ABSTRACT

There is an intimate connection between Mulberry and the silkworm. The silkworm or *Bombyx mori* reflects this close relationship with *mori* being derived from *Morus*. The commercially important silk generally refers to mulberry silk. Mulberry silk comes from the silkworm, *Bombyx mori* L. which solely feeds on the leaves of the mulberry plant. Although India is the second largest producer of silk, the difference in the world share is by a huge margin of 45%. There is thus a tremendous scope for increasing silk production in the country. The advancement of sericulture industry depends upon the quantitative and qualitative improvement of mulberry varieties and identifying better varieties. Mulberry leaves being the chief feed for the silkworm have a predominant influence on development of the worm and quality of the cocoon and so is the focal point for most of the studies carried out towards improvement of mulberry varieties. Different abiotic and biotic stresses are responsible for large decreases in crop productivity all over theworld. Drought and salt are the most severe environmental stresses affecting all plant functions, and cause serious problems for plant survival. The main use of mulberry globally is as feed for the silkworm. While nearly 40% of mulberry cultivation is under irrigated land, it is estimated that various abiotic stress conditions such as moisture (drought), salinity and alkalinity, result in 50-60 % yield loss, thereby affecting the economy of the sericulture industry. Mulberry leaf metabolism is severely affected by high temperature, water and salinity stresses. Therefore identification of superior mulberry cultivars with better biomass under stress conditions is extremely important. The identification of suitable mulberry species requires an efficient screening method. Genetic transformation is one of the most reliable and interesting method of introducing desired genes to mulberry. To improve the quality of existing varieties of mulberry, it is necessary to develop varieties which are resistant to different environmental stresses. By genetic transformation of mulberry with desirable genes, abiotic as well as biotic stress tolerant transgenic plants can be developed. Therfore, a collaborative study undertaken at department of Plant Molecular Biology, University of Delhi, South Campus, New Delhi to achieve the following objectives:

1. Screening and expression of three stress inducible genes HAL3a, NHX1 and Dehydrin of Different Genotypes under Simulated Drought and Salinity Stress.

2. Development of Transgenic Mulberry with *Osmotin* Gene under CaMV35S and stress inducible rd29A promoter and their growth performance under different abiotic and biotic stress condition.

3. Overexpression of *β-carotene hydroxylase* in Mulberry and their characterization under different abiotic stress conditions.

Screening of Different Genotypes under Simulated Drought and Salinity Stress

Both drought and salinity are the major osmotic stresses that dramatically limit plant growth and productivity. Mulberry is susceptible to both drought and salinity stresses. Free proline accumulation is one of the main stress characteristics and proline serves as a stress inducer molecule and a good cellular indicator of stress. Proline is a compatible osmolyte and accumulates during various kinds of abiotic stresses. Amongst ten genotypes screened for simulated drought stress, S13 accumulated more proline than other genotypes followed by AR12, S34 and S1, whereas K2 and RFS175 accumulated least proline. When salt stress was given for three hours S34 accumulated 5-fold higher proline than the basal levels followed by RFS175 and K2. Goshoerami, S1 and AR12 accumulated less proline than other genotypes. Relative Water Content (RWC) is a major factor in a plants capability to survive adverse water conditions. AR12 and S13 accumulating more proline under aerial stress also showed more RWC. Similarly, Goshoerami and S1, showed poor RWC and accumulated less proline. So, it can be concluded that there is a positive correlation between proline accumulation and relative water content under water deficit conditions. Any kind of environmental stress also affects the membrane integrity of the plants. Electrolyte leakage is thus used to measure the stress injury to cell membranes. S13 and K2, showed better membrane stability than other genotypes and C776 and C176 showed more susceptibilty to membrane damage under simulated drought stress. Under salt stress, RFS175 and K2 also showed better membrane stability than other

genotypes. K2 performed well both in salt and drought stress. Nitrate reductase activity is also affected in all the genotypes subjected to dehydration and salt stress. C776, DD and S34 showed least reduction in nitrate reductase activity, whereas S1 and RFS 175 were found sensitive to aerial drying stress with reduced nitrate reductase activity. Under salt stress conditions, like drought stress, S34 and DD performed well whereas C176 and Goshoerami showed reduced nitrate reductase activity. Chlorophyll fluorescence parameters (such as F0, Fv/Fm) are an indirect method to evaluate the photosynthetic performance of plants, since photosynthesis is often reduced in plants when exposed to adverse conditions. It also allows indirect study of the different functional levels of photosynthesis. Under simulated drought stress AR12 and Goshoerami were found to have better photosynthetic capacity than other genotypes tested. While C776 and K2 were recorded to possess less photochemical activity, after simulated salt stress, S1 and S34 were found to be resistant to photoinhibition and RFS175 and K2 observed to have least photosynthetic yield. S13, showed better relative water content, more proline content and also better cell membrane stability under simulated drought stress so can be categorized as drought tolerant, whereas RFS175, Goshoerami and S1 grouped into drought sensitive, and other genotypes moderately tolerant to drought. Under salt stress, in can be concluded that S34 was found more resistant with more proline accumulation, less reduction in nitrate activity and better photosynthetic yield value. So, S34, K2, RFS175 and S1 grouped into salt tolerant and among them S34 performed best. Goshoerami and AR12 can be categorized into salt sensitive genotypes.

Expression of four endogenous genes of mulberry HAL3A, dehydrin and *NHX1* were analyzed under simulated drought and salt stress with the same ten genotypes. These genes are reported to be involved in stress tolerance in other crops studied. S13, Goshoerami and AR12 accumulated more transcripts of HAL3A after three hours of aerial drying stress. Under salt stress RFS175, S1 accumulated more HAL3A transcript than other genotypes. Dehydrin belongs to the group II or D-11 family of LEA proteins. It is known to be one of the most abundant plant proteins produced during late embryogenesis or in response to drought, low-temperature, salinity, and ABA. In S1, expression of dehydrin is similar to the basal levels of expression after aerial drying stress but salt stress

up-regulates dehydrin expression. More dehydrin transcripts were found in RFS175, AR12 and K2 after salt stress. NHX-type transporters have a key function in maintenance of cytoplasmic Na^+ homeostasis by mediating a secondary active Na^+/H^+ antiport at the tonoplast for vacuolar Na^+ sequestration. Simulated drought stress did not cause any significant changes in all the genotypes. Whereas, salt stress up-regulates NHX1 in some genotypes, AR12 and S34 accumulated a very high amount of NHX1 after salt stress.

Development of Transgenic Mulberry with *Osmotin* Gene under 35S Promoter

Osmotin and osmotin-like proteins are stress proteins which are classified as members of the plant PR-5 proteins, Besides osmotic stress, osmotin is also induced in response to viral and fungal pathogen infections. Osmotin was amplified from *Nicotiana tabaccum* using *osmotin* specific primers, a band of 750bp was obtained by PCR amplification. *Nicotiana osmotin* shares the highest sequence homology with *osm* of *Solanum phureja* with 91% similarity. The encoded protein shows a good homology to the *osm* protein from other plants. All 16 cysteine residues present in thaumatin-like protein were highly conserved in *Nicotiana osmotin* and in osmotin of other plants. Osmotin was mobilized into pBI121 and used for mulberry transformation. Of the various explants used for co-cultivation, i.e., hypocotyls, cotyledons and leaf callus, it was observed that the leaf callus explants showed best transformation efficiency, i.e. almost 90% as compared to cotyledons (60%) and hypocotyls (20%) on the selection medium. Southern analysis confirmed the integration of osmotin in the mulberry genome, and one to two copies were integrated in the genome of mulberry. Confirmation of expression of *osmotin* gene in the transgenic plants were confirmed by northern analysis and RT-PCR. Expression analysis of *osmotin* under various abiotic stimuli showed that osmotin was upregulated by abscisic acid, aerial drying, salicylic acid, NaCl and wounding after 3 hours. Relatively more transcript accumulation was seen during aerial drying, NaCl and wounding stress. Transgenic plants were screened for simulated drought (2% PEG) and salt (200 mM NaCl) stress treatment and biochemical analyses like cell membrane stability

index, photosynthetic yield, proline accumulation were analyzed. Osmotin transgenics performed better than the non-transgenics in all the parameters tested.

Development of Transgenic Mulberry with *Osmotin* Gene under Stress Inducible rd29A Promoter

Rd29A promoter is known as a stress inducible promoter and reported to contain two major *cis*-acting elements, the ABA-responsive element (ABRE) and the dehydration-responsive elements (DRE), which are responsible for ABA and dehydration induced expression, respectively, and both are involved in stress-inducible gene expression. *Osmotin* was cloned in pBI121 under rd29A promoter and also used for transformation of mulberry. PCR and northern expression analysis confirmed the integration of *osmotin* under the stress inducible gene. To see the inducible expression of *osmotin* under drought and salt stress, transgenics of osmotin under both 35S and rd29A promoter were subjected to drought and salt stress for 20 days. After 20 days of drought stress real time expression analysis revealed that osmotin was induced to a very large extent under rd29A inducible promoter than the 35S promoter. Northern analysis also confirmed the same result. The transgenics of osmotin under 35S promoter accumulated a large amount of proline than osmotin under rd29A promoter, whereas both the transgenics accumulated more proline than the non-transgenic plants. Hence, in this study it can be suggested that *osmotin* may be involved in osmotic adjustment only where needed and in some other way also and not only in accumulating more proline like in case of *osmotin* transgenics with constitutive promoter. There was no significant difference in membrane stability index and photosynthetic yield between both the transgenics.

Osmotin transgenics with both the promoters were tested for resistance against various fungal infections, i.e. *Fusarium pallidoroseum, Collectotrichum dematium* and *C. gloeosporide* by using pure fungal spore suspensions. In all the cases, transgenic plants showed tolerance to fungal infection than the non-transgenics, while *osmotin* under 35S promoter performed better than *osmotin* under *rd29A* promoter. Biotic assay of *osm* protein has no negative effects on silkworm feeding and rearing, and the transgenics had no deleterious effect on

silkworm rearing during their entire life cycle which remains unaffected. Biotic assay with both the transgenics also proved that these transgenics have no deleterious effect on silkworm larvae. So, these improved and resistant varieties can be used for silkworm rearing in future. Hence, from this study it can be concluded that transgenic plants developed with both promoters can tolerate drought and salt stress efficiently.

Additionally, the *osm* transgenic under *rd29A* promoter can tolerate both the stresses more efficiently than under 35S promoter transgenics, whereas *osm* under 35S promoter transgenics can tolerate fungal infection better than those with the stress inducible promoter. So, the transgenics under both promoters are useful for tolerating various biotic and abiotic stresses.

Overexpression of *β-carotene hydroxylase* in Mulberry

Carotenoids which are present in membranes of all photosynthetic organisms, help protect plants against light-dependent oxidative damage caused by increased sunlight and high temperatures. β-carotene hydroxylases are present in the thylakoid membrane catalyzing the conversion of β-carotene to zeaxanthin. In plants, the xanthophyll cycle is responsible for the reversible inter-conversion of violaxanthin and zeaxanthin, which has a key photoprotective role and is therefore a promising target for genetic engineering to enhance stress tolerance in plants. The *bch1* cDNA clone was incomplete at 5' end, so by RACE-PCR full length clone was obtained. *bch1* shows 82% identity with *Coffea arabica* and *Capsicum annuum* and *w*ith *Brassica napus* it shows 79% homology. *Morus bch1* shows up to 45% identity with bacterial *bch1* and also up to 45% identity with blue-green algae. Southern analysis of *bch1* in mulberry proved that mulberry genome possess a single copy of *bch1.* Expression analysis of *bch1* gene in different organs like leaf, root, shoot, flower and shoot tips showed that the transcript of *bch1* was expressed at a very high level in the shoot tips than other organs. Also expression analysis of *bch1* in mulberry revealed that it is upregulated by ABA, salicylic acid, high temperatures and UV, and its expression increases with duration of high temp and UV stress. For overexpression of *bch1* in mulberry the full length gene was cloned into binary vector p*CAMBIA* 2301 and used for mulberry transformation. Transformation of leaf-induced callus explants was found

to be very efficient explants for transformation with 90% transformation efficiency. *Gus* histochemical and fluorometry assay also confirmed the transformation. Southern analysis confirmed that single integration had occurred in the plant genome in some transgenics. Expression of *bch1* transgenics showed comparatively more expression of *bch1* than non-transgenic plants. It was found that three hours of high-light radiation increases the transcript level to a very high amount in transgenic plants than the non-transgenic plants. Growth performance of transgenics under heat and UV stress, high temperature stress at 45°C and UV stress for five hours were studied. The *bch*1 transgenics showed less inhibition in photochemistry than the non-transgenics after high and moderate light stress. Chlorophyll and carotenoid content was also found to be high after both light stress treatments. The *bch1* transgenics are more tolerant to high-light and high temperature and to UV stress. So, it can be assumed that more tolerance of mulberry transgenic plants to high-light, high temperature and UV stress is due to more production of the xanthophyll pool by overexpressing a single enzyme in the carotenoid biosynthesis pathway. Biotic assay *of bch1 transgenics* showed that it has no negative effects on silkworm feeding and rearing, and that the transgenics had no deleterious effect on silkworm productivity. Also, it was found that larvae fed on transgenic mulberry were little healthier than feeding on non-transgenic larvae. So it can be concluded that transgenic mulberry with over-expressed *bch1* have better tolerance against abiotic stress and have improved quality of leaves.

Based on the above experiments conducted, the following major conclusions were drawn:

1. Screening under simulated abiotic stress -- Various economically important mulberry genotypes were subjected to drought and salt stress and biochemically characterized for relative water content, proline content, membrane stability index, nitrate reductase activity, photosynthetic yield. Expression analysis of three stress inducible genes of mulberry i.e. *HAL3a*, *NHX1* and *dehydrin* were analyzed under drought and salt stress.

2. Genetic transformation of mulberry with *tobacco osmotin* under constitutive *CaMV35S* and stress inducible *rd29A* promoter -- *Agrobacterium* mediated transformation was done by using various explants such as leaf calli,

hypocotyl and cotyledons with *osmotin* under the constitutive CaMV35S and stress inducible *rd29A* promoter for abiotic and biotic stress tolerance.

3. Overexpression of *β-carotene hydroxylase* in mulberry for high temperature and high irradiance stress induced damage -- Isolation and overexpression of *bch1* in mulberry conferring tolerance against high temperature, UV and high-light stresses.