1_len=640	AT3G14690.1	1.00E-123
30	CYP72A15	WsN_CYP734A1_C30	TR_GS5_N_DN18917_c0_g2_i4_len=973 TR_GS2_N_DN16026_c1_g4_i1_len=794	AT3G14690.1	3.00E-170
31	CYP72A15	WsN_CYP734A1_C31	TR_GS4_N_DN9813_c1_g1_i1_len=864 TR_GS3_N_DN7994_c1_g1_i1_len=950	AT3G14690.1	6.00E-173

Table4.2. Schematic representation of Blastx analysis of identified single sequence of CYP734A1

S.No.	CYP (Blast hits)	*Withania CYP* (Sinle Sequences)	Sequence IDs	*Arabidopsis* IDs	e.value
1	CYP72A15	WsN_CYP734A1_SS1	TR_GS4_N_DN17645_c0_g2_i1_len=1359	AT3G14690.1	1.00E-175
2	CYP714A1	WsN_CYP734A1_SS2	TR_GS2_N_DN22825_c0_g1_i1_len=1702	AT5G24910.1	1.00E-172
3	CYP72A15	WsN_CYP734A1_SS3	TR_GS3_N_DN14881_c2_g2_i9_len=1272	AT3G14690.1	2.00E-134
4	CYP72A15	WsN_CYP734A1_SS4	TR_GS3_N_DN15700_c1_g3_i2_len=922	AT3G14690.1	2.00E-117
5	BAS1	WsN_CYP734A1_SS5	TR_GS1_N_DN24231_c1_g1_i1_len=570	AT2G26710.1	9.00E-78
6	CYP72A15	WsN_CYP734A1_SS6	TR_GS2_N_DN16107_c1_g2_i3_len=795	AT3G14690.1	3.00E-93
7	BAS1	WsN_CYP734A1_SS7	TR_GS3_N_DN21967_c0_g1_i1_len=1252	AT2G26710.1	3.00E-159
8	CYP714A1	WsN_CYP734A1_SS8	TR_GS1_N_DN28390_c0_g1_i1_len=478	AT2G26710.1	0
9	BAS1	WsN_CYP734A1_SS9	TR_GS4_N_DN19857_c1_g4_i1_len=497	AT2G26710.1	3.00E-58
10	BAS1	WsN_CYP734A1_SS10	TR_GS1_N_DN376_c0_g1_i1_len=667	AT2G26710.1	7.00E-97
11	CYP72A15	WsN_CYP734A1_SS11	TR_GS1_N_DN24085_c1_g2_i1_len=1414	AT3G14690.1	1.00E-170
12	CYP72A15	WsN_CYP734A1_SS12	TR_GS3_N_DN15700_c1_g2_i1_len=2416	AT3G14690.1	0
13	CYP72A15	WsN_CYP734A1_SS13	TR_GS2_N_DN17182_c0_g5_i1_len=1827	AT3G14690.1	0
`14	CYP72A13	WsN_CYP734A1_SS14	TR_GS5_N_DN18917_c1_g1_i1_len=879	AT3G14660.1	1.00E-104
15	CYP721A1	WsN_CYP734A1_SS15	TR_GS4_N_DN19354_c1_g2_i2_len=1271	AT1G75130.1	6.00E-100
16	CYP721A1	WsN_CYP734A1_SS16	TR_GS1_N_DN21131_c0_g2_i1_len=834	AT1G75130.1	9.00E-70
17	CYP72A15	WsN_CYP734A1_SS17	TR_GS3_N_DN15700_c1_g1_i1_len=884	AT3G14690.1	1.00E-90
18	CYP72A11	WsN_CYP734A1_SS18	TR_GS3_N_DN14881_c2_g1_i3_len=942	AT3G14650.1	2.00E-107
19	BAS1	WsN_CYP734A1_SS19	TR_GS2_N_DN11894_c0_g2_i2_len=1014 _rc	AT2G26710.1	2.00E-149
20	CYP72A15	WsN_CYP734A1_SS20	TR_GS1_N_DN23242_c1_g1_i3_len=1607 _rc	AT3G14690.1	1.00E-173
21	BAS1	WsN_CYP734A1_SS21	TR_GS3_N_DN9050_c0_g1_i1_len=684 _rc	AT2G26710.1	9.00E-97
22	CYP72A15	WsN_CYP734A1_SS22	TR_GS2_N_DN16107_c1_g2_i1_len=1906	AT3G14690.1	0
23	CYP72A11	WsN_CYP734A1_SS23	TR_GS5_N_DN17015_c0_g1_i4_len=1396	AT3G14650.1	3.00E-165
24	CYP72A11	WsN_CYP734A1_SS24	TR_GS2_N_DN16026_c0_g1_i2_len=715	AT3G14650.1	4.00E-97
25	CYP72A15	WsN_CYP734A1_SS25	TR_GS2_N_DN16026_c1_g1_i2_len=1759	AT3G14690.1	0

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26	CYP72A15	WsN_CYP734A1_SS26	TR_GS1_N_DN22419_c0_g5_i2_len=971	AT3G14690.1	1.00E-115
27	CYP72A15	WsN_CYP734A1_SS27	TR_GS5_N_DN19992_c1_g1_i1_len=1245	AT3G14690.1	3.00E-159
28	CYP714A1	WsN_CYP734A1_SS28	TR_GS2_N_DN18202_c4_g11_i1_len=1785	AT5G24910.1	0
29	CYP714A1	WsN_CYP734A1_SS29	TR_GS5_N_DN22372_c0_g1_i1_len=1372	AT5G24910.1	0
30	CYP714A1	WsN_CYP734A1_SS30	TR_GS3_N_DN8513_c0_g1_i1_len=1076	AT5G24910.1	4.00E-157
31	CYP72A15	WsN_CYP734A1_SS31	TR_GS5_N_DN18917_c0_g2_i4_len=973	AT3G14690.1	1.00E-111
32	CYP72A15	WsN_CYP734A1_SS32	TR_GS2_N_DN16026_c1_g4_i1_len=794	AT3G14690.1	1.00E-95
33	CYP72A13	WsN_CYP734A1_SS33	TR_GS2_N_DN15795_c2_g1_i1_len=741	AT3G14660.1	1.00E-93
34	CYP72A15	WsN_CYP734A1_SS34	TR_GS4_N_DN9813_c1_g1_i1_len=864	AT3G14690.1	2.00E-76
35	CYP72A14	WsN_CYP734A1_SS35	TR_GS3_N_DN7994_c1_g1_i1_len=950	AT3G14680.1	2.00E-98
36	CYP72A14	WsN_CYP734A1_SS36	TR_GS4_N_DN9813_c0_g1_i1_len=709	AT3G14680.1	9.00E-92
37	CYP72A15	WsN_CYP734A1_SS37	TR_GS2_N_DN16026_c1_g7_i1_len=1791	AT3G14690.1	0
38	CYP72A8	WsN_CYP734A1_SS38	TR_GS4_N_DN1418_c0_g1_i1_len=678	AT3G14620.1	8.00E-82
39	CYP72A15	WsN_CYP734A1_SS39	TR_GS2_N_DN16026_c1_g5_i1_len=1093	AT3G14690.1	1.00E-123
40	CYP72A7	WsN_CYP734A1_SS40	TR_GS3_N_DN10848_c1_g3_i1_len=640	AT3G14610.1	3.00E-101
41	CYP72A15	WsN_CYP734A1_SS41	TR_GS2_N_DN16026_c1_g1_i2_len=1759	AT3G14690.1	0
42	CYP72A15	WsN_CYP734A1_SS42	TR_GS1_N_DN22419_c0_g5_i2_len=971	AT3G14690.1	1.00E-115
43	CYP72A15	WsN_CYP734A1_SS43	TR_GS2_N_DN16026_c1_g5_i1_len=1093	AT3G14690.1	1.00E-123
44	CYP72A7	WsN_CYP734A1_SS44	TR_GS3_N_DN10848_c1_g3_i1_len=640	AT3G14610.1	3.00E-101
45	BAS1	WsN_CYP734A1_SS45	TR_GS5_N_DN18759_c0_g2_i2_len=1875	AT2G26710.1	8.00E-107
46	CYP72A7	WsN_CYP734A1_SS46	TR_GS1_N_DN25323_c2_g1_i2_len=1493	AT3G14610.1	7.00E-102
47	CYP72A7	WsN_CYP734A1_SS47	TR_GS2_N_DN18368_c0_g2_i3_len=1132	AT3G14610.1	5.00E-93
48	CYP72A7	WsN_CYP734A1_SS48	TR_GS1_N_DN25323_c2_g4_i1_len=831	AT3G14610.1	8.00E-78
49	CYP721A1	WsN_CYP734A1_SS49	TR_GS1_N_DN21131_c0_g1_i1_len=1806	AT1G75130.1	2.00E-165
50	CYP721A1	WsN_CYP734A1_SS50	TR_GS3_N_DN10112_c0_g1_i1_len=1041	AT1G75130.1	9.00E-85
51	CYP72A15	WsN_CYP734A1_SS51	TR_GS1_N_DN24085_c1_g2_i1_len=1414	AT3G14690.1	1.00E-170
52	CYP72A15	WsN_CYP734A1_SS52	TR_GS3_N_DN15700_c1_g2_i1_len=2416	AT3G14690.1	0
53	CYP721A1	WsN_CYP734A1_SS53	TR_GS2_N_DN7310_c0_g1_i1_len=1917	AT1G75130.1	4.00E-178
54	CYP721A1	WsN_CYP734A1_SS54	TR_GS1_N_DN1325_c0_g1_i1_len=539	AT1G75130.1	9.00E-63
55	CYP721A1	WsN_CYP734A1_SS55	TR_GS3_N_DN476_c0_g1_i1_len=765	AT1G75130.1	2.00E-62
56	CYP72A15	WsN_CYP734A1_SS56	TR_GS2_N_DN17182_c0_g5_i1_len=1827	AT3G14690.1	0
57	CYP72A13	WsN_CYP734A1_SS57	TR_GS5_N_DN18917_c1_g1_i1_len=879	AT3G14660.1	1.00E-104
58	CYP72A15	WsN_CYP734A1_SS58	TR_GS5_N_DN18917_c0_g3_i1_len=1245	AT3G14690.1	4.00E-120
59	CYP72A9	WsN_CYP734A1_SS59	TR_GS4_N_DN6674_c1_g1_i1_len=862	AT3G14630.1	2.00E-82
60	CYP72A7	WsN_CYP734A1_SS60	TR_GS3_N_DN8919_c1_g3_i1_len=1100	AT3G14610.1	1.00E-107
61	CYP721A1	WsN_CYP734A1_SS61	TR_GS4_N_DN19354_c1_g2_i2_len=1271	AT1G75130.1	6.00E-100
62	CYP721A1	WsN_CYP734A1_SS62	TR_GS1_N_DN21131_c0_g2_i1_len=834	AT1G75130.1	9.00E-70
63	CYP72A15	WsN_CYP734A1_SS63	TR_GS3_N_DN15700_c1_g1_i1_len=884	AT3G14690.1	1.00E-90
64	CYP72A11	WsN_CYP734A1_SS64	TR_GS3_N_DN14881_c2_g1_i3_len=942	AT3G14650.1	2.00E-107
65	BAS1	WsN_CYP734A1_SS65	TR_GS2_N_DN11894_c0_g2_i2_len=1014 _rc	AT2G26710.1	2.00E-149
66	CYP72A9	WsN_CYP734A1_SS66	TR_GS3_N_DN19879_c0_g1_i1_len=1658 _rc	AT3G14630.1	2.00E-174
67	CYP735A1	WsN_CYP734A1_SS67	TR_GS1_N_DN20421_c0_g1_i1_len=1744 _rc	AT5G38450.1	0
68	CYP72A15	WsN_CYP734A1_SS68	TR_GS1_N_DN23242_c1_g1_i3_len=1607 _rc	AT3G14690.1	1.00E-173
69	BAS1	WsN_CYP734A1_SS69	TR_GS3_N_DN9050_c0_g1_i1_len=684 _rc	AT2G26710.1	9.00E-97
70	CYP72A15	WsN_CYP734A1_SS70	TR_GS2_N_DN17182_c0_g5_i2_len=932 _rc	AT3G14690.1	9.00E-126
71	BAS1	WsN_CYP734A1_SS71	TR_GS3_N_DN15238_c0_g5_i1_len=1675 _rc	AT2G26710.1	0
72	BAS1	WsN_CYP734A1_SS72	TR_GS2_N_DN11894_c0_g2_i2_len=1014 _rc	AT2G26710.1	2.00E-149
73	CYP72A9	WsN_CYP734A1_SS73	TR_GS3_N_DN19879_c0_g1_i1_len=1658 _rc	AT3G14630.1	2.00E-174
74	CYP735A1	WsN_CYP734A1_SS74	TR_GS1_N_DN20421_c0_g1_i1_len=1744 _rc	AT5G38450.1	0
75	CYP72A15	WsN_CYP734A1_SS75	TR_GS1_N_DN23242_c1_g1_i3_len=1607 _rc	AT3G14690.1	1.00E-173
76	CYP72A15	WsN_CYP734A1_SS76	TR_GS5_N_DN19992_c1_g1_i1_len=1245 _rc	AT3G14690.1	3.00E-159
77	BAS1	WsN_CYP734A1_SS77	TR_GS3_N_DN9050_c0_g1_i1_len=684 _rc	AT2G26710.1	9.00E-97
78	CYP72A15	WsN_CYP734A1_SS78	TR_GS2_N_DN17182_c0_g5_i2_len=932 _rc	AT3G14690.1	9.00E-126
79	CYP72A14	WsN_CYP734A1_SS79	TR_GS4_N_DN9813_c0_g1_i1_len=709 _rc	AT3G14680.1	9.00E-92
80	CYP72A11	WsN_CYP734A1_SS80	TR_GS2_N_DN16026_c0_g1_i2_len=715 _rc	AT3G14650.1	4.00E-97
81	CYP72A8	WsN_CYP734A1_SS81	TR_GS1_N_DN25323_c0_g1_i1_len=714 _rc	AT3G14620.1	7.00E-72
82	CYP72A13	WsN_CYP734A1_SS82	TR_GS2_N_DN15795_c2_g1_i1_len=741 _rc	AT3G14660.1	1.00E-93
83	CYP714A1	WsN_CYP734A1_SS83	TR_GS3_N_DN8513_c0_g1_i1_len=1076 _rc	AT5G24910.1	4.00E-157
84	CYP72A15	WsN_CYP734A1_SS84	TR_GS4_N_DN17645_c0_g2_i1_len=1359	AT3G14690.1	1.00E-175
85	CYP721A1	WsN_CYP734A1_SS85	TR_GS1_N_DN9474_c0_g1_i1_len=1320	AT1G75130.1	3.00E-140
86	CYP721A1	WsN_CYP734A1_SS86	TR_GS2_N_DN22825_c0_g1_i1_len=1702	AT5G24910.1	1.00E-172
87	CYP72A15	WsN_CYP734A1_SS87	TR_GS5_N_DN25033_c0_g1_i1_len=1053	AT3G14690.1	2.00E-134
88	CYP72A15	WsN_CYP734A1_SS88	TR_GS3_N_DN14881_c2_g2_i9_len=1272	AT3G14690.1	2.00E-117
89	BAS1	WsN_CYP734A1_SS89	TR_GS3_N_DN15700_c1_g3_i2_len=922	AT2G26710.1	9.00E-78
90	CYP72A9	WsN_CYP734A1_SS90	TR_GS1_N_DN24231_c1_g1_i1_len=570	AT3G14630.1	7.00E-76
91	CYP735A1	WsN_CYP734A1_SS91	TR_GS1_N_DN25323_c1_g1_i1_len=1029	AT3G14690.1	3.00E-93
92	CYP735A1	WsN_CYP734A1_SS92	TR_GS2_N_DN16107_c1_g2_i3_len=795	AT5G38450.1	2.00E-142
93	CYP735A1	WsN_CYP734A1_SS93	TR_GS2_N_DN11787_c1_g1_i2_len=946	AT5G38450.1	2.00E-142
94	CYP72A15	WsN_CYP734A1_SS94	TR_GS2_N_DN17182_c0_g4_i1_len=865	AT3G14690.1	5.00E-102
95	CYP72A15	WsN_CYP734A1_SS95	TR_GS5_N_DN25840_c0_g1_i1_len=669	AT3G14690.1	4.00E-57

Table4.3. Schematic representation of BLASTx analysis of identified contigs and single sequence of BAG-domain

S.No.	BAG domain	Sequence IDs	BAG domain (Blast hits)	*Arabidopsis* IDs	e.value
1	WsN_BAG1_C1	TR_GS5_N_DN19529_c0_g2_i2_len=1262_rc TR_GS4_N_DN21001_c0_g3_i4_len=1498_rc	BAG1	AT5G52060.1	2.00E-96
2	WsN_BAG1_C2	TR_GS2_N_DN16825_c3_g2_i1_len=2294 TR_GS5_N_DN19529_c0_g2_i3_len=1658_rc	BAG1	AT5G52060.1	2.00E-57
3	WsN_BAG1_C3	TR_GS2_N_DN16180_c2_g2_i1_len=825_rc TR_GS5_N_DN20337_c3_g1_i3_len=549	BAG1	AT5G52060.1	6.00E-100
4	WsN_BAG1_SS1	TR_GS3_N_DN14605_c1_g1_i3_len=2172_rc	BAG1	AT5G52060.1	2.00E-77
5	WsN_BAG1_SS2	TR_GS3_N_DN14605_c1_g2_i3_len=2223	BAG1	AT5G52060.1	4.00E-103
6	WsN_BAG2_C1	TR_GS4_N_DN14865_c0_g2_i1_len=1014 TR_GS4_N_DN14865_c0_g1_i1_len=713_rc	BAG2	AT5G62100.2	4.00E-66
7	WsN_BAG3_C1	TR_GS5_N_DN17913_c0_g2_i2_len=1510_rc+ TR_GS1_N_DN24842_c0_g2_i1_len=1099	BAG3	AT5G07220.1	2.00E-71
8	WsN_BAG4_C1	TR_GS2_N_DN11645_c0_g2_i3_len=1769 TR_GS5_N_DN15014_c0_g1_i1_len=996 TR_GS1_N_DN19645_c0_g2_i1_len=1316_rc	BAG4	AT3G51780.1	7.00E-48
9	WsN_BAG4_C2	TR_GS5_N_DN15460_c0_g2_i1_len=961_rc TR_GS4_N_DN14855_c0_g2_i2_len=402 TR_GS4_N_DN14855_c0_g2_i1_len=649	BAG4	AT3G51780.1	4.00E-56
10	WsN_BAG4_SS1	TR_GS1_N_DN19645_c0_g1_i1_len=1379	BAG4	AT3G51780.1	1.00E-53
11	WsN_BAG5_C1	TR_GS4_N_DN7782_c0_g1_i1_len=1036_rc TR_GS3_N_DN9099_c0_g2_i1_len=826_rc	BAG5	AT1G12060.1	9.00E-59
12	WsN_BAG5_C2	TR_GS4_N_DN20549_c0_g2_i1_len=646 TR_GS1_N_DN22530_c0_g1_i1_len=880_rc	BAG5	AT1G12060.1	2.00E-18
13	WsN_BAG5_SS1	TR_GS4_N_DN18637_c1_g3_i4_len=830	BAG5	AT1G12060.1	6.00E-14
14	WsN_BAG5_SS2	TR_GS1_N_DN9317_c0_g1_i1_len=379	BAG5	AT1G12060.1	4.00E-23
15	WsN_BAG5_SS3	TR_GS2_N_DN17848_c2_g12_i1_len=1491	BAG5	AT1G12060.1	1.00E-21
16	WsN_BAG5_SS4	TR_GS4_N_DN20549_c0_g5_i1_len=1108_rc	BAG5	AT1G12060.1	2.00E-21
17	WsN_BAG5_SS5	TR_GS5_N_DN19688_c0_g3_i6_len=1532	BAG5	AT1G12060.1	2.00E-19
18	WsN_BAG5_SS6	TR_GS4_N_DN20549_c0_g2_i3_len=1215	BAG5	AT1G12060.1	1.00E-18
19	WsN_BAG6_SS1	TR_GS4_N_DN15557_c0_g2_i2_len=4169	BAG6	AT2G46240.1	2.00E-30
20	WsN_BAG6_SS2	TR_GS4_N_DN15557_c0_g2_i1_len=2206	BAG6	AT2G46240.1	7.00E-21
21	WsN_BAG7_C1	TR_GS1_N_DN25670_c1_g3_i1_len=1243 TR_GS1_N_DN25670_c1_g1_i2_len=1837_rc	BAG7	AT5G62390.1	3.00E-75
22	WsN_BAG7_SS1	TR_GS1_N_DN25670_c1_g1_i4_len=1662_rc	BAG7	AT5G62390.1	5.00E-59

Figure4.1. Alignment of *WsN_CYP734A1* and *WsN_CYP72A15.*

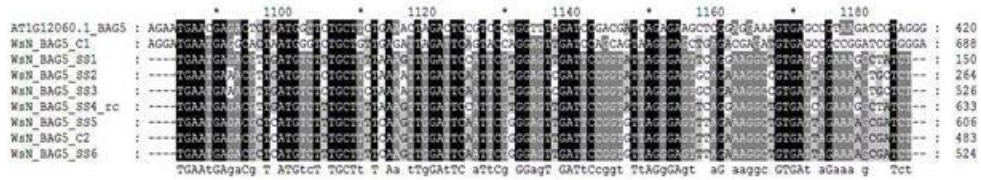

Figure4.2. Alignment showing Conserved region of BAG5

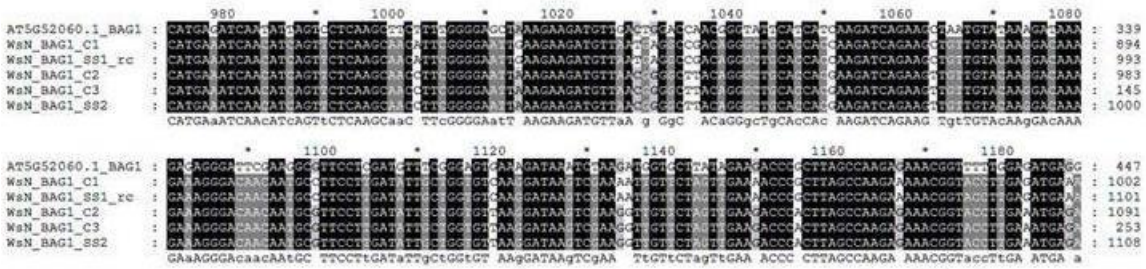

Figure4.3. Alignment showing Conserved region of BAG1.

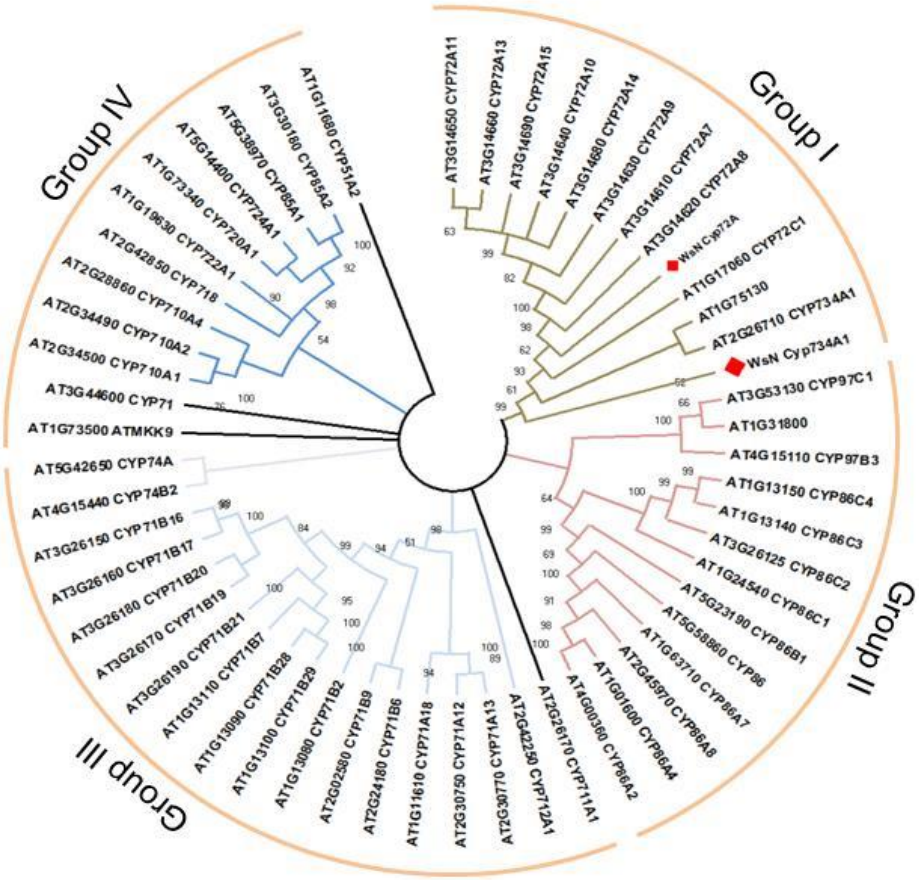

Figure.4.4. Phylogenetic tree analysis of *WsN_CYP734A1_C18* and *WsN_CYP72A15_C4* gene of *Withania somnifera*, (shown by tred dots) with similar sequences from Arabidopsis.

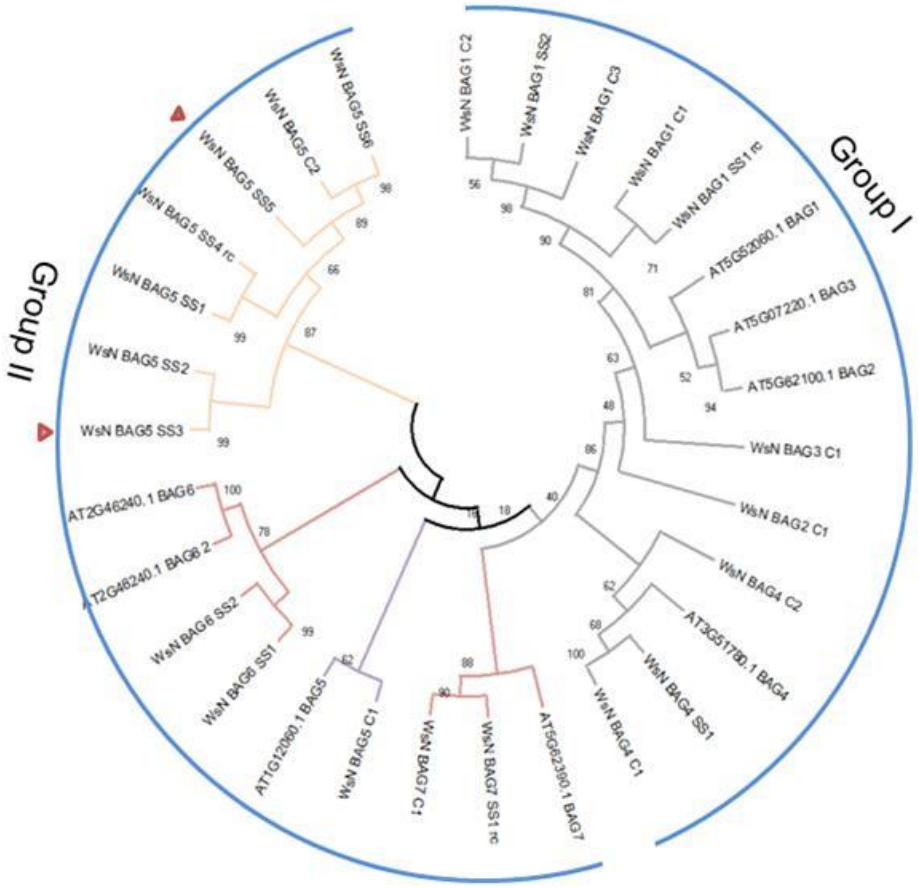

Figure4.5. Phylogenetic tree of *BAG-domain* genes found in *Withaniasomnifera* Nagori morphotype, cloned genes are represented by arrows.

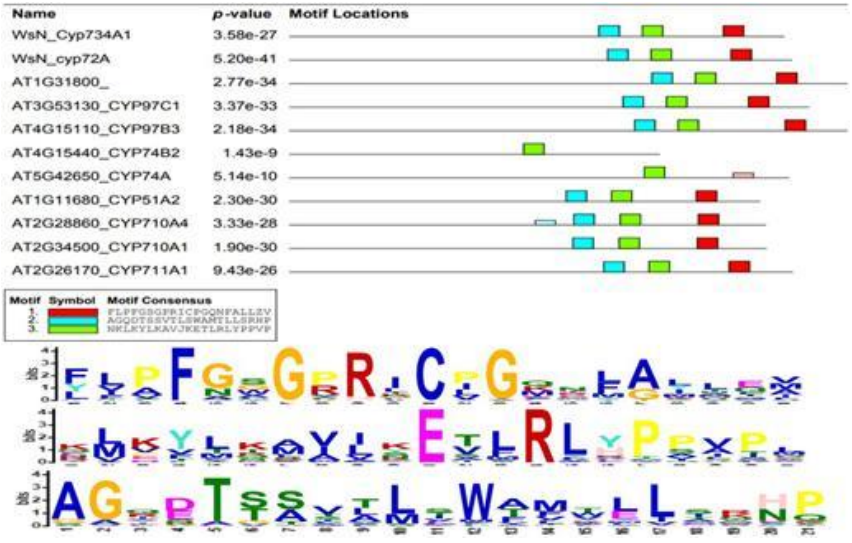

Figure.4.6. Conserved motifs of CYP734A1 and CYP72A15 Identified using MEME suits5.4.1, sequence logos of conserved motifs. The x-axis denotes the width of motifs and Y-axis represents the frequency of each letter.

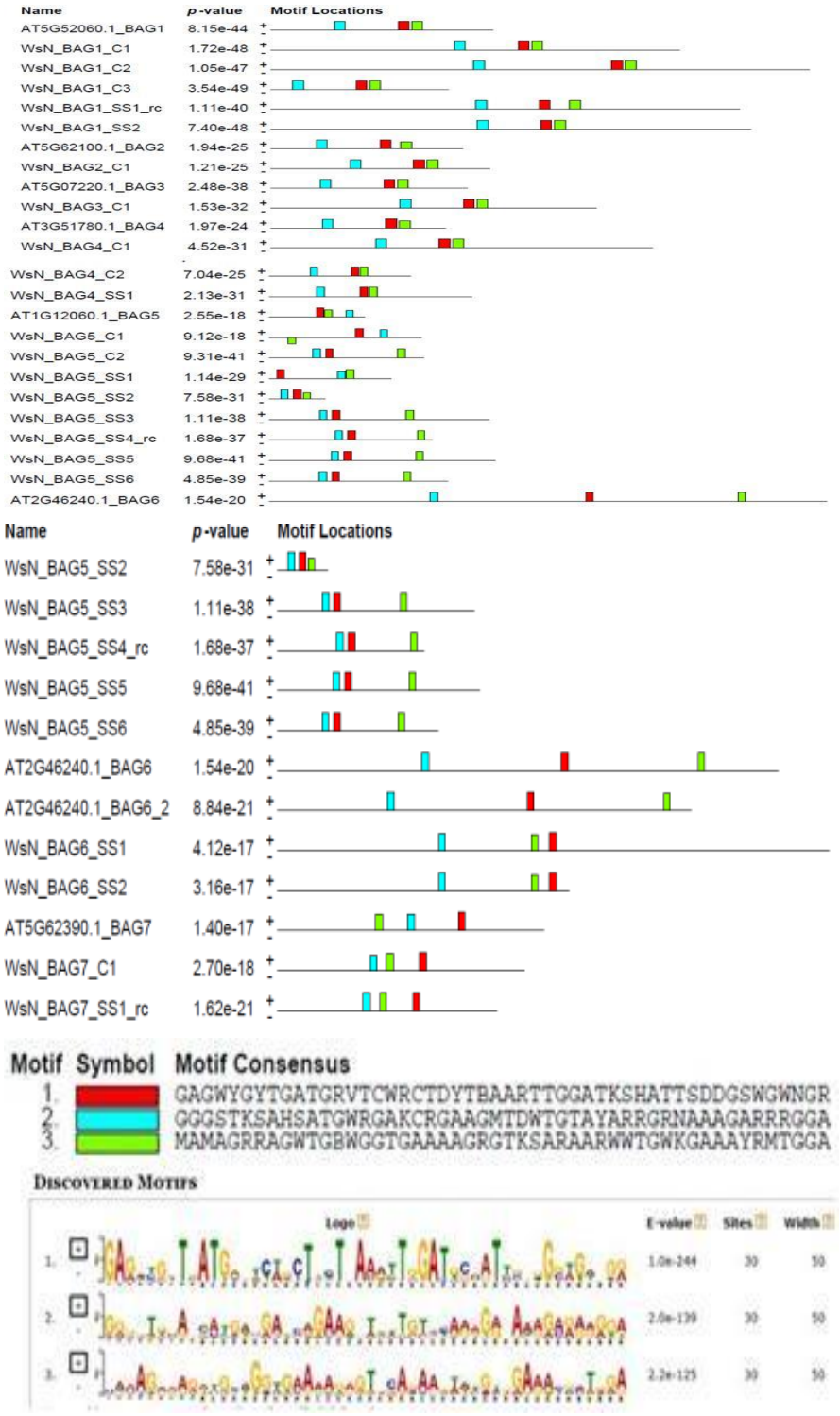

Figure4.7. Conserved motifs of BAG-domain Identified using MEME suits5.4.1, sequence logos of conserved motifs. The x-axis denotes the width of motifs and Y-axis represents the frequency of each letter.

4.2. Expression analysis of CYP and BAG-domain

For expression analysis, heat map of the above obtained assembly was generated by using MeV 4.9.0 Software (fig. 4.8, 4.9, 4.10 and 4.11) (https://sourceforge.net/projects/mev-tm4/files/mev-tm4/MeV%204.9.0/) and using TPM value of RNA-Seq analysis, genes were clustered using hierarchical clustering algorithm. Each TPM value is average of three independent replicates. Analysis was done for 45NP (45 DAS planting pots in greenhouse), 45F (45 DAS plant field grown), 85N (85 DAS field grown), 110N (110 DAS field grown), 125N (125 DAS field grown). In case of CAP3 generated contigs the average TMP values of the individual sequences were added to obtain the final value. Our in-silico analysis suggests that *WsN_CYP72A15_C4*expression is high in 145DAS cortex region than 145DAS pith region and for *WsN_CYP734A1_C18* and *WsN_BAG5_SS3* and *WsN_BAG5_SS5*, relative expression increases with increasing growth stages.

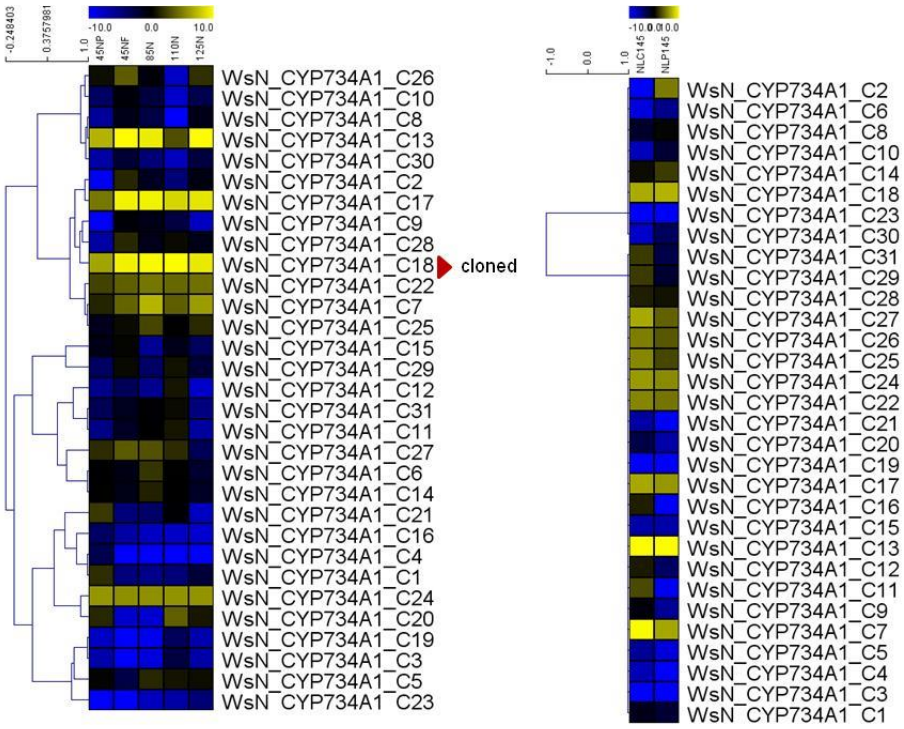

Figure.4.8. *WsN_CYP734A1*gene expression pattern of contigs at different root growth stages and NLC and NLP of 145DAS Nagori morphotype.

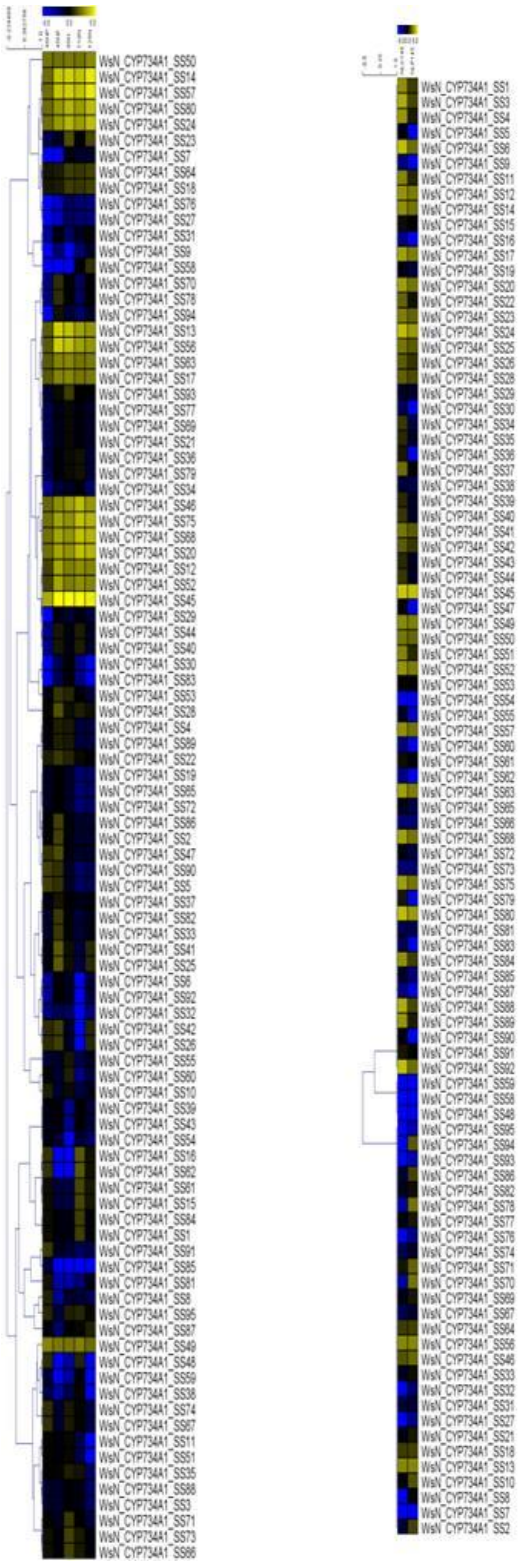

Figure.4.9 .*WsN_CYP734A1*gene expression pattern of single sequences at different root growth stages and NLC and NLP of 145DAS Nagori morphotype.

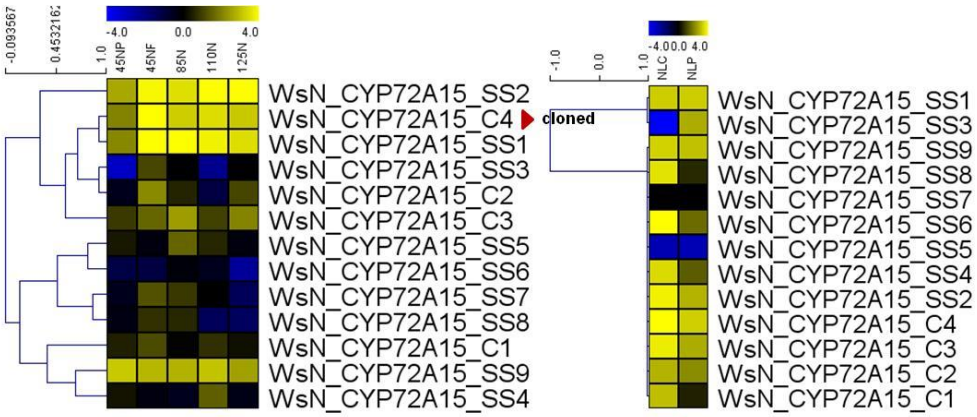

Figure4.10. *WsN_CYP72A15*gene expression pattern of contigs and single sequences at different root growth stages and NLC and NLP of 145DAS Nagori morphotype.

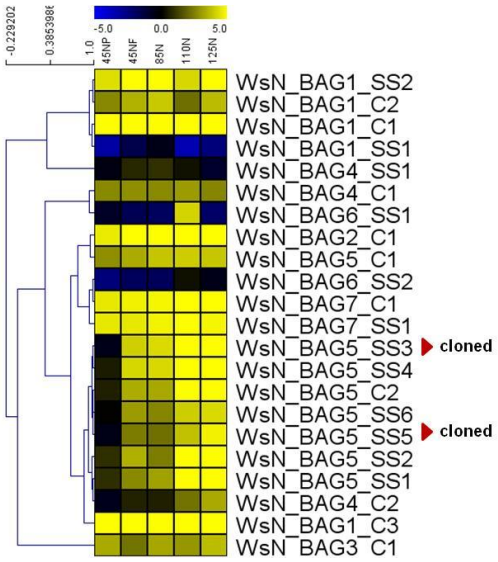

Figure4.11.*WsN_BAG* gene expression pattern of contigs and single sequences at different root growth stages of Nagori morphotype.

Quantitative real time (q-rt) PCR analysis of CYP and BAG-domain

To confirm the expression pattern of above mentioned genes we designed a set of qRTprimersusing primer express 2.0 software (Fig. 4.12) and performed real time PCR using Power SYBR green master mix and above

designed primers (Fig. 4.13, 4.14, and 4.15). The expression was found to be high in cortex region of 145DAS as compare to 145DAS pith based on ΔΔCt value for *WsN_CYP72A15_C4*. For rest of genes expressesion pattern was in accordance with TPM value of different growth stages.

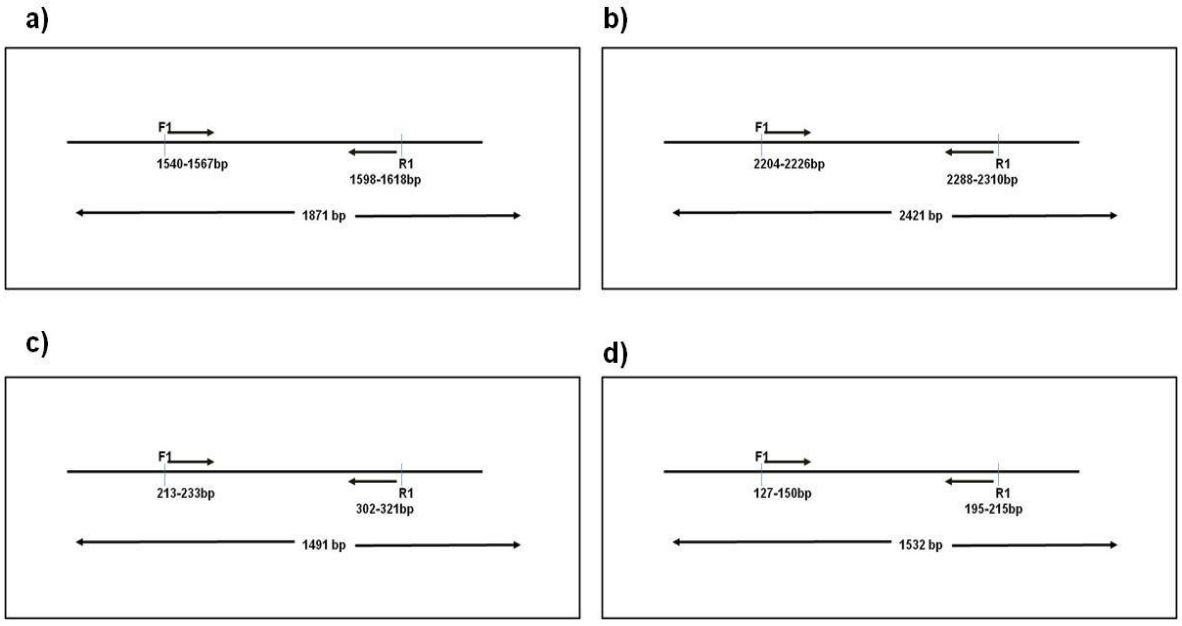

Figure.4.12. Forward and Reverse primer of qRT a) *WsN_CYP734A1_C18*; (b) *WsN_CYP72A15_C4*; (c) *WsN_BAG5_SS3*; (d) *WsN_BAG5_SS5*designed from primer express 2.0 software.

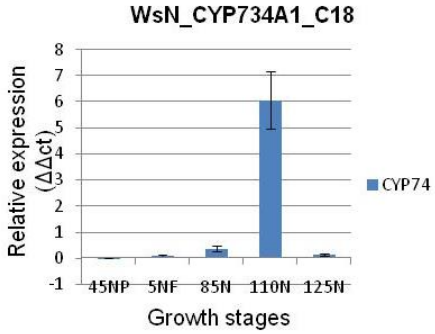

Figure4.13. Real Time expression profile of *WsN_CYP734A1_C18*.

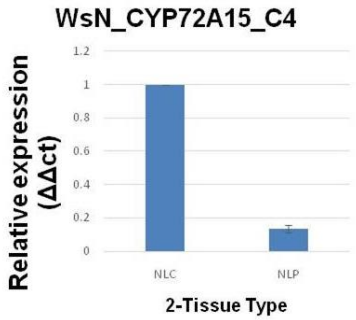

Figure4.14. Real Time expression profile of *WsN_CYP72A15_C4.*

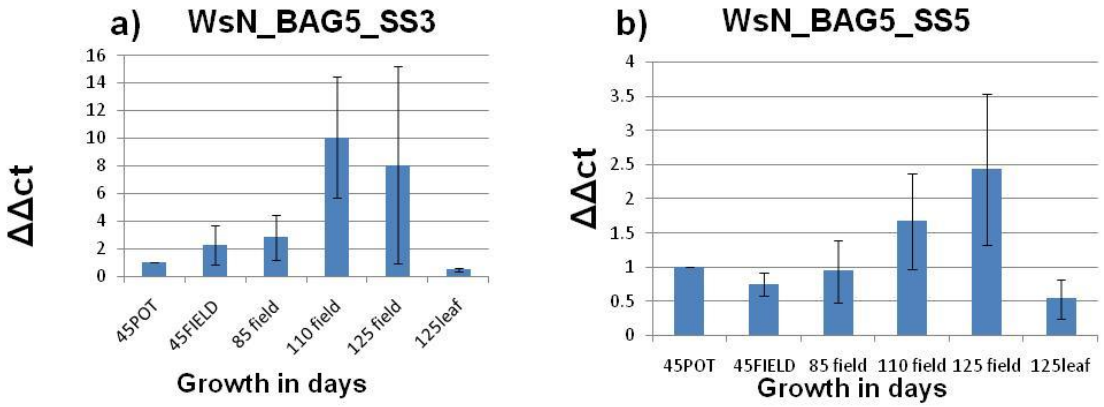

Figure4.15. Real Time expression profile of (a) *WsN_BAG5_SS3* and (b)*WsN_BAG5_SS5*.

4.3. PCR Amplification and Cloning of *CYP734A1, CYP72A15* and *BAG-domain* ORF

In silico Blastx analysis indicated WsN_CYP734A1_C18, *WsN_CYP72A15_C4*, WsN_BAG5_SS3 and WsN_BAG5_SS5 are full-length transcripts. Two set of primers were designed from the 5' and 3' UTR regions, and from start and stop codon of the gene, respectively, to PCR amplify the ORF region (Fig. 4.16). The nested PCR increases the specificity and the amplification level. For amplification of this gene cDNA prepared from RNA isolated from root samples of145 DAS growth stagesof plant was used as the DNA template for the PCR reaction (Fig.4.17). For amplification of WsN_CYP72A15_C4 and WsN_CYP734A1_C18 Phusion polymerase was

used under standard operative conditions because it has high fidelity and proofreading activities (Fig. 4.18). In case of WsN_BAG5_SS3 the amplification was not obtained with Phusion polymerase so PCR amplification was done using a mixture of *Taq: pfu* (4:1) and the desired application was obtained (fig. 4.18).The amplification of fragments by two polymerase and their buffers could be due to differential primer annealing under different buffer condition.

The annealing temperature of primers was calculated from the online NEB Tm calculator. PCR amplified band of expected size of *WsN_CYP72A15_C4* and *WsN_CYP734A1_C18* was obtained by using Phusion enzyme and incase of *WsN_BAG5_SS3 Taq-Pfu* polymerase mix (4:1) was used and expected band size was obtained. For *WsN_BAG5_SS5* we use following conditions as mentioned above but it was not amplified.

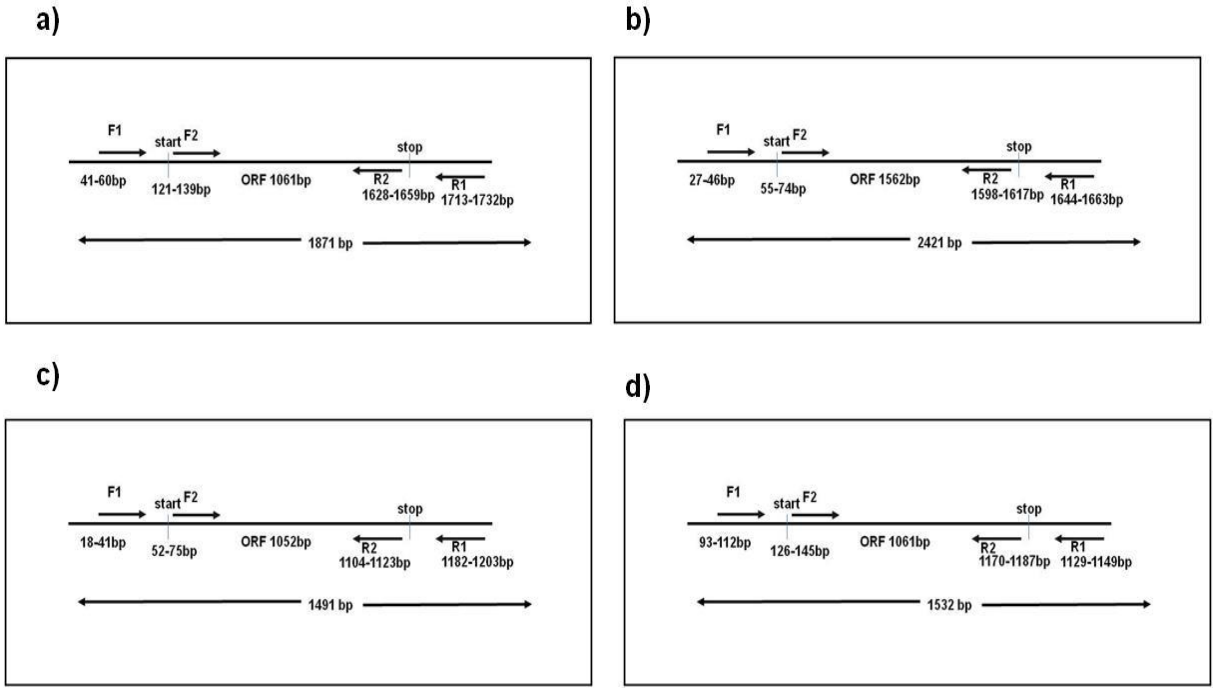

Figure4.16. Primer binding sites designed from UTR and start and stop codon of (a)*WsN_CYP734A1_C18*; (b) *WsN_CYP72A15_C4*; (c) *WsN_BAG5_SS3*; (d) *WsN_BAG5_SS5.*

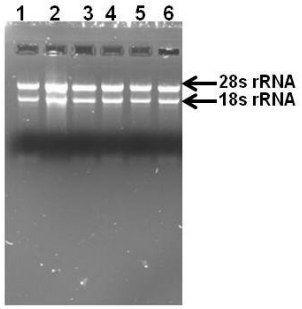

Figure.4.17. RNA of different development stage of root in *Withania somnifera* (Nagori)

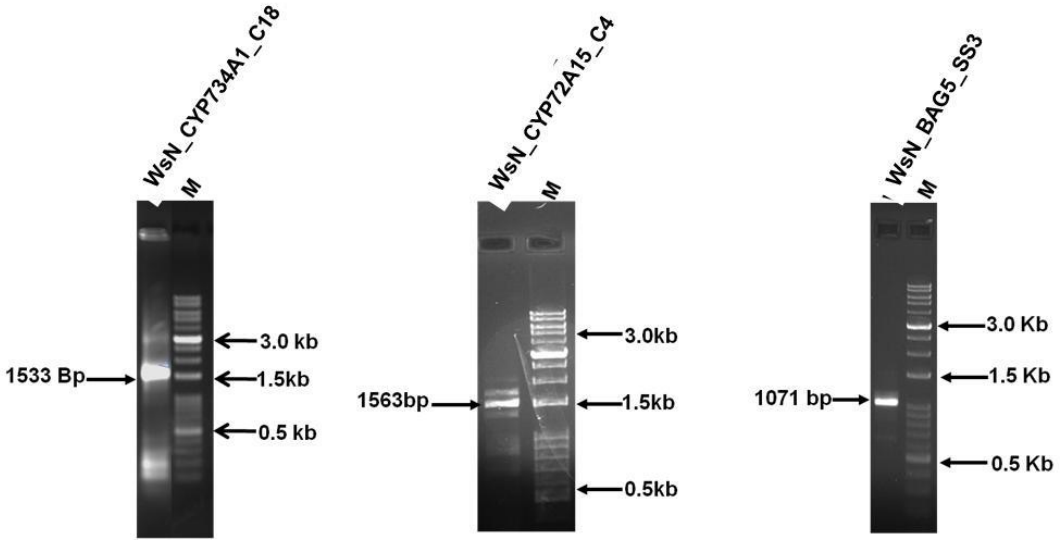

Figure.4.18. Agarose gel image showing *WsN_CYP734A1_C18, WsN_CYP72A15_C4* and *WsN_BAG5_SS3*genes have been amplified using Phusion polymerase (HF) enzyme and 10x diluted cDNA of the root of 145 days grown Nagori morphotype. M denotes Marker.

The eluted and purified PCR products (Fig. 4.19) again subjected to gel electrophoresis and quantified by eye estimation with respect to marker bands and also, by spectrophotometer, ligated into pJET1.2 vector at 1:3 (vector: insert) molar ratio and transformed into E. coli by chemical transformation and heat shock. Putative positive colonies were screened by colony PCR using *Taq* DNA polymerase (Fig. 4.20).

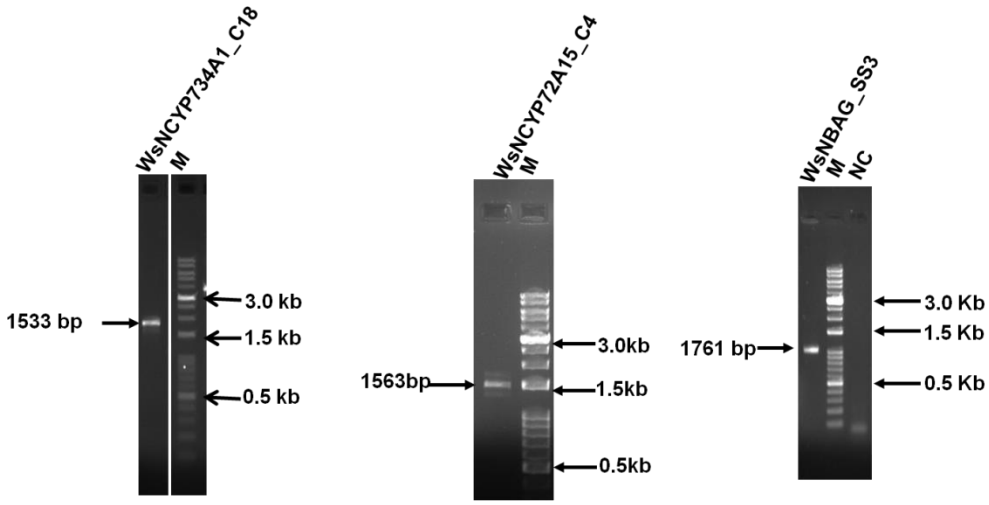

Figure.4.19. Agarose gel image showing; *WsN_CYP734A1_C18, WsN_CYP72A15_C4* and *WsN_BAG5_SS3*eluted PCR product Nucleospin purification kit.

Simultaneously, colonies were also streaked on a fresh plate and PCR positive colonies were grown in liquid medium, plasmid were isolated using alkaline lysis protocol and their quality was checked by Agarose gel electrophoresis (fig. 4.21). Highly dense bands corresponding to supercoiled DNA confirmed their good quality. A PCR was done from these isolated plasmids using insert specific primers further confirmed the presence of inserts.

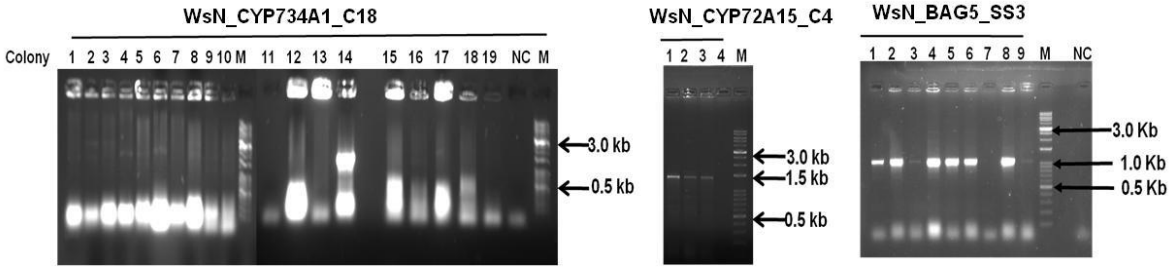

Figure4.20. Agarose gel image showing preliminary confirmation of *WsN_CYP734A1_C18, WsN_CYP72A15_C4* and *WsN_BAG5_SS3*gene by colony PCR using Taq polymerase enzyme.

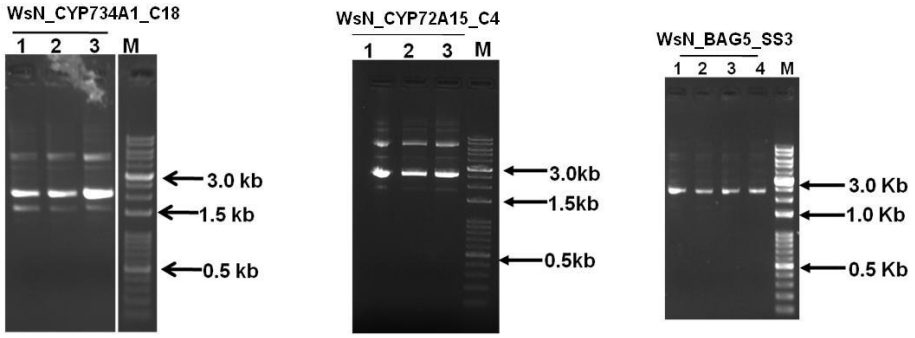

Figure4.21. Agarose gel image showing plasmid minipreparation of clone *WsN_CYP734A1_C18*_pJET1.2, *WsN_CYP72A15_C4*_pJET1.2 and *WsN_BAG5*_SS3_pJET1.2. Each lane represents different colonies for their respective clones.

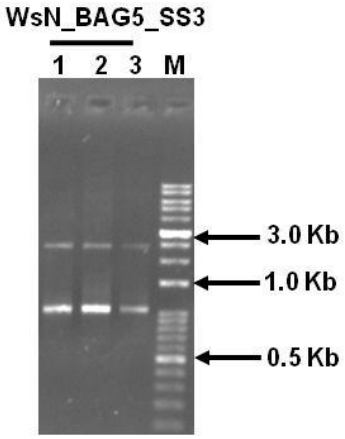

Figure4.22. Agarose gel image showing plasmid PCR of *WsN_BAG5_SS3*

5. CONCLUSION

Previous literature review suggests that various Cytochrome P450 gene are involved in withanolides biosynthesis, root development and phytohormone signaling. In this work, two genes from CYP family were chosen which are supposed to be involved in divergence as well as phytohormone biosythesis and shows expression correlation with non-fibrous tuberous root formation. Analysis was also done to identify different root expressed BAG-domain genes and from RNA-seq data their expression correlation with tuberous root formation was inferred. For all these genes conserved motif analysis was done and the position of the respective domain was established in the sequence and cloned for future characterization. Two Bag-domain proteins were selected for their estimated role in tuberous root formation in *W. somnifera* and their expression was further validated by qRT PCR. As per previous known literature BAG-domain has role in anti-apoptotic activity and growth and development related activity. To further functionally characterize these genes in future, the cloning of these genes were tried. However one of them was cloned.

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