DEVELOPMENT OF WATER USE EFFICIENT TRANSGENIC SUGARCANE THROUGH EXPRESSION OF WHEAT *TaVAP* TRANSGENE

Thesis

Submitted to the Punjab Agricultural University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE
in
BIOTECHNOLOGY
(Minor Subject: Plant Breeding and Genetics)

By

Jashandeep Singh Gill
(L-2018-A-168-M)

School of Agricultural Biotechnology
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CERTIFICATE I

This is to certify that the thesis entitled, **"DEVELOPMENT OF WATER USE EFFICIENT TRANSGENIC SUGARCANE THROUGH EXPRESSION OF WHEAT *TaVAP* TRANSGENE"** submitted to the Punjab Agricultural University, Ludhiana, in partial fulfillment of the requirements for the degree of Master of science in the subject of **Biotechnology** (Minor Subject: **Plant Breeding and Genetics**), is a bonafide research work carried out by **Jashandeep Singh Gill (L-2018-A-168-M)**, under my supervision and that no part of this dissertation has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE II

This is to certify that this thesis entitled, **"DEVELOPMENT OF WATER USE EFFICIENT TRANSGENIC SUGARCANE THROUGH EXPRESSION OF *TaVAP* TRANSGENE"** submitted by **Jashandeep Singh Gill (L-2018-A-168-M)** to the Punjab Agricultural University, Ludhiana, in partial fulfillment of the requirements for the degree of Master of Science in the subject of **Biotechnology** (Minor Subject: **Plant Breeding and Genetics**), has been approved by the student's Advisory committee after on oral examination on the same in collaboration with an External Examiner.

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ABSTRACT

The present study entitled “DEVELOPMENT OF WATER USE EFFICIENT TRANSGENIC SUGARCANE THROUGH EXPRESSION OF WHEAT *TaVAP* TRANSGENE” was carried out on Sugarcane variety Co238. pBI vector carrying *TaVAP* gene was mobilized into *Agrobacterium tumefacians* strain EHA 105. Transformation was carried out using *invitro Agrobacterium* mediated transformation. Sugarcane spindle leaf roll segments were raised on solid MS medium containing NAA (5.0 mg/l) + Kin (0.5 mg/l). The *Agrobacterium* broth at 1.0-1.2 was used for agroinfection of explants in presence of 9.8mg/l acetosyringone. The agroinfected explants were transferred on solid MS + 5 mg/l NAA + 0.5 mg/l Kin + 500 mg/l Cefotaxime for 21 days. Then explants were rooted on solid MS + 5 mg/l NAA + 0.5 mg/l Kin + 500 mg/l Cefotaxime for next 21 days. A total of 38 putative transgenic plants were developed. PCR verification of these 38 putative transgenic plants were analysed using *TAVAP* gene specific primers. The PCR did not detect the presence of *TaVAP* in putative plants.

Keywords: *TavAP*, Transgene, *Agrobacterium tumefacians,* PCR, NAA, Kin, Cefotaxime

Signature of Major Advisor

Signature of the Student

ਖੋਜ ਗ੍ਰੰਥ ਦਾ ਸਿਰਲੇਖ : ਪਾਣੀ ਦੀ ਕੁਸ਼ਲ ਵਰਤੋਂ ਵਾਲੇ ਟਰਾਂਸਜੈਨਿਕ ਗੰਨੇ ਨੂੰ ਕਣਕ ਦੇ ਟੀ ਏ ਵੀ ਏ ਪੀ ਟਰਾਂਸਜੀਨ ਰਾਹੀਂ ਤਿਆਰ ਕਰਨਾ

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ਮੌਜੂਦਾ ਅਧਿਐਨ “ਪਾਣੀ ਦੀ ਕੁਸ਼ਲ ਵਰਤੋਂ ਵਾਲੇ ਟਰਾਂਸਜੈਨਿਕ ਗੰਨੇ ਨੂੰ ਕਣਕ ਦੇ *ਟੀ ਏ ਵੀ ਏ ਪੀ* ਟਰਾਂਸਜੀਨ ਰਾਹੀਂ ਤਿਆਰ ਕਰਨਾ” ਪਾਣੀ ਦੀ ਕੁਸ਼ਲ ਵਰਤੋਂ ਵਾਲੇ ਟਰਾਂਸਜੈਨਿਕ ਗੰਨੇ ਨੂੰ ਕਣ ਕਦੇ *ਟੀ ਏ ਵੀ ਏ ਪੀ* ਟਰਾਂਸਜੀਨ ਰਾਹੀਂ ਤਿਆਰ ਕਰਨ ਲਈ ਗੰਨੇ ਦੀ ਸੀਓ 238 ਕਿਸਮ ਉਪਰ ਕੀਤਾ ਗਿਆ। *ਟੀ ਏ ਵੀ ਏ ਪੀ* ਜੀਨ ਨੂੰ ਪੀ ਬੀ I ਵੈਕਟਰ ਰਾਹੀਂ *ਐਗਰੋਬੈਕਟੀਰੀਅਮ ਟਿਊਮੀਫੇਸਿਅਨਸ* ਦੇ ਈ ਐੱਚ 105 ਸਟਰੇਨ ਵਿੱਚ ਲਾਮਬੰਦ ਕੀਤਾ ਗਿਆ। ਤਬਾਦਲੇ ਵਿੱਚ ਇਨਵਿਟਰੋ *ਐਗਰੋਬੈਕਟੀਰੀਅਮ* ਨਾਲ ਲਾਮਬੰਦ ਵਿਧੀ ਨੂੰ ਵਰਤਿਆ ਗਿਆ। ਗੰਨੇ ਦੇ ਸਪਿੰਡਲ ਪੱਤਾ ਮਰੋੜ ਹਿੱਸੇ (ਆਗ) ਠੋਸ ਐਮ ਐੱਮ ਮਾਧਿਅਮ ਜਿਸ ਵਿੱਚ 5.0 ਐੱਮ ਜੀ ਪ੍ਰਤੀ ਲੀਟਰ, ਐੱਨ ਏ ਏ ਅਤੇ 0.5 ਐੱਸ ਜੀ ਪ੍ਰਤੀ ਲੀਟਰ ਕਾਈਨਿਟੀਨ ਪਾਇਆ ਹੋਇਆ ਸੀ ਉਪਰ ਪੈਦਾ ਕੀਤੇ ਗਏ। ਐਕਸ ਪਲਾਂਟਾਂ ਦੀ ਐਗਰੋਇਨਫੈਕਸ਼ਨ ਲਈ 1.0 ਤੋਂ 1.2 ਤੱਕ ਦੀ ਮਾਤਰਾ ਵਾਲੇ *ਐਗਰੋਬੈਕਟੀਰੀਅਮ* ਬਰਾਥ ਨੂੰ 9.8 ਐੱਮ ਜੀ ਪ੍ਰਤੀ ਲੀਟਰ ਐਸੀਟੋਸਰਿੰਜੋਨ ਨਾਲ ਵਰਤਿਆ ਗਿਆ। ਐਗਰੋਇਨਫੈਕਟ ਕੀਤੇ ਐਕਸਪਲਾਟਾਂ ਦਾ ਠੋਸ ਐੱਸ ਐੱਸ ਨਾਲ 5 ਐੱਮ ਜੀ ਪ੍ਰਤੀ ਲੀਟਰ ਐੱਨ ਏ ਏ, 0.5 ਐੱਮ ਜੀ ਪ੍ਰਤੀ ਲੀਟਰ ਕਾਈਨਿਟੀਨ ਅਤੇ 500 ਐੱਮ ਜੀ ਪ੍ਰਤੀ ਲੀਟਰ ਸੀਫੋਟੈਕਸਾਈਮ ਉਪਰ 21 ਦਿਨਾਂ ਲਈ ਤਬਾਦਲਾ ਕੀਤਾ ਗਿਆ। ਇਸ ਤੋਂ ਮਗਰੋਂ ਐਕਸ ਪਲਾਟਾਂ ਨੂੰ ਠੋਸ ਐੱਮ ਐੱਸ ਨਾਲ 5 ਐੱਮ ਜੀ ਪ੍ਰਤੀ ਲੀਟਰ ਐੱਨ ਏ ਏ, 0.5 ਐੱਮ ਜੀ ਪ੍ਰਤੀ ਲੀਟਰ ਕਾਈਨਿਟੀਨ ਅਤੇ 500 ਐੱਮ ਜੀ ਪ੍ਰਤੀ ਲੀਟਰ ਸੀਫੋਟੈਕਸਾਈਮ ਨਾਲ ਅਗਲੇ 21 ਦਿਨਾਂ ਤੱਕ ਜੜ੍ਹਤ ਕੀਤਾ ਗਿਆ। ਕੁੱਲ 38 ਪੁਟੇਟਿਵ ਟਰਾਂਸਜੈਨਿਕ ਪੌਦੇ ਵਿਕਸਿਤ ਕੀਤੇ ਗਏ। ਜਿਨ੍ਹਾਂ ਦੀ ਪੀ ਸੀ ਆਰ ਤਸਦੀਕ ਦਾ ਵਿਸ਼ਲੇਸ਼ਣ *ਟੀ ਏ ਵੀ ਏ ਪੀ* ਜੀਨ ਖਾਸ ਪਰਾਈਮਰ ਰਾਹੀਂ ਕੀਤਾ ਗਿਆ। ਪੀ ਸੀ ਆਰ ਨੇ ਪੁਟੇਟਿਬ ਪੌਦਿਆਂ ਵਿੱਚ *ਟੀ ਏ ਵੀ ਏ ਪੀ* ਦੀ ਨਾ ਮੌਜੂਦਗੀ ਦਰਸਾਈ ।

ਮੁੱਖ ਸ਼ਬਦ: *ਟੀ ਏ ਵੀ ਏ ਪੀ*, ਟਰਾਂਸਜੀਨ, *ਐਗਰੋਬੈਕਟੀਰੀਅਮ ਟਿਊਮੀਫੇਸਿਅਨਸ*, ਪੀ ਸੀ ਆਰ, ਐੱਨ ਏ ਏ, ਕਾਈਨਿਟੀਨ, ਸੀਫੋਟੈਕਸਾਈਮ ।

ਮੁੱਖ ਸਲਾਹਕਾਰ ਦੇ ਹਸਤਾਖਰ **ਵਿੱਦਿਆਰਥੀ ਦੇ ਹਸਤਾਖਰ**

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ABBREVIATION

LBA	:	Luria Bertani Agar
DNA	:	Deoxy Ribonucleic Acid
PCR	:	Polymerase Chain Reaction
dNTP	:	Deoxy nucleotide triphosphate
RNA	:	Ribonucleic Acid
CTAB	:	Cetyl Trimethyl Ammonium Bromide
EDTA	:	Ethylene Diamine Tetra-acetic acid
TE	:	Tris EDTA
TBE	:	Tris Boric Acid

CHAPTER-I

INTRODUCTION

Sugarcane (*Saccharum* spp. L.) is a commercial cash crop belonging to family Poaceae. India is the second largest producer of sugarcane followed by Brazil. The crop accounts for about 85 per cent of the world's sugar production and in 2018-19, there was production of 188.25 million metric tonnes (World sugar statistics). Sugarcane products like sugar and fermented products are used in making various kinds of medicines like syrups, liquids, capsules, etc. It is also used for ethanol production which is used as a fuel blend with petrol. Also, green top of sugarcane is used as fodder and cattle feed. Sugarcane juice is used for making jaggery and many by-products like bagasse and molasses.

The sugarcane yield and production are affected by various biotic (Wayne *et al* 2010) and abiotic stresses (Azevedo *et al* 2011). Abiotic stresses include drought stress, water-logging stress, salt stress, temperature stress, pollution stress, nutritional stress, cyclone stress and some others. Drought is the abiotic stress that affects crop phenology and yield affecting growth and development, chemical composition, sugar synthesis and accumulation, aggravates other stresses and availability of seed cane which leads to loss in cane recovery and sugar productivity (Shrivastava and Srivastava 2006). Due to drought stress, the water potential of plant and its photosynthetic rate decreases. About 17 per cent potential yield loss is caused by drought alone (Ithape *et al* 2017). Drought also affects the anatomical structure and chloroplast ultrastructure which is present in leaves of sugarcane (Zhang *et al* 2014). Due to its glycophytic nature, sugarcane yield growth is largely affected under salt stress (Suprasanna 2010). In India, large sugarcane area is prone to drought (Shrivastava and Srivastava 2006).

Sugarcane development is divided into four growth phases i.e. germinative, formative, grand growth and maturation phase. Favourable moisture conditions affect the germination of sets (Hogarth *et al* 2000). Drought stress greatly affects the crop during germination phase because during the germination phase, there is a need for required moisture and after the germination phase, the primary shoot then develops secondary and then to tertiary shoots. Formative phase is also sensitive to various environmental factors in which tillering takes place and drought can cause an impact on leaf canopy. In formative phase, sugarcane crop is more affected by water stress (Venkataramana *et al* 1986). In grand growth phase, drought stress affects the yield of crop because there is no recovery of stalk length (Robertson *et al* 1999). In the maturation phase, there is an increase in sucrose content by drought stress (Barnes *et al* 1974). Drought conditions also affects the ratooning ability of sugarcane (Singles *et al* 2011). There is impact of water stress on post-harvest quality of sugarcane crop like cane weight, extraction per cent and juice quality (Misra *et al* 2016).

Various physiological and breeding approaches were used for controlling drought conditions. Physiological approaches have been used for drought tolerance in sugarcane for different characters like PSII photochemical efficiency, estimated chlorophyll content, leaf temperature and leaf relative water content (Naidu *et al* 1983). Drought stress resulted in the reduction of photochemical efficiency, chlorophyll content and leaf relative water content, transpiration rate, stomatal size and stomatal conductance and an increase in leaf temperature (Yordanov *et al* 2000). The impact of Nitric oxide was also studied to provide tolerance against drought stress (Silveira *et al* 2016). Nitric acid gave positive effect by improving root growth and photosynthesis. Nitric oxide reacts with glutathione to form S-nitrosoglutathione and it helps in improvement of relative water content, plant and leaf dry matter. Breeding practices can also be used but there is a problem in use of these methods because of its larger genome size, higher ploidy level, rare flowering and long breeding cycle (Ithape *et al* 2017). The genotypes of sugarcane germplasm lack a diversified structure so it creates problem in breeding practices (Dal-Bianco *et al* 2012).

Different genes (*AVP1, AtDREB2A CA, EaHSP70*, etc.) were used for conferring tolerance to drought in transgenic sugarcane (Ferreira *et al* 2017). The overexpression of *EaHSP70* gene was studied for drought tolerance in sugarcane and overexpression of this gene increased the water and nitrogen use efficiency, photosynthetic activity, chlorophyll content and improved membrane stability which helps in providing sugarcane crop tolerance against heat, water and salt stress (Augustine *et al* 2015). By overexpression of *AtDREB2A CA* in sugarcane crop, it was observed that this gene improved the bud sprouting and internode length and also helps in providing tolerance under water deficit conditions (Reis *et al* 2014). The *AVP1* gene encodes for Vacuolar Pyrophosphate protein enhanced root growth of sugarcane crop and provide resistance against drought and salt stress. It also helps in production of new leaves and improving growth as compared to natural conditions (Kumar *et al* 2013). Therefore, Biotechnological approaches like genetic transformation can be considered as an good alternate for integrating desired gene for inducing tolerance to drought stress.

Vesicle-associated membrane protein-associated protein (*VAP*) genes were also reported as a source for drought stress tolerance (Singh *et al* 2017). They are part of soluble N-ethylmaleimide sensitive fusion attachment protein receptor (SNARE) complexes. It helps in membrane fusion and cargo transport. *VAP* plays a role in various processes like microtubule organization, lipid metabolism and UPR response (Hosaka *et al* 1992).

In the secretory pathway, synthesis of proteins takes place in the endoplasmic reticulum and then after different processes of folding and proper oligomer assembly, they are transported to the golgi complex, where most of the protein sorting takes place and then further transported to the plasma membrane, lysosome or secretory vesicles (Hong *et al* 1996). Vesicle transport is transport of proteins between different compartments of membrane and is mediated by vesicles that bud from one compartment and then fuse with the subsequent compartment. Vesicle transport is important for maintaining homeostasis, cell growth and development, and polarity (Blatt *et al* 2003). Vesicle trafficking plays an important role in regulation of stress response (Levine *et al* 2001). The membrane vesicle transport machinery consists of various types of phospholipids and integral membrane proteins, for example vesicle-associated membrane proteins (VAMPs). VAMPs are major constituent of SNARE complexes. SNARE complexes are responsible for fusion of vesicles with the target membranes. SNAREs constitute superfamily of proteins with *Arabidopsis thaliana*, *Oryza sativa*, and *Populus tricocarpa* reported to contain 60, 57 and 69 members, respectively, as compared to 36 in humans and 21 proteins in *Saccharomyces cerevisiae* (Sanderfoot *et al* 2000, Sanderfoot *et al* 2007, Pratelli *et al* 2004, Uemura *et al* 2004, Sutter *et al* 2006 and Lipka *et al* 2007). Functionally SNAREs were classified into two categories- V-SNAREs (vesicle-associated) and T-SNAREs (target-membrane associated) (Sollner *et al* 1993). Structurally these proteins are classified into Q and R SNAREs which are based on the presence of glutamine and arginine in key position (Fasshauer *et al* 1998). The VAMP proteins belongs to V-SNAREs, which forms a biochemically stable trans complex with target membrane localized T-SNARE syntaxin. So they facilitate the fusion of membranes (Chen and Scheller 2001).

Several members of SNARE family have been implicated in stress response in plants. In *Arabidopsis*, one of the SNARE proteins, AtSec20, homolog of yeast Sec20p and mammalian BNIP1, was shown to promote Golgi to ER retrograde trafficking, and was regulated by salt and osmotic stress, and gibberellin signalling (Zhao *et al* 2013). Recent studies demonstrated that over-expression of *SYP71* (t-SNARE) in rice enhanced tolerance to oxidative stress and rice blast disease through elimination of the H_2O_2 or other reactive oxygen species from the cell (Bao *et al* 2012). Overexpression of *OsNPSN11*, *OsNPSN12* and *OsNPSN13* genes in tobacco and yeast enhanced susceptibility to salt and osmotic stress but conferred tolerance to oxidative stress but (Bao *et al* 2008). Other members of SNARE gene family, *OSM1/SYP61*, were reported to regulate stomatal movements in *Arabidopsis thaliana* through modulation of phytohormone abscisic acid (ABA) levels (Zhu *et al* 2002). Suppression of expression of a v-SNARE, *AtVAMP71*, resulted in enhanced tolerance to salt stress in *Arabidopsis*, which was attributed to inhibition of fusion of H_2O_2-containing vesicles with the tonoplast (Leshem *et al* 2006). On the contrary, the reduced expression of *AtVAMP7* gene conferred reduces tolerance to drought stress in the *Arabidopsis* transgenic plants, which was caused by inhibition of ABA-dependent stomatal closure (Leshem *et al* 2010). These studies shown the role of different SNAREs in regulation of stress-response in plants, also underline the need to identify proteins that interact with different SNAREs.

A protein interacting with a v-SNARE, VAMP, was first identified in *Aplysia californica* (VAP-33) (Skehel *et al* 1995). *VAP-27*, the first plant ortholog of *VAP-33* and highly homologous to the inositol regulator SCS2 of yeast (Kagiwada *et al* 1998), was cloned in *Nicotiana tabaccum* (Laurent *et al* 2000). The *VAP*s are conserved proteins, its homologs present in all eukaryotes (Weir *et al* 1998, Nishimura *et al* 2004 and Lipka *et al* 2007). The characteristic features of *VAP*s comprise a motile sperm protein (MSP-VAP) domain, a linker that contains a coiled-coil (cc), and a carboxyl-terminal transmembrane domain containing a GXXXG dimerization motif (Russ and Engalman 2000). *VAP* homologs have very prominent *VAP* consensus sequence (VCS), a 16-residue segment that is highly conserved in VAPs but not in MSP1. The homologs of VAP in *S. cerevisiae* (SCS2) and other fungi have a similar domain structure, but lack coiled-coil domain in the linker, and the GXXXG motif.

Studies on *Aplysia californica* implicated the role of *VAP-33* in exocytosis of neurotransmitters, thus, providing the first insight into the role of these proteins (Skehel *et al* 1995). Though later studies hinted at the role of these proteins in several other fundamental cellular processes like microtubule organization, lipid metabolism, inositol auxotrophy and UPR response, etc. (Hosaka *et al* 1992, Skehel *et al* 2000, Lev 2004, Rutkowski and Kaufman 2004, Ron and Walter 2007). In plants, though information on the role of *VAP* is scanty, a membrane-associated mannitol-induced protein (AtMAMI-30), which is a structural homolog of *VAP-33*, was reported to be induced by osmotic stress in *A. thaliana* (Galaud *et al* 1997).

TaVAP gene is the ortholog of *VAP*. The gene coding for vesicle-associated membrane protein-associated protein (*TaVAP*) was checked for differential expression in drought tolerant and susceptible cultivar of wheat and difference in expression leads to changed leaf water potential of tolerant and susceptible cultivars (Singh *et al* 2007). The overexpression of *TaVAP* gene maintains high level of proline content, photosynthetic efficiency and antioxidant activity. *TaVAP* is tissue specific and responsive against multiple stresses. The transgenic *Arabidopsis thaliana* showed good survival level under drought and decreased yield loss under moisture deficit conditions (Singh *et al* 2017). The physiological role of this gene is still not understood.

As is evident from the documented literature, there is lack of information about the functions of *TaVAP* in plants.

The present study was understand the role of *TaVAP*, particularly for drought tolerance in sugarcane undertaken to "DEVELOPMENT OF WATER USE EFFICIENT TRANSGENIC SUGARCANE THROUGH EXPRESSION OF WHEAT *TaVAP* TRANSGENE" with the following objectives:

- Introduction of wheat *TaVAP* in sugarcane through *Agrobacterium* mediated transformation
- Characterization of putative transgenic plants

CHAPTER-II

REVIEW OF LITERATURE

Sugarcane is a tropical, perennial crop belonging to grass family, Poaceae. Sugarcane accounts for about 80% of sugar globally. Brazil produce 40% of world's sugar (746.8 million tonnes) (FAOSTAT 2018) while India is second largest producer of sugarcane. Sugarcane crop is used in making alcohol, biofuel, beverages, bagasses, jaggery, rum and juice etc. Various biotic and abiotic stresses affect the sugarcane yield and production. Drought is the major abiotic stress in sugarcane. About 17 per cent potential yield loss is caused by drought alone (Ithape *et al* 2017).

2.1 Overview of drought stress

2.1.1 Types of drought stress

2.1.2 Effect of drought stress on various crops

2.1.3 Effect of drought stress on sugarcane crop

2.1.4Various physiological, biochemical and morphologicalchanges induced by drought

2.2 Approaches to deal with drought stress tolerance

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2.3.4 Role of *TaVAP* in drought stress tolerance

2.1 Overview of Drought stress

All crops are affected by various abiotic and biotic stresses. Out of these stresses, drought stress is major abiotic stress which cause an impact on these crops which requires large amount of water. Drought stress is the factor which affect different physiological parameters like radiation capture, leaf temperature (LT), transpiration, stomatal conductance, electron transport, photosynthesis and respiration and these factors cause impact on yield (Qing *et al* 2001). Drought stress increases the level of reactive oxygen species. Due to this increase, crop requires a higher level of antioxidant activity for stress compensation. Drought stress reduces the photosynthetic rate by stomatal closure and lower CO_2 activity (Singh *et* *al*2012).

2.1.1 Types of drought stress

Various types of drought stress experienced by the crops:

- Occasional drought
- Frequent drought
- Drought in rainfed conditions
- Drought experienced by crop during dry spells in its growth cycle

2.1.2 Effect of drought stress on various crops

Drought stress cause major impact on rice, sugarcane, maize, tabacco, wheat, sorghum and some vegetable crops. In wheat, drought stress decreased the LEA proteins, proline and glycine content. In 2002, drought stress affected rice production by 20%. (Pandey *et al* 2007). Severe drought conditions affected rice production by 40% (Pandey *et al* 2005). In tropical countries, 17- 60% loss in maize yield was recorded due to drought stress. When moisture stress is accompanied with heat stress, 100% yield loss occurred in silking phase of maize. (Anami *et al* 2009). In sorghum, pre-flowering drought stress affects panicle size, grain yield, biomass and grain number. (Sanchez *et al* 2002). In tabacco, drought stress enhanced the ROS (Sengupta *et al* 1993) and decreased the unsaturated fatty acids and trehalose content (Romero *et al* 1997).

2.1.3 Effect of drought stress on sugarcane crop

Sugarcane requires 1250-2500 mm water for proper growth and yield (Khan *et al* 2004). As per data of Punjab, irrigation water requirement (m^3/ha) of paddy (transplanted before 10th June), basmati, sugarcane and wheat were 13710, 9653, 11914 and 3661 respectively (Kaur *et al* 2010).

Sugarcane growth is determined by four development phases (Roberts *et al* 1990) – germinative phase, formative phase, grand growth phase and maturity phase.Favourable moisture conditions affect the germination of sets (Hogarth *et al* 2000). Sufficient water is required in the germination phase for the development of primary shoots and shoot roots (Barnes 1974). Formative phase along with early grand growth stage is most critical stage which is affected by drought conditions (Ramesh 2000) because, in this phase, about 80 % of yield is produced (Singh *et al* 1987). Inman-Bamber *et al* 2004 showed that the drought stress during formative phase affect the cane yield, sucrose content and number of cane stalks. In formative phase, drought stress cause reduction of the above ground biomass, stalk numbers, leaf area and tillering in sugarcane (Robertson *et al* 1999). In case of grand growth phase, drought affect the internode length. Proper moisture level give rise to longer internodes (Robertson *et al* 1999). Drought stress results in reduction of stalk biomass takes place in maturation phase and therefore photosynthesis is directed to sucrose accumulation. Hence the positive effect of sufficient drought is measured in maturation phase (Inman-Bamber *et al* 2002).Drought stress also affects photosynthetic activity, dry matter accumulation and cane yield (Qing *et al* 2001).Misra et al (2016) studied the post-harvest losses due to drought stress in sugarcane for various characteristics like sucrose per cent, purity coefficient, commercial cane sugar per cent, total soluble solids, pH etc. They also showed that loss in purity coefficient was greater in drought affected canes as compared to normal canes in march harvest (after 10 days of harvest) and in November harvest, loss of purity coefficient was more in normal canes than drought affected canes. They also revealed that post-harvest loss of commercial cane sugar % was similar in normal as well as drought affected crop (in November harvest) and more in drought affected crop (in March harvest). In November harvest, there was more loss of total soluble sugars in drought affected crop. Similarly, the decrease of pH in November harvest is more in drought canes.

2.1.4 Various physiological, biochemical and morphological changes induced by drought

Drought stress induces various physiological, biochemical and morphological changes in different cropsas given in Fig 2.1

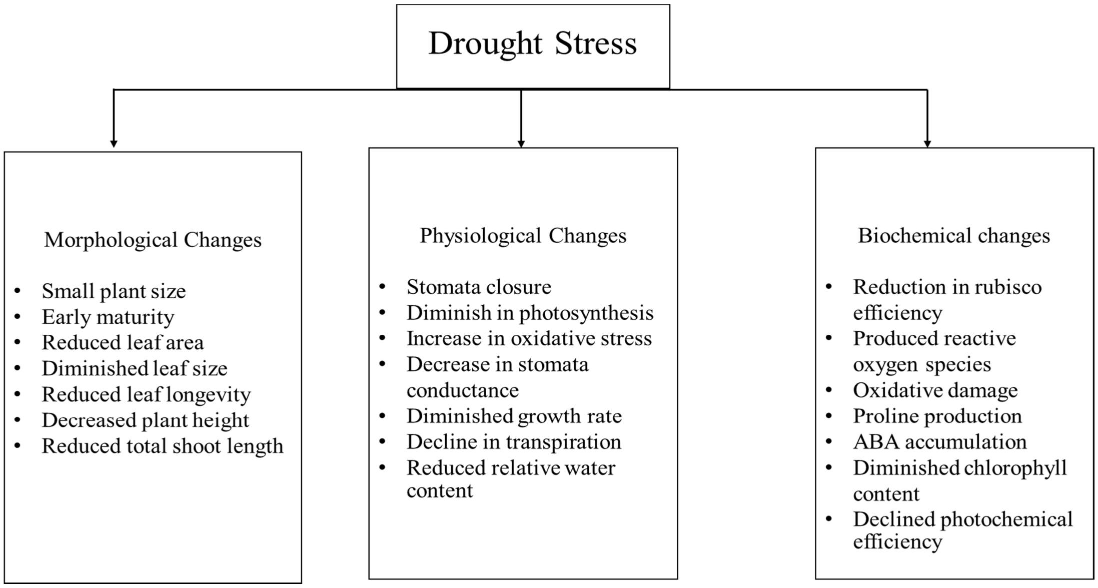

Fig 2.1 Various physiological, biochemical and morphological changes induced by drought

The morphological responses to a deficit of water include leaf wilting, reduction in leaf area, leaf abcission,early maturity and stimulating growth of root by channeling nutrients to underground part of plants.

Physiological changes at the cellular level which are associated with drought stress include stomata closure. Stomatal closure causes a decrease in CO2 availability. Other physiological changes include increase in oxidative stress, decline in transpiration, reduced relative water content etc.

During drought stress, the xylem vessels give up some contents like ABA to leaf apoplast, which increases the hormone concentration in the compartment. The plant defence response to water stress is related with synthesis of osmolytes, osmoprotectants, or some compatible solutes.

2.2 Approaches to deal with drought stress tolerance

Various approaches like physiological, biochemical, breeding and molecular approaches were used to deal with the drought stress tolerance (Fig 2.2).

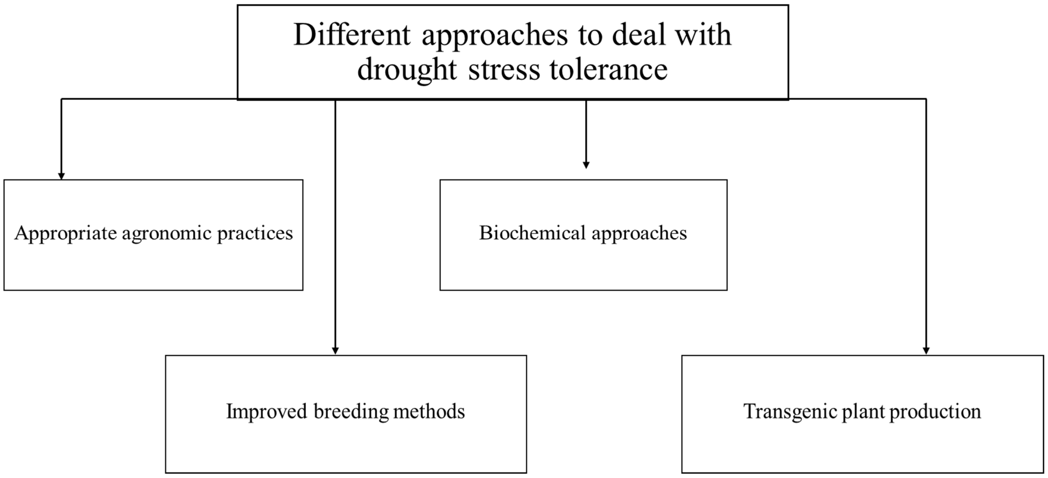

Fig. 2.2 Approaches to deal with drought stress tolerance

2.2.1 Cultural approaches

Different Cultural approaches were implicated for drought stress tolerance in sugarcane.

Naidu *et al*(1983) reported drought stress tolerance by spraying urea + 2.5 % KCl at 60,90 and 120 days after planting. Jayabal *et al* 1990 improved drought condition by spraying 2.5% K2SO4 during summer.

Durai *et al*(1996) used moisture absorber in soil which ensures availability of some amount of moisture during drought stress.

Sato *et al* (2010) reported that supply of phosphorus reduced effect of drought stress by enhancement of proline content.

Li *et al*(2003) noticed that foliar spray of low concentration of ethrel (100 ppm) before drought improved drought stress tolerance.

2.2.2 Biochemical approaches

There are different biochemical approaches for drought stress. Free proline content is correlated with water stress. Transgenic sugarcane plant which expressed heterologous *P5CS* increased proline content, increased biomass yield and photochemical efficiency of photosystem II (Molinari *et al* 2007). Proline acts as an osmolyte, metal chelator, an antioxidant defence molecule and a signal molecule. Proline mainly plays an important role in antioxidant defence system as compared to other amino acids (Hayat *et al* 2012).

Exogenous supply of proline help in reducing the level of ROS (Matysik *et al* 2002). Rezaei *et al*(2011) observed that exogenous supply of glycine betaine helps in tolerance to drought stress

During drought stress, there is an accumulation of reactive oxygen species (ROS) which cause damage to cell membrane (Mittler 2002). Low ROS concentration takes part in signal transduction processes.

2.2.3 Breeding approaches

Some varieties of sugarcane were tolerant to drought stress. These could be used as parents for breeding.Krishnamurthi (1989) reported some indigenous canes which are possessing tolerance to drought stress, which could be utilized in directed breeding.

All India Coordinated Research project on Sugarcane has released some varieties of sugarcane which are tolerant to drought stress and used for commercial cultivation.

Misra and Chaudhary (2003) reported some sugarcane varieties from different locations of India which are drought tolerant. Co 94008, Co 99004, Co 87025, Co 8371, Co 2001-13 and Co 91010 are the varieties from peninsular region, CoC 01061 is from eastern region. Co 87268, CoLk 94184 is from north-central zone and CoPant 90223 is from north-west zone.

2.2.4 Genetic transformation for drought stress tolerance

Cultural, biochemical and breeding approaches are short term approaches for conferring drought tolerance. However, new molecular and biotechnological approaches may go for a long way approach in developing transgenic plants for drought stress tolerance.

Overexpression of target genes, transcription factors and enzymes led to water stress tolerance in various crops (Table 2.1, 2.2 and 2.3). The regulation of trehalase activity occurs by expression of trehalose synthesis-related genes like *trehalose phosphate synthase* (*TPS*) and *trehalose-6-phosphate phosphatase* (*TPP*) improves tolerance to abiotic stresses in rice (Garg *et al* 2002).

Kishor et al (1995) reported *P5CS* gene for conferring drought tolerance in tabacco. First, they introduced pBI 121 containing *Vigna aconififolia* P5CS cDNA into the *Agrobacterium tumefacians* and then tabacco leaf discs were transformed with that *Agrobacterium* strain under the control of CaMV 35S promoter. Further they conducted drought and salinity treatments and southern, northern and westerns blot assays were also analysed. They also estimated amino acid content and osmotic potential of transgenic plants in comparison to wild type plants. The transgenic plants showed high level of proline and also accumulation of proline maintained the osmotic potential. Therefore, accumulation of proline accelerated the ability of tabacco plants to tolerate drought stress.

Chauhan and Khurana (2010) studied the expression of barley *HVA1* gene for conferring drought tolerance in wheat. They used anther culture derived haploids as explants for transformation. pCAMBIA 2301 vector was used in which *HVA1* gene was inserted. Then pCAMBIA-*HVA1* was mobilized into *Agrobacterium* strain LBA 4404 under the control of Act1 promoter. After that, southern analysis and RT-PCR experiments were done. Then physiological and biochemical analysis were experimented. The transgenic plants showed high level of drought tolerance with respect to wild type plants. There was increase in per cent germination, seedling growth and biomass accumulation in transgenic plants compared to non-transgenic plants.

Hu (2008) reported *OsLEA3* gene for conferring drought tolerance in transgenic rice. pHOS-*OsLEA3* plasmid vector was used which was mobilized into *Agrobacterium* strain AGL0 under the control of CamV35 S promoter. Southern analysis investigated the presence of single copy insertion of *OsLEA3* gene in transgenic rice. Northern analysis was also done for confirmation of *OsLEA3* transcript in transgenic rice. Western analysis was also performed for the confirmation of exogenous *OsLEA3* proteins in transgenic rice. Accumulation of *OsLEA3* gene in transgenic rice showed good performance of crop under salt and water deficit conditions. After 15 days of drought stress some non-transformed plants were gradually died whereas transgenic plants survived.

Lal *et al* (2007) studied the expression of barley *HVA1* gene for conferring drought and salinity stress in transgenic mulberry. Mulberry variety K2 was utilized as explant for transformation. pCAMBIA 2301 vector was used which was containing *HVA1* under the control of Act1 promoter. The resultant plasmid was then mobilized into *Agrobacterium tumefacians* LBA4404 strain. After transformation, PCR and southern blot analysis were performed for the confirmation of gene in transgenic plants. Transgenic mulberry plants showed drought stress tolerance as compared to wild type plants. Transgenic plants also showed high level of relative water content, cell membrane stability, proline content and photosynthetic yield as compared to non-transformed plants.

De Ronde *et al* (2004) compared the transgenic and non-transgenic lines with respect to drought stress tolerance. For this, they used six independent sense and one antisense transgenic line. Non-transgenic soyabean cultivar was used as a control. Further various analysis was performed like relative water content, proline estimation, carbohydrate and molecular analysis. PCR analysis detected the presence of *P5CR* gene in 3rd generation transgenic plants with their respective copy numbers. Sense transgenic plants showed significant increase in relative water content as compared to control and antisense transgenic plants. The proline dehydrogenase activity was greatly decreased in sense transgenic plants but antisense transgenic plants showed decreased level activity of PDH enzyme. There was also increased level of proline and *P5CR* enzyme activity in sense transgenic plants as compared to control and antisense transgenic plants.

Romero *et al* (1996) studied expression of yeast *trehalose-6 phosphate synthase* gene in transgenic tabacco plants. *TPS1* gene was cloned into pBin 19 under the control of CaMV 35S promoter. *Agrobacterium* strain LBA4404 was transformed with plasmid by high voltage electroporation and then used for plant transformation experiments. For confirmation of genomic DNA in transgenic tabacco, southern blot analysis was done by probing DNA with yeast *TPS1* gene. Further northern analysis was also performed for confirmation of gene transcripts. There was presence of trehalose content in transgenic plants and also showed high level of drought tolerance in those transgenic plants.

Gao *et al* (2013) studied the overexpression of *TaLEA* Gene from *Tamarix androssowii* for drought tolerance in transgenic poplar. *TaLEA* gene was cloned into pROKII under the control of CaMV 35S promoter. The leaves were used as explants for experiment. The leaves were first pre-cultured on MH solid medium (MS added with 0.1 mg/l folacin and 0.1 mg biotin) supplemented with 0.05 mg/l NAA and 0.5 mg/l BA (differentiation medium) for two days. Then pre-cultured explants were agro-infected. The infected explants were co-cultivated on differentiation medium in dark for 2-3 days. Then explants were transferred to differentiation medium supplemented with 200 mg/l cefotaxime for two days. Then explants were placed on selective medium and finally transferred to rooting medium. PCR and RNA gel blot experiment was performed for the confirmation of gene and its transcript in transgenic plants. Then malonyldialdehyde levels and relative rate of height growth were measured. Relative electrolyte leakage assay was also prerformed. The transgenic poplar plants showed decreased level of malonyldialdehyde content and electrolyte leakage as compared to non-transgenic plants. The transgenic plants contained lower number of wilted leaves after stress. Some transgenic lines grew faster than non-transgenic plants under stress conditions.

Certain genes like *TSase*, *DREB*, *PDH 45*, *AVP1* and *HSP70* etc. were used for conferring drought tolerance in sugarcane (Table 2.4). This approach also helps in studying the function of particular gene (Ramanjulu and Bartels 2002, Ferreira *et al* 2017).

The *TSase* gene accumulate trehalose and showed high chlorophyll content, antioxidant activity, lower permeability of plasma membrane and reduce the malondialdehyde content and its overexpression from *Grifola frondosa* resulted in biomolecule stabilization and improved drought tolerance in sugarcane plants (Zhang *et al* 2006). pBBBt plasmid which contains *TSase* gene was constructed and mobilized into EHA 105. ROC 10 cultivar of sugarcane was used as an explant. First, they cultured explants on M1 medium (MS + 1 mg/L 2,4-D) for induction of callus. Then calli were agro-infected and transferred to MR medium (1/5 MS + 1 mg/L 2,4-D + 200 µmol/LAS + 10 mmol/L glucose + 25 g/L sucrose). After co-cultivation, calli were dried and put on M2 medium (MS + 1 mg/L BA + 0.5 mg/L KT + 30 g/L sucrose + 500 mg/L carbenicillin + 0.75 mg/L phosphinothricin) for shoot regeneration. Then shoots were rooted on M3 medium (1/2 MS + 1 mg/L NAA + 0.5 mg/L PPT + 20 g/L sucrose + 300 mg/L cefoxitin).

DREB genes are family of transcription factors which helps in regulating different activities under abiotic stress conditions (Wang *et al* 2016). Overexpression of *AtDREB2A CA* gene led to increased relative water content, photosynthetic rate and also improved the sprouting of buds and internode length and helps in tolerance to drought and salt stress in sugarcane (Reis *et al* 2014). Sugarcane variety RB855156 was used. pBract302 expression vector was used which contains *DREB2A CA* coding sequence with Rab17 promoter sequence (from maize). Explants (transverse segments 2-3 mm) were initially cultured on solid MS media supplemented with 20g/l sucrose and 3 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D). Transformation was carried out using particle bombardment method. After bombardment, calli were transferred to basal MS media for 10 days and then on MSC3 with glufosinate ammonium. PCR analysis were done for confirmation of 674 bp product. After this, western blot analysis, water deficit assays, Rhizotron trials, Realtime PCR assays were done and measurement of sucrose content, biomass, root growth, gas exchange took place. A total of 24 plantlets contains the gene with transformation efficiency of 3.3 %. RT PCR results reported the upregulation of proline, raffinose content, two *Saccharum* drought responsive genes expect for late embryogenesis abundant (LEA) genes.

AtDREB2A genes were co-expressed by *PDH45* gene and provide greater tolerance to salt stress and less tolerance to drought stress. *PDH45* gene encodes DNA helicase 45 which unwinds the nucleic acids duplex. *PDH45* plays an important role in expression of downregulated stress related genes. It upregulates the stress related genes by 50fold and above by various mechanisms like DNA replication, transcription, translation, recombination, repair and ribosome biosynthesis (Wu *et al* 2012).

Overexpression of *PDH45* gene helps in nucleic acid metabolism and enhanced drought and salt stress in sugarcane (Augustine *et al* 2015). CO 86032 sugarcane variety were used. pCAMBIA1305.1 vector was initially used which were restricted with *BamHI* and *NcoI* enzymes for release of CaMV 35S promoter and Ubi 2.3 promoter was inserted to make pPORT-Ubi2.3-GFP. *PDH45* gene was amplified from pRT101 vector and cloned into pPORT-Ubi2.3-GUS to make the new construct which was named pSBI-*PDH45* and by freeze thaw method, it was transferred to *Agrobacterium* strain LBA4404. MS media was used for culturing supplemented with 3 mg/l 2,4-D, 10% filtered fresh coconut water, 100 mg/l myo-inositol and 20 g/l sucrose. *Agrobacterium* mediated transformation method was performed. PCR analysis was initially done to confirm the product size of 1.2 Kb and further confirmed by using hygromycin gene to generate a size of 800 bp. Later electrolytic leakage, relative water content, chlorophyll content, photosynthetic efficiency, qRT-PCR, bud germination assays were done. Report showed that there was increase in relative water content, membrane stability, chlorophyll content and photosynthetic efficiency in transgenic sugarcane as compared to wild type plants.

AVP1 gene encodes a vacuolar membrane protein which take the H^+ ions from the cytoplasm and transfer it into vacuoles and increases vacuolar solute content. The *AVP1*gene encode for enhanced root growth of sugarcane crop and provide resistance against drought and salt stress. It also helps in production of new leaves and improving growth as compared to natural conditions (Kumar *et al* 2013). Sugarcane variety CP-77-400 was used. *Agrobacterium tumefaciens* strain EHA105 was used for transformation. pGreen 0029 binary vector which was containing *AVP1* gene was utilized under the control of CaMV 35S promoter. Culturing was done on basal MS media. After co-cultivation, buds were transferred into regeneration media containing solid MS + BAP(1mg/l) + Kinetin(1mg/l) + GA3(1mg/l) + NAA (0.25mg/l). Molecular analysis was done which includes PCR and RT-PCR analysis. The overexpression of *AVP1* increased the relative water content, maintains osmotic and turgor pressure and improved drought tolerance in transgenic sugarcane.

HSP70 plays an important role in abiotic stress conditions. HSPs are the molecular chaperones which stabilizes the protein and membrane structure. *HSP70* helps in transport of subcellular proteins, degradation of unwanted proteins and folding of proteins (Evans *et al* 2010). The *chrysanthemum* overexpressing *HSP70* conferred drought tolerance in *Arabidopsis* (Song *et al* 2014). *EaHSP70* exhibit high membrane thermostability, relative water content, chlorophyll content and photosynthetic efficiency under drought stress conditions and provide tolerance to drought stress (Augustine *et al* 2015). They used Co 86032 for genetic transformation. From construct pCAMBIA3301-*cry1Ab*, they made construct pPORT- UBI2.3-3301 (restricted with *SacI* and *Sbf1*) and finally they formed the pSBI-*HSP70*. CaMV 35S promoter was used for regulation of gene expression. Gene was transferred through *Agrobacterium* mediated transformation method. Then PCR analysis were done to confirm the gene size of 1.2 Kb. Various analysis was done which includes morphological analysis, cell membrane thermostability analysis, plant water status, leaf chlorophyll and qPCR analysis etc.

Ramiro *et al* (2016) studied the expression of *Arabidopsis* Bax inhibitor-1 (*AtBI-1*) for conferring drought tolerance in transgenic sugarcane. *AtBI-1* increases the stomatal conductance, CO_2 assimilation rate, relative water content. This gene also increases the activity of antioxidant enzymes and osmoprotectant level. Sugarcane variety RB835089 was utilized for transformation. Apical region of sugarcane was sterilized and placed on solid MS medium supplemented with 5% coconut water, 13.5 µM 2,4-dichlorophenoxyacetic acid(2,4-D), 150 mg/l of citric acid and 30g/l sucrose. Transformation was done by particle bombardment method. Ubi promoter which was incorporated in plasmid pHA9 was used for co-transformation. Further they analysed the drought tolerance experiments, proline content, antioxidant enyme activity, relative water content, total soluble and reducing sugars content.

Saravanan *et al* (2018) studied the *BcZAT12* gene expression for drought and salinity tolerance in sugarcane. *BcZAT12* was isolated from *Brassica carinata* using yeast one hybrid system. *BcZAT12* encodes C2H2 binding proteins and study showed that the expression of gene leads to higher photosynthetic rate, transpiration rate, relative water content, stomatal conductance, chlorophyll content, proline and glycine betaine content in stress tolerant transgenic plants. Shoot tops of Co86032 was used. *Agrobacterium* strain LBA4404 containing pCAMBIA1300-*BcZAT* was streaked and used as co-cultivation and then calli were transferred to regeneration media. PCR analysis was done to confirm the amplified product of 258 bp internal sequence. Further physiological parameters like leaf temperature, Relative water content, chlorophyll content and biochemical attributes like proline content, Glycine betaine content and statistical and RT-PCR expression were analysed.

Raza *et al* (2015) demonstrated the *H+-PPase* gene overexpression for drought tolerance in sugarcane. The gene was isolated from *Arabidopsis*. Sugarcane cultivar CSSG-668 was used for transformation. Transformation was conducted by particle bombardment method. pZSI plasmid which was harbouring *H+PPase* was used under the control of CaMV 35S promoter. On callus induction medium, apical discs were culture for callusing. The plasmid DNA was precipitated into gold particles and bombardment was done, then survived calli were transferred to regeneration selection medium. PCR analysis was performed using gene specific primers. Western blot analysis and water deficit experiment was also performed. Transgenic plants showed drought stress tolerance. In addition to it, transgenic plants also showed greater root growth, high Brix %, high relative water content, high sugar and amino acid content as compared to wild type plants.

Mbambalala *et al* (2020) analysed the overexpression of *AtBBX29* against drought tolerance in sugarcane. *AtBBX29* is B-box zinc finger protein. In this experiment, they cultured apical inner leaf roll of sugarcane cultivar NCo310 on MSC3 medium (4.43 g/L MS with vitamins + 20 g/L sucrose + 0.5 g/L casein + 3 mg/L 2,4-D and 2.22 g/L gelrite). They incubated cultures in dark at 26°C and sub-cultured on fresh media every 2 weeks. Before bombardment, callus was placed on osmoticum medium (MSC3 + 0.2 M mannitol + 0.2 M sorbitol). pUBi510-*AtBBX29*vector was mixed with tungsten particles and used for bombardment. PCR analysis was done for confirmation of transgenic plants. After that, they analysed some physiological parameters and measurement of ROS, antioxidants, proline and ABA were taken. The transgenic plants showed great amount of tolerance against drought. The proline content was increased in transgenic plants and also decrease in ROS, antioxidants content as compared to wild type plants.

2.3 Molecular mechanism of *VAP*

2.3.1 Protein trafficking and transport

The primary objective of cell is to interact with its environment. There are secretory and transmembrane proteins. The role of various organelles like Golgi apparatus, Endoplasmic reticulum (ER), lysosomes and endosomes, and the plasma membrane in the transport of proteins is referred to as the secretory pathway. Once a new protein is synthesized, the ER represents the entry point for newly-made proteins (Palade 1975, Rothman and Wieland 1996, Schekman *et al* 1996 and Schekman *et al* 1997). After proper folding and oligomer assembly, the ER-resident proteins are retained in the ER while the majority of newly-made proteins are selectively incorporated into transport vesicles budding off from the ER.

In a eukaryotic cell, Vesicle traffic is maintained to transport membrane material, proteins and soluble cargo between endomembrane compartments, the plasma membrane, and the extracellular space. By budding, formation of vesicles takes place and further constricted at the donor compartment membrane surface. The delivery of vesicles is achieved by fusion and interaction with the lipid bilayer of the target membrane (Pratelli *et al* 2004). Specific delivery of molecular cargo is very important for cell function and its survival. For example, this specificity is vital for the release of neurotransmitters into the presynaptic region of a nerve cell to transmit a signal to a neighbouring nerve cell (Chen and Scheller 2001).

The known components which are involved in the transport of cargo are the vesicles but the specific mechanism is under study that how these vesicles reach their destinations and how these vesicles fuse with the different organelles or plasma membrane. Dr. James E. Rothman, Dr. Randy W. Schekman, and Dr. Thomas C. Sudhof (Nobel Prize in Physiology or Medicine, 2013) were three Nobel laureates who pioneered an in-depth study of the machinery that regulates vesicle traffic, a major transport mechanism in eukaryotic cells. Imposition of stress factors has an impact on the mechanism of vesicle transport (Mazel *et al* 2004).

SNARE proteins are the proteins that involved in vesicle transport and maintaining the cell homeostasis (Surpin and Raikhel, 2004). SNAREs are Soluble NSF Attachment Protein Receptors and are conserved among all eukaryotes (Jahn *et al* 2003, Pratelli *et al* 2004, Surpin and Raikhel 2004). Rothman and Fries coined the term SNARE in 1981 for describing the processes where binding of NSF and Soluble NSF Attachment Proteins (SNAPs) takes place (Sollner *et al* 1993).

On the basis of their localization, there are two categories of SNAREs. v-SNAREs are present on the vesicle or donor compartment and t-SNAREs are present on the target or acceptors compartments (Gerald 2002). There are some proteins which display regulatory functions and those proteins are termed as SNARE-masters (Gerst 1999). SNARE proteins consists of three major families: (1) syntaxins (2) SNAP-25 (3) VAMP (Vesicle-Associated Membrane Protein)/Synaptobrevin. In case of mammalian SNAREs, homologs were present in other organisms (Lewis *et al* 1997). All these three SNARE protein families are conserved through evolution (Bennett and Scheller 1994). In plants, the syntaxins (Pratelli *et al* 2004) are the best characterized among the core SNAREs and they are functionally most interesting. Fusion particle is formed by association of SNAPs and NSF that plays an important role in vesicle trafficking among sub-cellular compartments (Nichols and Pehlam 1998). There are Complementary SNAREs which are identified by their core residues (either Arg or Glu), are localized to different membrane compartments and interact in such a way that they form a tetrameric bundle structure of coiled helices that helps in drawing the membrane surfaces together and facilitate fusion. In reconstituted membrane preparations, this complex forms a minimal set of proteins required for fusion (Weber *et al* 1998). The specificity of SNARE interactions is a most determining factor in recognition of membrane and targeting of vesicle (Paumet *et al* 2004).

2.3.2 Role of SNARE proteins in plants under abiotic stress conditions

The SNARE proteins are known to function in vesicle trafficking and some of these proteins have also been implicated in stress response (Zhao *et al* 2013, Bao *et al* 2012 and Leshem *et al* 2010). AtSec20, *Arabidopsis* homolog of yeast Sec20p and mammalian BNIP1, was shown to facilitate Golgi-to-ER retrograde trafficking, and its expression was regulated by salt and osmotic stress, and gibberellin signalling (Zhao *et al* 2013).

Over-expression of *SYP71* (t-SNARE) conferred tolerance to oxidative stress in rice through exclusion of H_2O_2 or other reactive oxygen species from the cell (Bao *et al* 2012). Overexpression of plant specific t-SNARE genes, *OsNPSN11*, *OsNPSN12 and OsNPSN13* in tobacco and yeast resulted in susceptibility to salt and osmotic stress but conferred tolerance to oxidative stress.

When H_2O_2-containing vesicles get fused with the tonoplast, then it caused susceptibility of transgenic phenotypes towards stress (Bao *et al* 2008). On the contrary, suppression of expression of a v-SNARE, *AtVAMP71*, resulted in enhanced tolerance to salt stress in *Arabidopsis*, which was attributed to inhibition of fusion of H_2O_2-containing vesicles with the tonoplast (Leshem *et al* 2006).

In another study, the downregulation of expression of *AtVAMP71* gene resulted in inhibition of ABA-dependent stomatal closure which leads to decrease in tolerance to drought stress in the *Arabidopsis* transgenic plants (Leshem *et al* 2010). An osmotic stress-sensitive mutant gene (*OSM1*/*SYP61*) was reported to regulate stomatal movements in *A. thaliana* through modulation of phytohormone abscisic acid (ABA) levels (Zhu *et al* 2002).

These studies highlighting the role of different SNAREs in regulation of stress-response in plants, but there is need to identify such proteins that interact with different SNAREs.

VAMPs are SNARE proteins which are reported to be involved in fusion of vesicles. There are eight different types of VAMPs reported in eukaryotes which are categorized as synaptobrevins (VAMP1 and VAMP2), cellubrevin (VAMP3), VAMP4, VAMP5 and VAMP7, endobrevins (VAMP8), etc. VAMP1 and VAMP2 plays an important role in release of neurotransmitters (Archer *et al* 1990). They are constituents of synaptic vesicles and expressed in brain. VAMP3 acts in exocytosis which is mediated by secretory vesicles and secretory granules (Annaert *et al* 1997). VAMP5 and VAMP7 also participates in constitutive exocytosis. VAMP5 is part of myotubes, tubulovesicular structures and secretory vesicles. VAMP7 is also part of endosomes and secretory granules (Zeng *et al* 1998). VAMP8 plays an role in endocytosis but sometimes participate exocytosis in pancreatic acinar cells. Transport from golgi is mediated by VAMP4 (Steegmaier *et al* 1999).

The proteins which attaches to the VAMPs are known as VAMP-associated proteins (*VAP*s). The first *VAP* was cloned from *Aplysia californica* and size of that clone is 33kDa, so it was designated as *VAP-33* (Skehel *et al* 1995). It codes a protein that helps in synaptic hameostatis. Inhibition is caused by injection of *antiVAP33*, so it also plays its putative role in the exocytosis of neurotransmitters (Skehel *et al* 1995). Homologs of *VAP-33* is present in humans and othe mammals (Nishimura *et al* 1999). *VAP-A*, *VAP-B* and *VAP-C* homologs are present in mammals (Weir *et al* 1998, Skehel *et al* 2000). *VAP-A* and *VAP-B* are two homologs of *VAP33* present in humans designated as *h-VAP-A* and *h-VAP-B* respectively. *h-VAP-A* and *h-VAP-B* are 60% and 46% homologous respectively to *VAP-33* at amino acid level. Both *h-VAP-A* and *h-VAP-B* formed complex association with each other through their transmembrane domains. These findings showed that formation by different *VAPs* may be important in vesicles trafficking of mammals. Rat homologs of *VAP-33* were also reported and designated as *r-VAP-A* and *r-VAP-B* respectively. *r-VAP-A* and *r-VAP-B* is 95% and 88% homologous to *h-VAP-A* and *h-VAP-B*, respectively at amino acid level. *VAPs* consist of a motile sperm protein (MSP-VAP) domain, a linker that contains a coiled-coil (cc) and a carboxyl-terminal transmembrane domain containing a GXXXG dimerization motif (Russ and Engalman 2000). Similar domain is present in homolog of *VAP* in *S. cerevisiae* (Scs2) and other fungi but here found no coiled coil structure and also GXXXG motif is absent. *VAP* consensus sequence (VCS) is prominent sequence present in homologs, which is a 16-residue segment that is highly conserved in VAPs but not in MSP1. It also has been reported that some *VAP*-interacting proteins have a FFAT motif consisting of two phenylalanines (FF) in an acidic tract (FFAT) (Loewen *et al* 2003).

2.3.3 Functions of *VAPs* in eukaryotes

The expression of *VAPs* is present almost all compartments of the cells (Nishimura *et al* 1999, Skehel *et al* 2000). In eukaryotes, *VAPs* are reported to be localized in the ER, intracellular membranes of golgi apparatus and the ER-golgi intermediate compartments, recycling endosomes, neuromuscular junctions and the plasma membrane (PM) (Lapierre *et al* 1999, Soussan *et al* 1999 and Pennetta *et al* 2002). The diverse and specific cellular and subcellular localizations identify the role of these proteins in different cellular processes. The first function for *VAP* was proposed from its initial identification that it interacts with the membrane fusion protein synaptobrevin/VAMP in *Aplysia*. Further studies also reported that *VAP* plays an role in membrane traffic by binding with different membrane fusion proteins and also inhibiting traffic from Golgi to ER (Weir *et al* 1998, Soussan *et al* 1999).

In humans, there is a single missense mutation results in familial motor neuron disease (MND) caused in the *VAP-B* gene (Nishimura *et al* 2004). This condition is characterized by the formation of insoluble aggregates of the *VAP-B* protein. *VAPs* have been reported in the maintenance of cellular organelles and its organization. (Skehel *et al* 2000). *VAPs* helps in membrane trafficking by binding to the microtubules (Pennetta *et al* 2002). Studies in *Drosophila* have revealed their interaction with microtubules, decoding their requirement for microtubules cytoskeleton in the presynaptic terminals (Pennetta *et al* 2002). Choline-sensitive dominant mutation (CSE1) mutants of yeast showed inositol auxotrophy in the presence of choline and also when the Scs2 protein (yeast homolog of *VAP*) was overexpressed. Therefore, choline and SCS2 were demonstrated as suppressors of inositol auxotrophy of CSE1. In yeast, inositol auxotrophy results due to impaired expression of *INO1* gene, which encodes an enzyme inositol-1-phosphate synthase required for the synthesis of inositol. The expression of *INO1* is regulated by cis-acting promoter UASINO (inositol-sensitive upstream activating sequence) which is activated by a heteromeric basic helix-loop-helix transcription factor (Ino2p and Ino4p). When inositol or choline are in abundance in the cells, the *INO1* expression is repressed. On the contrary, the deficiency of these two compounds results in the de-repression of this gene. Another player Opi1p, a basic leucine zipper and FFAT-motif containing protein, acts as a negative regulator of *INO1* expression. Scs2p interacts with the Opi1p through the FFAT motif (Loewen *et al* 2003), thereby, inhibiting its translocation to the nucleus and promoting the activation of *INO1*. Scs2p was also reported to play a role in signal transduction pathways. Scs2-deficient mutants showed reduced levels of *INO1* and phosphatidylinositol (PtdIns), whereas, increased level of phosphatidylcholine (PtdCho) were observed through cytidine diphosphocholine (CDP-choline) pathway (Kagiwada and Zen 2003). Hyperactivation of PtdCho biosynthesis resulted in the expression of the *INO1* gene through Scs2p, thus, implying that Scs2p acts as a component of CDP-choline signal transduction pathway. In another pathway, inositol starvation activates unfolded protein response (UPR) that is regulated by Ire1 (inositol requiring enzyme 1), which activates the transcription activator Hac1p of the UPR pathway genes (Cox and Walter 1996). Therefore, despite Scs2-Opi1p and UPR-Hac1 pathways being different, Scs2 and Hac1 proteins are indispensable in the cell for the activation of *INO1* gene. Since, *VAP*s are SNARE-binding proteins and their overexpression may sequester the corresponding SNARE molecules, therefore, *VAP*:SNARE ratio is very crucial for the cell (Lev *et al* 2008). For instance, overexpression of *VAP-A* blocks the glucose transporter 4 (GLUT4) in response to insulin treatment by sequestering the VAMP-2 (Lev *et al* 2008). In another study, overexpression of *VAP-B* and N-terminal domain-interacting receptor 3 (Nir3) in mammalian cells resulted in changes in the ER microtubule structure, thereby, implying the interaction of *VAP-B* with microtubules, where Nir3 acts as a bridging molecule (Lev 2004).

VAMPs interact with t-SNAREs plays an important role in docking of vesicles in the target membranes. The *VAP*s (VAMP-associated protein) interacts with VAMPs are also conserved proteins and their homologues found in all eukaryotic cells (Nishimura *et al* 1999). *VAPs* helps in linking membranes with microtubules and lipid trafficking inside and outside of the endoplasmic reticulum by the CopI vesicles (Soussan *et al* 1999).

The first member of *VAP-33* family identified in plants was *VAP-27* from *Nicotiana tabaccum* (Laurent *et al* 2000). VAP-27 is highly homologous to the inositol regulator SCS2 of yeast (Kagiwada *et al* 1998). In Arabidopsis, a membrane-associated mannitol-induced protein (*AtMAMI-30*), a proposed structural homolog of *VAP-33*, was shown to be developmentally regulated and its expression was enhanced under osmotic stress (Galaud et al 1997). *VAP-27* and the yeast SCS2 share in their N-termini a common highly conserved MSP-VAP domain (120 residues). Along with the MSP-VAP domain, the *VAP-27* from *Nicotiana tabaccum* also possesses the coiled-coil region and a strongly hydrophobic stretch of 22 amino acid residues at the C-terminal, which represents the transmembrane region (Laurent *et al* 2000). The formation of homo and heterodimers is facilitated by the presence of hydrophobic residues in the coiled-coil region. The model suggests that *VAP-27* might form a dimer or a multimer in its C-terminus.

2.3.4 Role of TaVAP in drought stress tolerance

Singh *et al* (2007) analysed the expression of *TaeIf3g* and *TaVAP* in drought tolerant and susceptible cultivar of wheat. They used one drought tolerant variety (C-306) and one susceptible variety (HD-2329). Expression analysis was done in flag leaf and developing grains of wheat using real time PCR and actin was used as an internal control. In flag leaf, the expression of *TaVAP* was increased after 2 days stress and decreased after 5 days of drought stress in drought tolerant cultivar whereas in drought susceptible cultivar expression was increased after 2 days of stress and remained constant after 5 days of stress. In developing grains, after 2 and 5 days of drought stress there was increase in expression in drought tolerant cultivar but decreased in drought susceptible cultivar of wheat.

Singh *et al* (2017) studied the overexpression of *TaVAP* for drought tolerance in *Arabidopsis thaliana.* The overexpressing *TaVAP* transgenic *Arabidopsis* plants showed an increase in relative water content, proline, soluble sugars, peroxidase and photosynthetic activity Increase in relative water content and photosynthetic activity helps in maintaining turgor pressure. Increase in proline helps in antioxidant defence system. Here they used *Arabidopsis thaliana* ecotype Columbia and were plated on MS media in petri dishes. First, they amplified the *TaVAP* using gene specific primers and finally they made the recombinant construct (pYES-*TaVAP*). Overexpression was done in *Arabidopsis* plants and after, various seed germination, abiotic stress and biochemical assays were done. Therefore, the overexpression of *TaVAP* conferred tolerance to drought conditions.

Table 2.1 Differential gene expression for drought stress in other crops than sugarcane

Gene	Protein	Transgenic system	Promoter	Mechanism	References
Cox	Choline oxidase	Tabacco	CaMV35S	Accumulation of glycine betaine	Huang *et al* 2000
TPS1	Trehalose-6-phosphate synthase	Tobacco	CaMV35S	Accumulation of trehalose	Romero *et al* 1997
BetB	Trehalose-6-phosphate synthase 1	Tobacco	Ats1A	Accumulation of glycine betaine and trehalose	Holmstrom *et al* 1996
P5CS	Pyrroline carboxylate synthase	Tobacco	CaMV35S	Accumulation of proline	Kishor *et al* 1995
HVA1	late embryogenesis abundant protein	Wheat	Ubi1	Accumulation of LEA proteins	Sivamani *et al* 2000
HVA1	Late embryogenesis	Wheat	Act1	Accumulation of LEA proteins	Chauhan and

Gene	**Protein**	**Transgenic system**	**Promoter**	**Mechanism**	**References**
	abundant protein				Khurana 2011
HVA1	Late embryogenesis abundant protein	Morus indica	Act1	Accumulation of LEA proteins	Lal *et al* 2008
mt1D	Mannitol-1-phosphate dehydrogenase	Wheat	Ubi1	Improvement in fresh and dry weight, plant height and flag leaf length	Abebe *et al* 2003
AtP5C1	Pyrroline carboxylate synthase	Soyabean	AIPC	Proline accumulation, delay in wilting	De Ronde *et al* 2004
TaLEA	Late embryogenesis abundant protein	Poplar	CaMV35S	Protection of cell membranes by lowering electrolyte leakage and MDA content	Gao *et al* 2013
OsLEA3	Late embryogenesis abundant protein 3	Rice	CaMV35S	Accumulation of LEA	Hu 2008
AtPARP1	poly(ADP-ribose) polymerase	Canola	CaMV35S	Reduction in ROS, low mitochondrial respiration	De Block *et al* 2005
Dadc	Datura arginine decarboxylase	Rice	Ubi1	Accumulation of spermine/spermidine, increase in chlorophyll	Capell *et al* 2004
CodA	Choline oxidase	Potato	SWPA2	Accumulation of glycine betaine, increase in leaf water potential, reduced membrane damage,	Cheng *et al* 2013

Gene	Protein	Transgenic system	Promoter	Mechanism	References
				photosynthetic	

Table 2.2 Transcription Factors

DREB1A	Dehydration responsive element binding protein 1A	Rice	Ubi1	Maintenance of Fv/Fm ratio	Oh *et al* 2005
OsDREB2B	Dehydration responsive element binding protein 2B	Rice	Rd29A	Improvement in survival rate under stress	Matsukura *et al* 2010
AtDREB2A	Dehydration responsive element binding protein 2A	Arabidopsis	Rd29A	Accumulation of LEA proteins	Sakuma *et al* 2006
ZmDREB2A	Dehydration responsive element binding protein 2A	Maize	CaMV35S, Rd29A	Accumulation of LEA proteins	Qin *et al* 2007
CBF4	C-repeat binding factor 4	Arabidopsis	CaMV35S	ABA-dependent, improved survival rate	Haake *et al* 2002
DST	Zinc finger protein	Rice	-	Accumulation of proline and sugars, better H O2 2 homeostasis	Huang *et al* 2009
WXP2	Wax production2	Arabidopsis	CaMV35S	Higher water retaining capacity, maintenance of fresh weight	Zhang *et al* 2007
HARDY (HRD)	AP2/ERF-like transcription factor	Rice	CaMV35S	Increase in water use efficiency, reduced	Karaba *et al* 2007

				transpiration rate, high photosynthesis	
SNAC1	Stress-responsive NAC 1	Rice	CaMV35S	Increase in ABA mediated stomatal closure, more biomass accumulation	Hu *et al* 2006
WXP1	Wax production1	Medicago sativa	CaMV35S	Reduced epidermal permeability and water loss, maintenance of chl content	Zhang *et al* 2005
WIN1	Wax inducer 1	Arabidopsis	CaMV35S	Increased cuticle permeability, reduced stomatal density	Aharoni *et al* 2004
GmERF3	AP2/ERF transcription factor3	Tobacco	CaMV35S	Accumulation of proline and carbohydrate	Zhang *et al* 2009
OsWRKY11	WRKY11	Rice	HSP101	Reduced transpiration rate and protection of photosystem	Wu *et al* 2009
TsCBF1	Dehydration responsive element binding protein	Maize	Ubi1	Reduced membrane damage, accumulation of osmolytes	Zhang *et al* 2010
DREB1A	Dehydration	Pea	Rd29A	Retention of	Bhatnagar-

	responsive element binding protein			more water	Mathur *et al* 2014

Table 2.3 Enzymes

NPK1	Nicotiana protein kinase 1	Rice	Hva22	Maintaining leaf water potential	Xiao *et al* 2009
ERA1	Farnesyl transferase	Canola	Rd29A	ABA-mediated reduction in stomatal conductance	Wang *et al* 2005
MnSOD	Manganese superoxide dismutase	Rice	SWPA2	Improvement in the ROS scavenging system	Wang *et al* 2005
BetA	Choline dehydrogenase	Cotton	CaMV35S	Accumulation of glycine betaine, higher RWC, membrane protection	Lv *et al* 2007
CDPK1	Calcium-dependent protein kinase 1	Tobacco	CaMV35S	Maintenance of photosynthetic machinery and leaf water conten	Vivek *et al* 2013
ZmPIS	Phosphatidylinositol (PtdIns) synthase	Maize	Ubi1	Alteration in membrane lipid composition, synthesis of ABA	Liu *et al* 2013
AtDHAR1	Dehydroascorbate	Potato	CaMV35S	Reduction in	Eltayeb *et*

	reductase 1			ROS and ion leakage, maintenance of chlorophyll content	*al* 2011
ZmSIMK1	Mitogen-activated protein kinase 1	Tobacco	CaMV35S	Reduced MDA and transpirational rate, Increased levels of antioxidants, including ascorbic acid, proline, SOD, POD, CAT and APX	Wang *et al* 2014
GS1a	Glutamine synthetase 1a	Populus	CaMV35S	Alterations in the ROS genes expression	Molina-Rueda *et al* 2013
γ-TMT γ-Potato RNAi Seedling	tocopherol methyltransferase	Tobacco	CaMV35S	Reduced membrane damage, increase in biomass, accumulation of γ-tocopherol instead of α	Abbasi *et al* 2007

Table 2.4 Genes that are expressed in sugarcane for drought stress tolerance

S No.	Gene	Time of stress (days)	Promoter Used	Function	References
1.	*HSP70*	10	Ubi	High RWC, photosynthetic activity and membrane stability	Augustine *et al* 2015
2.	*DREB2A CA*	6	Rab17	Increase RWC and photosynthetic activity	Reis *et al* 2014
3.	*PDH45*	10	Ubi	Nucleic acid metabolism	Augustine *et al* 2015
4.	*AVP1*	15	35S enhanced	Increased RWC and leaf water potential	Kumar *et al* 2013
5.	*TSase*	15	35S enhanced	High chlorophyll content and antioxidant activity	Zhang *et al* 2006
6.	*AtBI*	21	Ubi	Increased RWC, antioxidant activity and Osmoprotection level	Ramiro *et al* 2016
7.	*BcZAT12*	15	CaMV 35S	High proline, glycine betaine, RWC, stomatal conductance and transpiration rate	Saravanan *et al* 2018
8.	*H+-PPase*	15	CaMV 35S	High amino acid, RWC	Raza *et al* 2015
9.	*AtBBX29*	21	CaMV 35S	High proline content	Mbambalala *et al* 2020

CHAPTER-III

MATERIALS AND METHODS

The experiments performed in present study entitled "DEVELOPMENT OF WATER USE EFFICIENT TRANSGENIC SUGARCANE THROUGH EXPRESSION OF WHEAT *TaVAP* TRANSGENE" were taken out in the Tissue Culture and Transformation Laboratories, SOAB, PAU, Ludhiana during the year 2018-2021. This chapter describes materials utilized and methods conducted for different experiments

3.1 Plant material

Sugarcane variety Co238 was used as source of plant material for genetic transformation. Spindle leaf roll segments are used as an explant for tissue culture which were collected from Regional research station, Kapurthala (Fig 3.1).

Fig. 3.1 Sugarcane crop at Kapurthala

3.2 *In vitro* raising of sugarcane spindle leaf rolls

3.2.1 Culture media

The spindle leaf segments were raised on basal MS (Murashige and Skoog 1962) media which contains five MS stocks i.e. stock solutions 1, 2, 3, 4 and 5, myo-inositol (100 mg/l) and sucrose/maltose (3 %). The stock solutions were prepared by adding their constituent salts (HiMedia, India) in distilled water as given in Table 3.1. To prepare one litre of basal MS media 50 ml, 20 ml, 20 ml, 10

Table 3.1 Composition of MS medium

Stock no.	Stock strength	Constituent salts	Quantity (g/l)	Stock volume used (ml/l)	Actual amount in the culture medium (g/l)
For 2 litre					
1.	20X	Ammonium nitrate (NH_4NO_3)	66.0	50	1.65
		Potassium nitrate(KNO_3)	76.0		1.90
		Potassium dihydrogen orthophosphate (KH_2PO_4)	6.80		0.17
		Boric acid (H_3BO_3)	0.248		0.0062
		Manganese sulphate ($MnSO_4.7H_2O$)	0.892		0.0223
		Zinc sulphate ($ZnSO_4.7H_2O$)	0.344		0.0086
		Potassium iodide (KI)	0.033		0.000825
		a. Copper sulphate ($CuSO_4.5H_2O$)	1.0 ml		0.000025
		b. Sodium molybdate ($Na_2MoO_4.2H_2O$)	1.0 ml		0.000250
		c. Cobalt chloride ($CoCl_2.6H_2O$)	1.0 ml		0.000025
½ litre					
2.	50X	Calcium chloride ($CaCl_2.2H_2O$)	11.0	20	0.44
3.	50X	Magnesium sulphate ($MgSO_4.7H_2O$)	9.250	20	0.37
1 litre					
4.	100X	Ferrous sulphate ($FeSO_4.7H_2O$)	2.780	10	0.0278
		Ethylene diamine tetra acetic acid and disodium salt (Na_2 EDTA)	3.728		0.03728
1 litre					
5.	100X	Thiamine HCl	0.010	10	0.0001
		Nicotinic acid	0.050		0.0005
		Pyridoxine HCl	0.050		0.0005
		Glycine	0.200		0.002
		*Myoinositol			0.1
6		Sucrose			30

a. Dissolve 100 mg $CuSO_4.5H_2O$ in dH_2O and make final conc. of 100 ml
b. Dissolve 1000 mg $Na_2MoO_4.2H_2O$ in dH_2O and make final conc. of 100 ml
c. Dissolve 0.1 g $CoCl_2.6H_2O$ in dH_2O and make final conc. of 100 ml
Note: 1. Conc. HCl (1 ml) was dissolved in Stock No.1 to avoid precipitation.
2. Stock No. 4 was slightly heated to avoid precipitation.
*While preparing final medium, Myoinositol and sucrose were dissolved

ml and 10 ml of stock I, II, III, IV and V respectively were mixed in 700 ml of distilled water (Millipore, USA) along with 100 mg of myo-inositol and 30 g of sucrose and making final volume of 1L (Table 3.1). The pH of the medium was then adjusted to 5.8 by adding 1 N NaOH/HCl using pH meter (Mettler-Toledo AG, Switzerland). Agar (8 g/l) was added as a gelling agent in the medium after heating the media on hot plate to around 70 °C to avoid clump formation. The mixture was then cooled at room temperature for few minutes and about 50 ml of media was poured in 500 ml jam jar. After pouring, the culture vessels were sealed with polyurathene caps. The sterilization of culture media was done by autoclaving at 121 °C temperature, 15 psi pressure for 20 min.

3.2.2 Raising of spindle leaf roll segments

The spindle leaf roll segments of sugarcane genotype Co238 were extracted by hand cuttings (Fig. 3.2). The segments were washed with 1 % Tween-20 for 5 min. Then they were washed with sterile water for removal of detergent. Subsequently, they were treated with 10 gL^{-1} Bavistin and kept on rotary shaker (New Brunswick Scientific, USA) at 150 rpm for 30 minutes and 0.1 % $HgCl_2$ for 9-10 min. Then three washings of sterile water were given to explants. The laminar airflow cabinet was sterilized with UV rays for 20 min, followed by wiping with cotton soaked in spirit. The forceps and scalpel were dipped in absolute alcohol and flamed briefly on Bunsen burner placed inside laminar air flow cabinet and cooled before using for inoculation. The culture vessels were cleaned with spirit before

Fig. 3.2 Sugarcane genotype Co238 explants

keeping them on laminar airflow cabinet and their rims were slightly flamed before and after every inoculation. The sterile ex-plants were then put on basal MS media in jam jars and incubated at 25 ± 2° C under a 16 h photoperiod for generation of shoots. Pre-culture was done on MS and NAA (5 ppm) and Kin (0.5 ppm) and agar (0.8%) for 96 h.

3.3 Maintenance of gene construct

pBI 121 vector containing *TaVAP* gene (Accession number = DQ054792) was mobilized in *Agrobacterium* strain EHA 105 under the control of CaMV 35S promoter (Fig. 3.3). Streaked plates of pBI121 were procured from Dr. Prabhjeet lab, Guru Nanak dev University, Amritsar. The *Agrobacterium* culture carrying the gene construct was grown on Luria Bertani (LB) medium (Table 3.2) supplemented with kanamycin (50 mg/l) and streptomycin (50 mg/l). The media was then autoclaved and different antibiotics were added to the lukewarm media in the laminar flow. Following is the procedure of preparing Kanamycin and streptomycin stock solutions:

Kanamycin: A stock solution of 50 ppm concentration was made by dissolving 500 mg of kanamycin sulphate (HiMedia, India) in 10 ml of distilled water. The solution was filter sterilised using 0.2 m nylon membrane filters attached on a syringe and poured into eppendorf tubes, which were then stored at -20 °C.

Streptomycin: A 50 ppm concentration of stock solution was made by dissolving 500 mg of streptomycin (HiMedia, India) in 10 ml of distilled water. After filter sterilisation with a 0.2 m nylon syringe filter, the solution was poured into eppendorf tubes and stored at -20 °C.

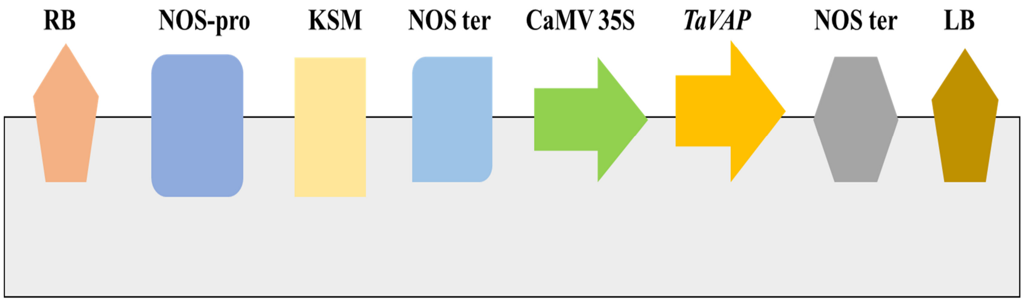

Fig. 3.3 T-DNA region of binary vector pBI121

Table 3.2 Composition of LB medium (pH7.0)

Components	**Amount (g/L)**
Trypton	10.0
Yeast extract	5.0
NaCl	10.0
Agar	14.0

3.4 Isolation of plasmid DNA

The EHA105 *Agrobacterium* strain was streaked on solid LB medium carrying the plasmid. For 48 hours, the temp. used to kept streaked plates was 28°C. For primary culture generation, an isolated colony was picked and was suspended in 10 ml liquid LB media which was supplemented with 50 mgl-1 conc. of kanamycin and then incubated at temp. of 28 °C for 24 hours at continuous shaking of 200 rpm. This primary culture was used as inoculum for making secondary culture. For this, some amount of primary culture was picked and transferred to 100 ml of fresh liquid LB media which contains antibiotics resistant to desired plasmid. This culture was then incubated at 28 °C for 24 hours at constant shaking of 200 rpm. After incubation for 48 h, isolation of plasmid DNA was done from the broth by 'alkaline lysis method' (Birnboim and Doly 1979) with some minor modifications and as given below:

1. In eppendorf tube, 1.5 ml of culture was poured. Then centrifuged at maximum speed (14000 rpm) for 5 min at 4°C in microcentrifuge tube. Store unused culture medium.
2. After centrifugation, medium was removed by aspiration and leaving pellet behind.
3. Then resuspension of pellet was done in 100 microlitre of ice cold solution I (Resuspension Buffer) (Table 3.3) by vigorous vortexing.
4. Then 200 µl of freshly prepared solution II (Lysis buffer) (Table 3.4) was mixed to each tube of bacterial suspension. Tightly tubes were closed and contents were used to mix gently by inverting tubes for 4-5 times. Here, vortexing was not performed. Tubes were stored on ice.
5. Then addition of 150 µl ice cold solution III (Neutralization Buffer) (Table 3.5) was done. Gently mix the contents and stored on ice for 10 min.
6. After time period of 10 min, lysate was kept on centrifugation at max speed at 4°C for 15 minutes.
7. Centrifugation was performed again for 15 min at 4 °C.

8. The retained supernatant was put in fresh tube and 2 volume conc. of isopropanol was mixed. The contents were allowed to stand for 15 minutes of time.
9. The nucleic acid (precipitated) was centrifuged at maximum speed of rpm for 5 min at room temperature.
10. After this, addition of 70 % conc. of ethanol (1 ml) was performed. Recovery of DNA was done by centrifuging it at maximum rpm for 2 min at 4 degree Celcius
11. Removal of supernatent by aspiration and then tubes were kept open for some sort of time for evaporation of ethanol.
12. The pellet was liquefying in 50 µl conc. of Tris EDTA buffer.

Table3.3 Resuspension Buffer (Autoclaving at 121°C temp. at 15 psi)

Components	Quantity
Tris HCL (25 mM) (pH 8.0)	605 mg
EDTA (10 mM) (pH 8.0)	744 mg
Glucose (50 mM)	1800 mg

Table3.4 Lysis Buffer

Components	Quantity
NaOH (10 N)	0.2 N
SDS	1 %

Table3.5 Neutralization Buffer (Autoclave at 121°C temp. at 15 psi)

Components	Quantity
potassium acetate (5 m) (pH 5.5)	60.00 ml
Double distilled water	28.50 ml
Glacial acetic acid	11.50 ml

3.5 Gene construct verification in plasmid through analysis of PCR

The plasmid vector was isolated and then *TaVAP* gene was verified using PCR with *TaVAP* specific primers (Table 3.6) (Table 3.7).

Table3.6 Nucleotide sequence of *TaVAP* primers

Open reading frame	Primer	Sequence (5'–3')	Annealing temperature	Expected amplicon size (bp)
TaVAP	Forward F	TCTAGAATGAGCGCGG GAAGCGCGA	65.9	717
	Reverse R	GGATCCCTATGTCTTCT TGAGGATGAA	60.8	

Table3.7 PCR reaction mixture (10 µl)

Components	Volume
Master mix	5 µl
100 ng template DNA	2 µl
Forward primer	1 µl
Reverse primer	1 µl
Sterile water	1 µl
Total	10 µl

Amplification was done in a PCR thermocycler machine (Mastercycler Gradient-eppendorf^{TM}, Germany) by running it on following program:

Process	Temp.(°C)	Time(min.)
Initial denaturation	95	05:00
Final denaturation	95	01:00
Annealing	60	00:45
Initial extension	72	01:00
Final extension	72	07:00
Storage	4	∞

3.5.1 PCR product analysis

The obtained PCR product used to analysed on 1.5 % agarose gel. 1.5 g of agarose (MP Biomedical, USA) was dissolved in 100 ml of 1X TBE buffer to make the 1.5 percent agarose gel (Table 3.8). The mixture was heated until the agarose was entirely dissolved, or until the solution was clear. After cooling the mixture at 60 °C, six µl of EtBr (10 µg/ml) were added at a final conc. of 0.5 µg/ml of buffer. Then solution was used to put on a casting tray of gel and allowed to solidify at room temperature for period of 30-40 minutes. The comb was separated after solidification. Each PCR product (10 µl), together with a standard 1 kb plus DNA ladder, was loaded into individual wells. (SMOBIO Ladder). The amplicons were determined by running the gel at 100 V for 1 h. The gel bands were visualized in the presence of UV light and photographed by the help of photo gel documentation system (Avegene, USA).

Table 3.8 Preparation of TBE solution

Components	Conc.
Tris Base	45mM
Boric Acid	45mM
EDTA	1mM

3.6 Genetic transformation of Sugarcane

3.6.1 Raising of plasmid broth for agroinfection

The *Agrobacterium* culture co0ntaining the desired gene in its plasmid was maintained on solid LB-agar petri plates. A colony was picked and were dipped in 50 ml of liquid LB medium containing kanamycin (50 mg/l) (Table 3.9). The inoculated media was then incubated at 28 °C with 230 rpm shaking on a rotary shaker until the culture reached A_{600}=1-1.2. The culture was pelleted at density of 5,000 rpm and the pellet was then resuspended in 50ml of MS basal media (pH 5.6) to achieve the final optimum density of 5 x10^8 cells/ml (A_{600}=1). To this 50 µM of acetosyringone (HiMedia) was added.

Preparation of Acetosyringone solution

The acetosyringone stock solution was made by dissolving 196.2 mg acetosyringone salt in some drops of ethanol and then volume was raised to 10 ml. Filter sterilization of stock solution was done with the help of nylon membrane (0.2 µm) (syringe mounted) and stored at 4 °C.

3.6.2 Agroinfection of explants and cocultivation

Genetic transformation experiment was carried out using *In-vitro Agrobacterium*-mediated transformation. The explants were pre-cultured for 96 hours in pre-culture media and then explants were immersed in *Agrobacterium* culture for 10-25 min in different experiments. After infection, the segments were blot dried on autoclaved Whatman no.1 filter paper and were subsequently transferred to cocultivation media (MS basal). The cocultivation was carried out for 72 hours under dark conditions at 25 ± 2 °C. Agro-infected spindles were washed after co-cultivation with autoclaved distilled water supplemented with cefotaxime solution (500 mg/l) (HiMedia, India) and transferred on regeneration media on MS and NAA (5mg/l) and Kin (0.5 mg/l) and agar (8g/l) and cefotaxime (500 mg/l) (Table 3.9). The cultures were maintained under 16 h photoperiod at 25 ± 2 °C.

Preparation of cefotaxime solution

The cefotaxime stock solution was made by dissolving one gram of cefotaxime in few drops of sterile water and volume was raised to 4 ml (250mg/ml). This stock solution was then filter sterilized with the help of nylon membrane (syringe mounted) (0.2 µm) and stored at 4 °C.

Preparation of Timentin solution

The timentin stock solution was made by dissolving 2 g of timentin salt in 10 millilitre of sterile water and then stock was sterilized through the 0.2 µm syringe filter and kept on storage at 4°C for further use.

3.7 Shoot regeneration and elongation

The agroinfected explants cultured on MS containing NAA (5mg/l) and Kin (0.5 mg/l) and agar (8g/l) and cefotaxime (500 mg/l) regeneration media designated as M1. Second shoot regeneration media (M2) contained MS + 3 mg/L NAA + 0.5 mg/L Kinetin + 30 g/l sucrose + 8 g/l agar. Third media (M3) contained MS supplemented with 7 mg/l NAA + 1 mg/l Kinetin + 30 g/l sucrose + 8 g/l agar. They were regularly subcultured on fresh media after 15-20 days (Table 3.9). The agroinfected spindles developed shoots after 40-45 days. The properly emerged shoots were then transferred to their respective media. In the process of elongation the explants were regularly transferred to fresh media after every 15-20 days.

3.8 *In-vitro* root induction and hardening of the regenerated explants

When the elongated shoots reached the height of 4-5 cm, they were transferred to MS supplemented with NAA (5mg/l) and Kin (0.5 mg/l) and agar (8 g/l) and cefotaxime (500 mg/l) designated as M1 and MS supplemented with 0.5 mg/L IAA and 0.5 mg/L IBA and 0.5 mg/L BAP and 30 g/l sucrose and 8 g/l agar designated as M4 for root formation. After 10-15 days, plantlets with roots were hardened by thoroughly washing in normal water and then placing them into jars containing water soaked cotton. The jars were incubated for 8-10 days in incubation room and covered with polythene in order to retain moisture.

3.9 Preparation of different growth regulators

Various growth regulators like Kinetin, IAA, IBA, BAP and NAA were used in different media. The stock solutions of these growth regulators were prepared by dissolving 50 mg of each growth regulator in some volume of 1N NaOH and then raising final volume to 50 ml. Distilled water was added gently for avoiding of precipitation. Growth regulators were prepared fresh every time whenever we used.

Table 3.9 Media used for genetic transformation of sugarcane

Type of medium	Basal medium	Kinetin (mg/l)	NAA (mg/l)	Sucrose (g/l)	Acetosyringone (mg/l)	Cefotaxime (mg/l)	Kanamycin (mg/l)
Pre-culturing	MS	0.5	5	30	-	-	-
Co-cultivation medium	MS	0.5	5	30	9.8	-	30
Regeneration medium	MS	0.5	5	30	-	500	30
Rooting medium	MS	0.5	5	30	-	500	

3.10 Transfer of plantlets to soil

The hardened plantlets were first put in polythene bags which are containing field soil and FYM (3:1) and then to earthen pots. The plants were then kept in glass house, where 30 °C temp. and 80% RH was maintained. Plants were used to watered two times during summer season and once during winter season. They were not given any fertilizer application.

3.11 Characterization of putative transgenic plants

3.11.1 Genomic DNA isolation of regenerated transformed plants

Genomic DNA was extracted from the leafs of *in-vitro* grown plants using CTAB extraction procedure (Saghai-Maroof *et al 1984*):

1. Young leaves were taken from well grown putative transgenic sugarcane plants.
2. The leaves used to grinded in liquid N2 with pestle and morter to form a fine powder. The sample was placed in centrifuge tubes.
3. Addition of 800 µl of CTAB buffer containing 1 % mercaptoethanol) was done. (Table 3.10)
4. The sample tubes were kept at 65°C for nearly 1 hour in the water bath.
5. 800 microlitre of phenol:chloroform:isoamyl (25:24:1) was mixed after incubation and put at shaker for 40 minutes of time at 60 rpm rotation.
6. Centrifugation of samples was done at about 10000 rpm rotation for 10-15 min.
7. The supernatant (with pippette) was disparate from the tissue in a new centrifuge tube
8. The 10 µl of RNase was added to each tube and incubating at temp. of 37 °C for 28-35 minutes.
9. 800 µl conc. of chilled isopropanol was used to add in this solution and mixed with the help of some gentle inversions. Samples were kept at -20 °C for 15 min.
10. The tubes were then centrifuged at 10,000 rpm for 15 min.
11. The pellet had rinsed with 70% ethanol after the supernatant was discarded.
12. By inverting tubes on clean filter paper, the pellet was air dried and dissolved in 100 µl of TE (Table 3.11) buffer.
13. Storage of DNA samples were done at 4 °C overnight
14. After samples were properly dissolved, then placed samples at -20 °C.

Table 3.10 Preparation of CTAB Buffer

Components	Conc.
CTAB	2%
NaCl	1.4M
Tris HCL	100mM
EDTA	20mM
Sodium bisulphate	0.5%

Table 3.11 Preparation of TE Buffer

Components	Conc.
Tris HCL	10mM
EDTA	1mM

3.11.2 PCR analysis

Genomic DNA isolated from regenerated plants was analysed for the confirmation of *TaVAP* gene by PCR using *TaVAP* specific primers (Table 2.6). A PCR reaction of 10 µl was made (Table 3.12)

Table 3.12 PCR rxn mixture (10 µl)

Components	Volume
Master mix	5 µl
100 ng template DNA	2 µl
Forward primer	1 µl
Reverse primer	1 µl
Sterile water	1 µl
Total	10 µl

The PCR was performed using amplification profile given above. The amplified product so obtained was visualized on 1 % agarose gel.

CHAPTER IV

RESULTS AND DISCUSSION

The present investigation was carried out on “DEVELOPMENT OF WATER USE EFFICIENT TRANSGENIC SUGARCANE THROUGH EXPRESSION OF WHEAT *TaVAP* TRANSGENE”. This chapter describes results of the experiments conducted towards achieving the objectives. The results are discussed under following sub headings:

4.1Introduction of wheat *TaVAP* in sugarcane through *Agrobacterium* mediated transformation

4.1.1 *TaVAP* gene in *Agrobacterium* strain EHA 105

4.1.2 Plasmid verification of *TaVAP* gene by colony PCR

4.1.3 Regeneration of transgenic explants

4.1.4 Estimation of number of shoots per explant, shoot length, rooting percentage and root length

4.2 Characterization of putative transgenic plants

4.2.1 Genomic DNA extraction and its quantification

4.2.2 PCR analysis of putative transgenics for *TaVAP* gene

4.1Introduction of wheat *TaVAP* in sugarcane through *Agrobacterium* mediated transformation

In the present study, sugarcane was transformed through in vitro *Agrobacterium*-mediated genetic transformation using *TaVAP* gene

4.1.1 *TaVAP* gene in *Agrobacterium* strain *EHA* 105

pBI vector containing *TaVAP* gene was mobilized into *Agrobacterium* strain EHA 105 (Fig 4.1).

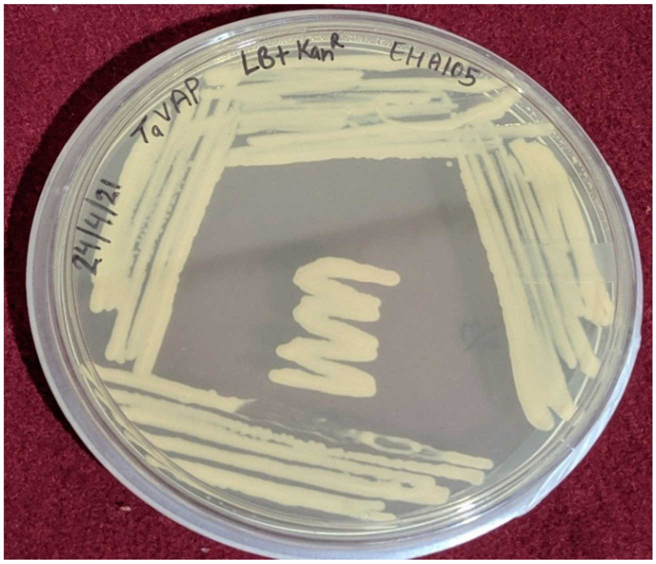

Fig. 4.1 *TaVAP* gene in *Agrobacterium* strain EHA 105

4.1.2 Plasmid verification of *TaVAP* gene by colony PCR

Colony PCR was performed. The amplification of transgene, i.e. *TaVAP* was carried out using gene specific primers (Table 3.6). A fragment of 717 bp on 1.5% agarose gel confirmed the gene of interest in plasmid as shown in Fig 4.2

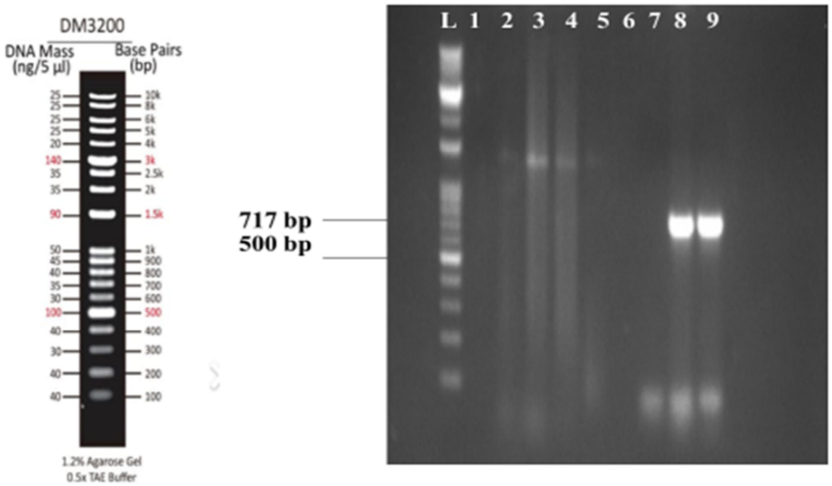

Fig. 4.2 Plasmid verification of *TaVAP* gene

4.1.3 Regeneration of transgenic explants

First explants were pre-cultured for 96 hours. Explants were then agro-infected and put on co-cultivation medium for 72 hours. Agro-infected explants were then transferred to shoot regeneration media containing cefotaxime (500 mg/l) (Fig. 4.3). Then regenerated shoots (Fig. 4.4) were rooted on elongation media. Total 58 shoot clusters were regenerated (Table 4.1). Further 58 shoot clusters were put on hardening and 80 individual plantlets were transferred to soil in glass house (Fig 4.5). Out of these 80 plantlets, only 38 plantlets were survived (Table 4.1).

Pre-culturing sugarcane spindle leaf roll segments

After agro-infection

After co-cultivation

Shoot regeneration and root induction

Hardening of plantlets

Transfer of plants to glass house

Fig. 4.3 Diagrammatic representation of Co238 sugarcane transformation

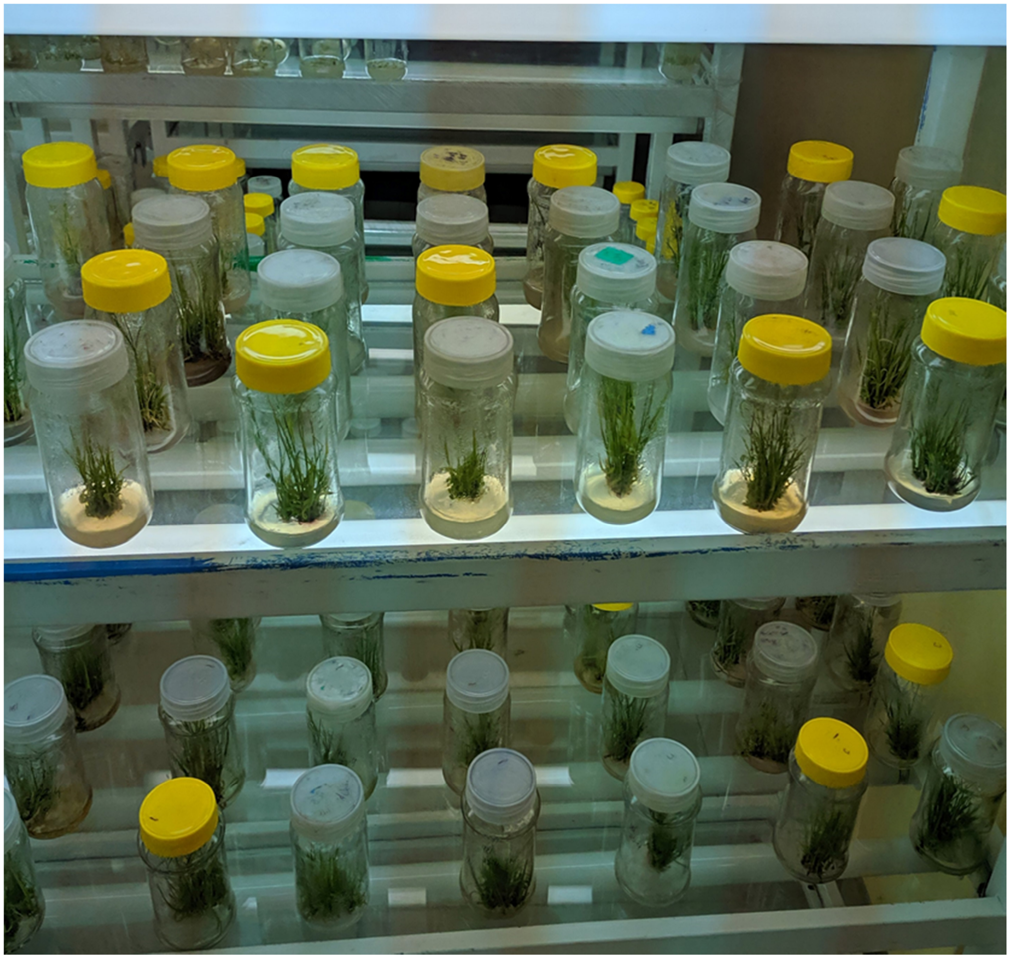

Fig 4.4 Regenerated sugarcane explants

Fig. 4.5 Plants transferred to glass house

Table 4.1 No. of Regenerated plants

Experiment No.	No. of Pre-cultured explants	Culture O.D	No. of Agro-infected and co-cultivated plants	No. of plants showing regeneration	No. of shoot clusters put on hardening	No. of plants transferred to soil
1.	10	0	0	0	0	0
2.	50	1.3	37	0	0	0
3.	30	1.0-1.2	25	0	0	0
4.	28	1.0-1.2	0	0	0	0
5.	33	1.0-1.2	12	0	0	0
6.	20	0.8-1.0	13	0	0	0
7.	55	1.0-1.2	40	0	0	0
8.	57	1.0-1.2	53	0	0	0
9.	65	1.0-1.2	47	5	5	8
10.	47	1.0-1.2	0	0	0	0
11.	52	1.0-1.2	39	0	0	0
12.	64	1.0-1.2	34	3	3	7
13.	55	1.0-1.2	48	4	9	6
14.	56	1.0-1.2	0	0	0	0
15.	60	1.0-1.2	24	0	0	0
16.	63	1.0-1.2	37	7	7	12
17.	48	1.0-1.2	48	0	0	0
18.	41	1.0-1.2	34	3	3	8
19.	63	1.0-1.2	42	0	0	0
20.	61	0.8-1.0	56	8	0	2
21.	65	1.0-1.2	40	4	4	5
22.	74	1.0-1.2	63	6	8	8
23.	62	1.0-1.2	46	0	0	0

Experiment No.	No. of Pre-cultured explants	Culture O.D	No. of Agro-infected and co-cultivated plants	No. of plants showing regeneration	No. of shoot clusters put on hardening	No. of plants transferred to soil
24.	35	1.0-1.2	16	0	0	0
25.	66	1.0-1.2	41	0	0	0
26.	84	1.0-1.2	72	8	8	10
27.	72	1.0-1.2	0	0	0	0
28.	66	1.0-1.2	40	0	0	0
29.	30	1.0-1.2	27	0	0	0
30.	42	1.0-1.2	37	2	2	4
31.	48	1.0-1.2	48	0	0	0
32.	41	1.0-1.2	34	3	3	5
33.	37	1.0-1.2	32	0	0	0
34.	51	0.8-1.0	46	0	0	0
35.	65	1.0-1.2	40	0	0	0
36.	82	1.0-1.2	63	5	5	5
37.	72	1.0-1.2	46	0	0	0
38.	35	1.0-1.2	16	0	0	0
39.	68	1.1-1.3	50	0	0	0
Total	2053	-	1346	58	57	80

Out of total 2053 cultured explants, 1346 were agroinfected. Out of these 1346 agro-infected explants, 887 were transferred to regeneration media. Only 58 shoot clusters showed regeneration. 57 shoot clusters were put on hardening and 80 individual plantlets further transferred to soil (Table 4.1). There was 2.82% plant regeneration and 1.85% survived plantlets.

Kumar *et al* (2013) reported the regeneration frequency of 77.5% using MS media containing 1mg/l kinetin, 1mg/l BAP, 1mg/l GA3, 0.25mg/l NAA, 50 µM acetosyringone, 500mg/l cefotaxime and 150 mg /l kanamycin on 72 hours of co-cultivation.

4.1.4 Estimation of number of shoots per explant, shoot length, rooting percentage and root length

The leaf roll segments showed unwhorling after 5-10 days of incubation. The unwhorling was observed on sixth day of incubation on M1 medium, on ninth day of incubation on M2 medium and on seventh day of incubation on M3 medium. The direct shoot regeneration was observed from the cut ends after third week of incubation in cluster of 2-3 shoots (Fig. E). The shoots were excised from the explants and subcultured on same media for another passage. The shoot proliferation increased in the clusters after 2 weeks of incubation (Fig. F, G). After six weeks of culture initiation, data for no. of shoots per explant and length of shoots were recorded. The number of shoots per explant were maximum on M1 (7.68 ± 0.86) as compared to M2 (6.04 ± 0.54) and M3 (4.5 ± 0.33). Maximum mean shoot length (cm) was recorded in M1 (8.13 ± 1.15) followed by MS2 (6.19 ± 0.74) and M3 (4.78 ± 0.47). The elongated shoots clumps were then rooted on M1 and M4 media. Roots were observed after two weeks of incubation. Rooting percentage was maximum on M1 (100%) followed by M4 (96.43%). Root length was maximum in M1 (3.8 ± 0.42 cm) followed by M4 (2.9 ± 0.17 cm).

Fig 4.6 Explant unwhorling and regeneration

Table 4.2 Effect of media on no. of shoots per explant, shoot length

M1	Shoot no.	Shoot length (cm)	M2	Shoot no.	Shoot length (cm)	M3	Shoot no.	Shoot length (cm)
1	8	7.1	1	5	6.3	1	5	5.6
2	7	7.5	2	7	7.2	2	4	5.4
3	7	7.4	3	6	6.5	3	4	4.2
4	8	10.4	4	5	6.7	4	5	4.5
5	9	9.9	5	6	6.3	5	4	4.1
6	7	7.2	6	7	5.4	6	5	5.2
7	8	7.3	7	6	6.8	7	5	5.3
8	7	7.8	8	6	6.1	8	5	4.6
9	9	9.6	9	6	7.4	9	4	5.2

M1	**Shoot no.**	**Shoot length (cm)**	**M2**	**Shoot no.**	**Shoot length (cm)**	**M3**	**Shoot no.**	**Shoot length (cm)**
10	7	7.1	10	7	6.5	10	5	4.9
11	7	8.2	11	7	4.4	11	5	4.3
12	7	6.2	12	6	5.3	12	4	4.4
13	8	8.2	13	6	6.6	13	4	4.4
14	9	9.1	14	6	6.3	14	4	4.9
15	8	9.9	15	6	6.3	15	5	4.3
16	9	7.6	16	6	6.4	16	4	4.8
17	6	7.8	17	6	6.1	17	4	5.3
18	7	7.6	18	6	5.5	18	5	4.5
19	8	8.6	19	6	7.2		**4.5±0.51**	**4.78±0.47**
	7.68±0.88	**8.13±1.15**	20	6	5.4			
			21	5	5.3			
				6.04±0.59	**6.19±0.74**			

Table 4.3 Effect of media on root length

M1	**Root length**	**M4**	**Root length**
1	3.4	1	2.8
2	4.1	2	3.1
3	3.4	3	2.6
4	3.7	4	3.2
5	3.9	5	3.1
6	4.1	6	3.1
7	3.5	7	2.9
8	3.6	8	3.1
9	4.3	9	2.9
10	3.7	10	2.7
11	4.2	11	2.9
12	3.5	12	2.8
13	3.7	13	3
14	4.6	14	2.7
15	4.2	15	2.7
16	3.6	16	2.9
17	4.2	17	2.8
18	4.1	18	2.7
19	3.4	19	2.8
20	3.7	20	2.7

M1	Root length	M4	Root length
21	4.8	21	3
22	4.4	22	2.8
23	3.1	23	3.2
24	3.6	24	2.8
25	4.3	25	3.1
26	3.4	26	3.1
27	3.5	27	2.9
28	4.2	28	2.8
29	3.7		**2.9±0.17**
	3.8±0.42		

4.2 Characterization of putative transgenic plants

4.2.1 Genomic DNA extraction and its quantification

A total of 38 putative transgenic plants were generated. Genomic DNA was extracted using CTAB method and then quantification was done on NANODROP instrument (Table 4.1).

Table 4.4 DNA Quantification on NANODROP

Sample No.	Conc. (ng/µl)	Sample No.	Conc. (ng/µl)
1	1694	20.	1731
2.	1406	21.	1873
3.	1172	22.	1499
4.	1371	23.	1855
5.	1711	24.	1389
6.	1739	25.	1160
7.	2446	26.	1076
8.	1651	27.	1135
9.	1636	28.	1213
10.	898.5	29..	1621
11.	1705	30.	1797
12.	1511	31.	1521
13.	1386	32.	1001
14.	600.43	33.	913.3
15.	1196	34.	1767
16.	1889	35.	1505
17.	1240	36.	1212
18.	1421	37.	1770
19.	1253	38.	1794

4.2.2 PCR analysis of putative transgenics for *TaVAP* gene

The putative transformed sugarcane plants were analyzed by PCR using *TaVAP* gene specific primers (Table 3.6). Genomic DNA of 38 putative transgenic plants of T_0 generation of sugarcane variety Co238 was extracted using modified CTAB method of DNA extraction. PCR amplification was performed using *TaVAP* gene specific primers. During PCR analysis, plasmid DNA harbouring *TaVAP* gene sequence was used as positive control. Thus, out of 38 putative transgenic plants, 0 plants showed the presence of *TaVAP* gene (Fig. 4.6,4.7 and 4.8) (Table 4.3). To verify the genomic DNA and PCR reaction, further PCR amplification was also carried out using actin as a primer (Fig. 4.9). The plants showed amplification when we used actin as a primer. Hence we verified genomic DNA and PCR reaction.

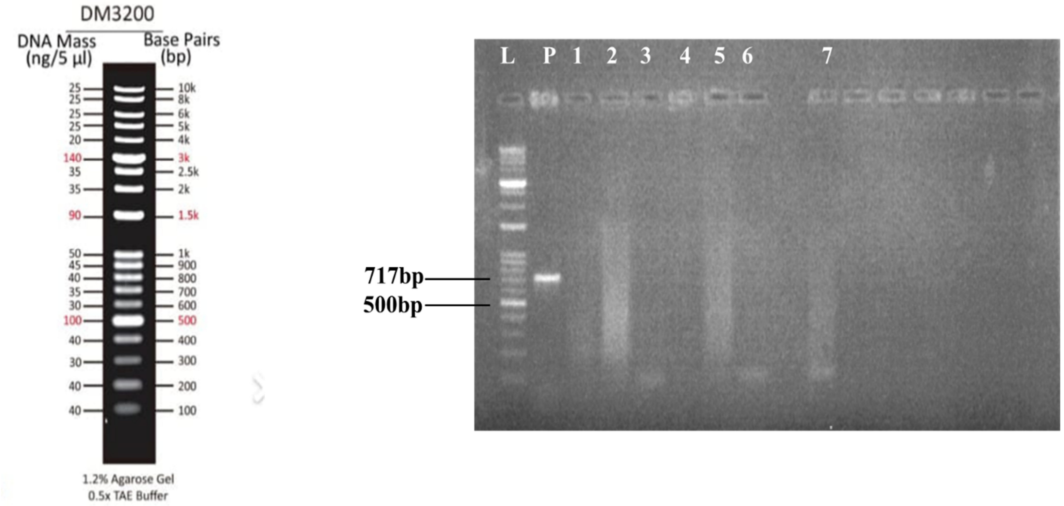

Fig. 4.7 PCR verification of 7 putative transgenic sugarcane plants

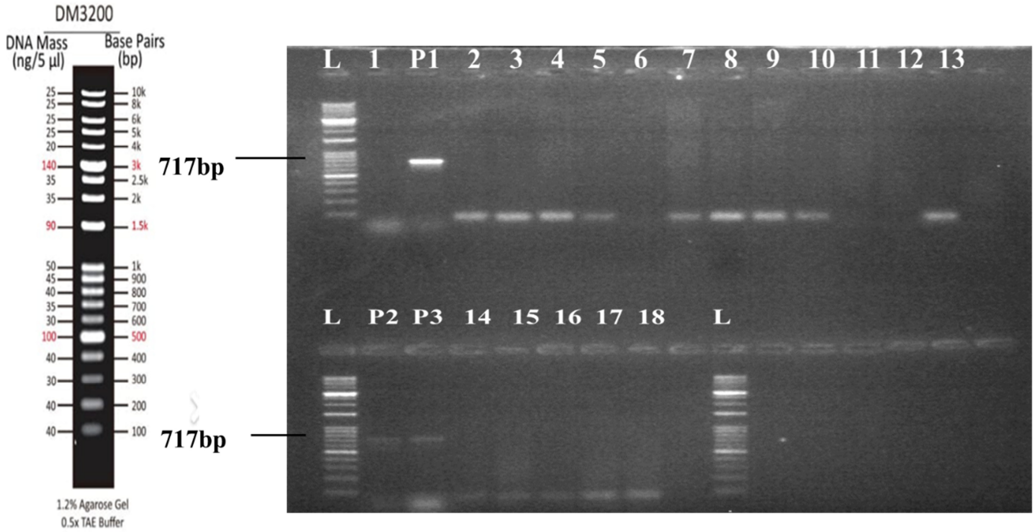

Fig. 4.8 PCR verification of 18 putative transgenic sugarcane plants

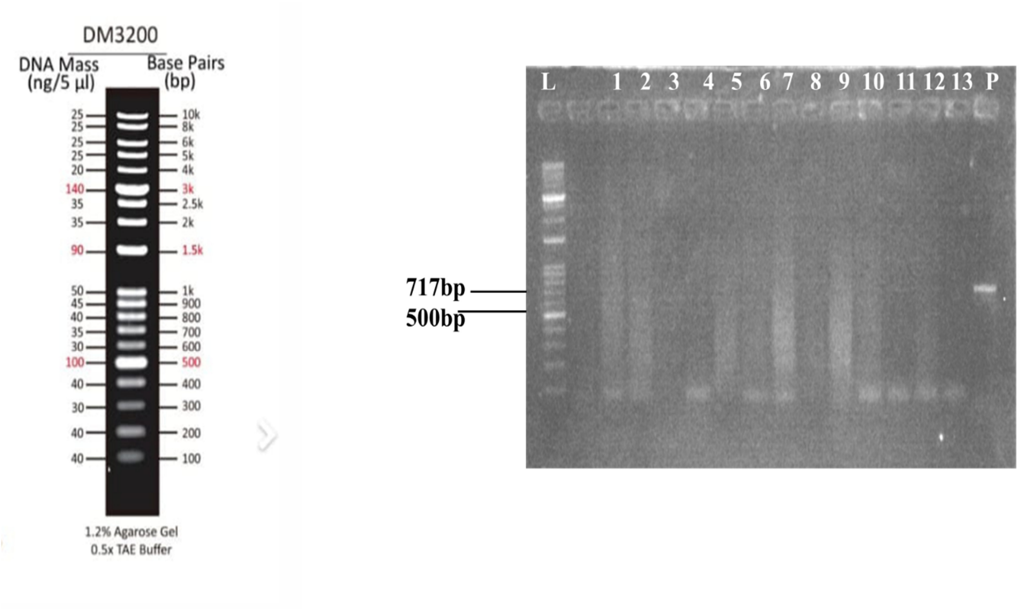

Fig. 4.9 PCR verification of 13 putative transgenic sugarcane plants

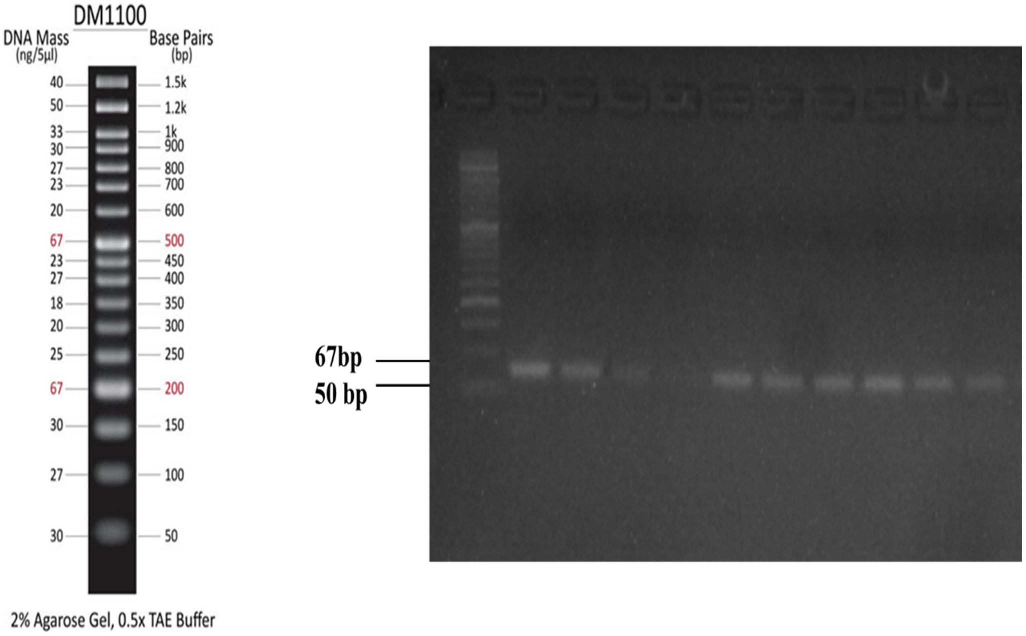

Fig. 4.10 PCR verification of putative plants using actin as a primer

There was absence of *TaVAP* gene in these 38 putative transgenic plants. This may be due to incompatibility of *Agrobacterium* strain EHA 105 with sugarcane genotype Co238.

Kalunke *et al* (2009) achieved the transformation efficiency of 28% which were containing gene *Cry1 Aa3*. CoC671 variety of sugarcane was used. Explants were raised on MS medium which was supplemented with 3% sucrose, 0.6mg/l 2,4-

D, 100mg/l PVP, 500mg/l CH and 0.8% agar. Regenerated shoots were raised on same medium and further transferred to MS medium + 2mg/l NAA for initiation of roots. Transformation vector pBinAR-*Cry1Aa3* was used which was mobilized into *Agrobacterium* strain EHA105 under the effect of CaMV 35S promoter. Out of 44 putative transgenic plants, 20% plants were PCR positive.

Zhang *et al* (2006) reported 30% of PCR positive plants. First they incubated commercial sugarcane variety on M1 medium (MS + 1mg/L 2,4-D) for callus induction, Then they transferred callus into M2 medium (MS + 1mg/L 6-BA + 0.5 mg/L KT) for regeneration of shoots and then were placed on M3 medium (1/2 MS + 1mg/L IAA) for root induction. pBBBT vector containing *TSase* was utilized which was inserted into *Agrobacterium* strain EHA105 using triparental mating.

Wang *et al* (2009) estimated the transformation rate of 10.5 % in putative transgenic sugarcane plants. In this experiment, they use the variety ROC22. They constructed the plasmid pBI-*antiaco* and pBI-*aco*, and further mobilized into *Agrobacterium* strain EHA 105. For callusing, M1 medium (MS + 1 mg/l 2,4-D) was used. For shoot regeneration, they used M2 medium (MS + 1.0 mg/l 6-BA + 0.5 mg/l KT). Then shoots were rooted in M3 medium (MS + 1 mg/l IAA). PCR analysis revealed that 2 plants were PCR positive out of 19 plants.

Khan *et al* (2013) achieved the 31.6% regeneration efficiency and 30% transformation efficacy. They used sugarcane shoot tips for culturing. EHA 101 *Agrobacterium* strain was used which containing plasmid pIG121Hm. First, explants were cultured on MS medium supplemented with Gibberellic acid (GA3) + Kinetin (kin). Then explants were co-cultivated on basal MS medium + AA (amino acid) + 50 µM acetosyringone. For pre-selection, MS + 1000mg/L cefotaxime + 50 mg/L hygromycin and for selection, MS + 1000mg/L cefotaxime + 50 mg/L hygromycin + 0.1 mg/L GA3 + 1.0 mg/L BAP + 0.5 mg/L IBA were used during experiments of transformation. 4 plants were PCR positive out of 13 putative transgenic plants.

Sandhu *et al* (2016) achieved maximum transformation efficiency of 1.65 % in sugarcane using *β-1,3-glucanase* gene specific primers. CoJ 83 variety of sugarcane was used. The spindle leaf roll segments were precultured on solid MS medium + NAA (5 mg/l) + Kinetin (0.5 mg/l). Cultures were agro-infected and placed on co-cultivation medium containing basal MS medium + 9.8 mg/l acetosyringone for 72 hours. Agro-infected explants were cultured on regeneration media (MS + 5.0 mg/l NAA + 0.5 mg/l kinetin + 500 mg/l cefotaxime) and rooted on same media for three weeks. pBI plasmid was constructed which was containing *β-1,3-glucanase* and CaMV 35S promoter was used as control. pBI- *β-1,3-glucanase* cassette was mobilized into *Agrobacterium tumefacians* strain LBA 4404. 12 plants out of 1019 cultured plants were PCR positive.

Dessoky *et al* (2020) reported 22.2% transformation efficiency in sugarcane using *cry1 Ac* gene specific primers. Sugarcane variety GT 54-9 was used. Cultures were transferred to co-cultivation medium (MS + 2 mg/l NAA + 1 mg/l BAP) and then placed on selection media containing solid MS medium supplemented with 300 mg/l carbenicillin and 100mg/L kanamycin. Shoot elongation was done on MS medium + 0.1 mg/l BAP and 2 mg/l kinetin. Then shoots were rooted on MS medium + 2 mg/l NAA. pART-*cry1Ac* plasmid was used which was mobilized into *Agrobacterium tumefacians* strain GV 1303 and CaMV 35S promoter was used as control. Out of 90 plants, 20 were PCR positive.

Gao *et al* (2018) revealed transformation efficiency of 47.6 % out of putative transgenic plants. Sugarcane cultivar ROC 22 was used. They used the recombinant plasmid pGcry-*cry 2A*. Transformation was carried out by particle bombardment method. First, they induced callusing on MS medium + 3.0 mg/L 2,4-D + 30 g/L sucrose + 6 g/L agar and then bombardment was done using tungsten particles. Sub-culturing was done on MS medium + 2.0 mg/L 2,4-D + 30 g/L sucrose + 6 g/L agar. In differential medium, MS medium + 1.5 mg/L 6-BA + 1.0 mg/L Kinetin + 0.2 mg/L NAA + 30 g/L sucrose + 6 g/L agar was used. Then plantlets were rooted on ½ MS + 0.2 mg/L 6-BA + 3.0 mg/L NAA + 60 g/L sucrose + 5.5 g/L agar. Out of 21 regenerated putative transgenic plants, 10 were PCR positive.

Rani *et al* (2012) achieved transformation efficiency of 2.04% in sugarcane out of putative plants using *Osgly II* gene specific primers. CoJ 83 sugarcane cultivar was employed. Cultures were initially placed on basal MS medium supplemented with 30 g/L sucrose + 4.0 mg/L 2,4-D + 0.5 mg/L kinetin. Transformation was carried out using particle bombardment method. pCAM 1304 – gII plasmid mobilized in *E.coli* strain JM 109 was used. Sugarcane calli were placed on osmoticum MS medium (MS + 0.4 M mannitol) before bombardment. Bombarded calli were cultured on MS medium + 2.5 mg/L 2,4-D + 0.5 mg/L kinetin + 35 mg/L hygromycin for selection. Then well-defined colonies were placed on shoot regeneration medium (MS + 5 mg/L IAA + 0.5 mg/L kinetin + 0.5 mg/L BAP + 335 ppm hygromycin). Shoots were rooted on ½ MS + 7% sucrose. Out of 98 shoots, two shoots were PCR positive.

Wang *et al* (2017) inserted *EPSPS* and *Cry1 Ab* gene into sugarcane for development of insect and herbicide resistant sugarcane. *EPSPS* and *Cry1 Ab* genes were under the control of Ubi-1 promoter. Expression vector was mobilized into *Agrobacterium* strain EHA 105. ROC 22 sugarcane cultivar was used as a source cultivar for embryogenic callus induction. Transverse sections were put on MS + 30 g/L sucrose + 2 mg/l 2,4-D + 8 g/L agar. Then they co-cultivated calli into 1/5 MS + 30 g/L sucrose + 30 g/L glucose + 100 mM acetosyringone. Then infected calli were placed on resting medium (MS + 1.0 mg/L 2,4-D + 30 g/L sucrose + 8 g/L agar + 300 mg/L timentin). Then calli were transferred to selection medium (MS + 2.0 mg/L 2,4-D + 8 g/L agar + 300 mg/l timentin + 5 g sucrose + 8 g mannose). The resistant calli were transferred to regeneration medium (MS + 1.0 mg/L 6-BA + 8 g/L agar + 300mg/L timentin + 5 g sucrose + 8 g mannose). Regenerated calli were elongated on MS + 8 g/L agar + 300mg/L timentin + 5 g sucrose + 8 g mannose. Out of 33 shoots, 32 were PCR positive for *EPSPS* gene and 30 were PCR positive for *Cry1 Ab* gene.

This study clearly indicate that transformation efficiency is dependent upon genotypes, correct combination of growth hormones and vitamins.

CHAPTER V

SUMMARY

Sugarcane (*Saccharum* spp. L.), a commercial cash crop belongs to family Poaceae. It originated in New Guinea and is grown throughout in the tropical and sub-tropical countries especially in Brazil because canes can directly be used to produce ethyl alcohal. Sugarcane can be consumed as raw and also used for production of jaggery, biofuel, medicines, juices and alcohol. The crop accounts for about 85 per cent of world's sugar production and in 2018-19, there was production of 188.25 million metric tonnes (World sugar statistics). India ranks second in production of sugarcane.

The overexpression of *TaVAP* gene maintains high level of proline content, photosynthetic efficiency and antioxidant activity. *TaVAP* is tissue specific and responsive against multiple stresses. The transgenic *Arabidopsis thaliana* showed good survival level under drought and decreased yield loss under moisture deficit conditions (Singh *et al* 2017). So, in the present study, role of *TaVAP* was observed for drought tolerance in sugarcane.

Using the *in vitro* transformation method, putative transgenic material of sugarcane variety Co238 was generated in Tissue Culture and Genetic Transformation laboratories, School of Agricultural Biotechnology, PAU Ludhiana during the year 2018-21. Thus, the present study was proposed to be undertaken on "DEVELOPMENT OF WATER USE EFFICIENT TRANSGENIC SUGARCANE THROUGH EXPRESSION OF WHEAT *TaVAP* TRANSGENE". For this study, firstly the shoots were regenerated after agro-infection. 6.54 % of regeneration was observed. During the study, 38 putative transgenic plants were generated and PCR analysis was carried out of all the putative transgenic plants of sugarcane. PCR reaction was carried out using *TaVAP* gene specific primers which gives an amplicon of 717 bp in 38 putative transgenic plants. 38 putative transgenic plants were analyzed and none of the plants were found to be PCR positive, so transformation efficiency was found to be 0%. This probably might be due to non-compatibility of *Agrobacterium* strain EHA 105 with sugarcane genotype Co238.

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