Chapter 5

Watermelon bud necrosis virus (WBNV) (genus *Tospovirus*; family *Bunyaviridae*) is an emerging *Tospovirus* in India. The disease is widespread in watermelon growing areas in Karnataka, Andhra Pradesh and Maharashtra with an estimated yield loss of 60-100% (Singh *et al.*, 1996). The virus has also been reported in ridge gourd (*Luffa acutangula* Roxb.) (Mandal *et al.*, 2003) and cucumber (*Cucumis sativus* L.) (Jain *et al.*, 2007). WBNV causes mottling and yellowing of leaves, stunting of vines and dieback of buds and shoots in watermelon (Singh *et al.*, 1996 and Kumar *et al.*, 2010). WBNV is an isometric virus with single-stranded RNA genomes. It is closely related with *Watermelon silver mottle virus* (WSMoV) and *Ground nut bud necrosis virus* (GBNV). The present work has been carried out with the major objectives of molecular characterization of M segment of WBNV genome and transgene expression in watermelon by developing transgenic construct from the viral genome sequence. WBNV is a tentative member and complete genome sequence was not available till 2010. Molecular characterization of WBNV is expected to lead to better understanding of its taxonomy and to establish the relationship of WBNV with other tospoviruses reported worldwide. The study is also expected to have high practical value, as the gene construct might be helpful in extending the genetically engineered resistance to other commercially important crops that are affected by tospoviruses.

Natural occurrence of watermelon bud virus necrosis was observed in farmers as well as experimental fields in Delhi, Haryana, UP and Bihar. The virus was transmitted to several test species by mechanical sap inoculation. *Nicotiana benthamiana* was found to be a good host assay for WBNV. Host range assay revealed that the virus isolated from watermelon induced characteristic symptoms on different host species. The chlorotic local spots on *Nicotiana benthamiana*, *V. unguiculata* cv. Pusa Komal or necrotic lesion on *C. amaranticolor*, *N. glutinosa* and *N. tabacum*, chlorotic ring on *V. unguiculata* cv. C-152 were observed. The watermelon virus isolate also induced systemic symptoms on leaves of *N. glutinosa* (necrotic spot), *N. tabacum* (veinal necrosis) and *N. benthamiana* (chlorosis, mottling and wilting). Inoculated watermelon plant produced bud necrosis symptoms similar to as those observed in the naturally infected field plants. The other inoculated plants *Cucumis sativus*, *Cucurbit moschata*,...
Lagenaria siceraria, Arachis hypogaea, Lycopersicum esculentum cv. Pusa Ruby did not produce any symptoms.

w-Del isolates was more similar to the south Indian isolates (TSVV-W) of WBNV (Singh and Krishnareddy, 1996). However w-Del produce different symptom from TSVV-W isolates on C-152 and N. tabacum (Table 4.1.1). The isolates were different from GBNV (Thien et al., 2003) with respect to host range and reaction. On cowpea cv. Pusa Komal, WBNV and GBNV could be differentiated based on expression of local and systemic symptoms. WBNV produced only local symptom whereas GBNV produced both local and systemic symptoms. WBNV and GBNV were further differentiable based on RT-PCR. Mixed infection of GBNV and WBNV can be identified by duplex PCR by using specific primers (BM 67wf & BM182R for WBNV and BM67F & BM181R for GBNV). Physical properties of the crude sap was available for GBNV (Ghanekar et al., 1979) but not for WBNV. In the present report, studies of physical properties revealed that the virus remained infective in sap dilution end point 1:100 and at a temperature of 40º C.

The virus isolate showed serological relationships with a Tospovirus group as it reacted strongly with antisera of GBNV/WSMoV. The electron microscopic study of the leaf dip preparations revealed isometric cored virus particles of 100-120 nm (in diameter), which resembled with the size of Tospovirus described earlier. Biological, serological, electron microscopic and molecular studies confirmed that the isolate causing bud necrosis was a Tospovirus. Genome characterization is essential for the authentic identification of virus isolate. Only NP gene sequence was available (Jain et al., 1998) and no information on complete genome sequence of WBNV was available. Therefore, in the present study, M RNA genome of WBNV-wDel isolate was sequenced and characterized. The full length M RNA genome of WBNV-wDel isolate was amplified by RT-PCR using their overlapping primers. The expected length of amplicons were successfully cloned in pGEM-T Easy vector system, sequenced and joined for obtaining their consensus sequence data.
Sequence analysis of M RNA genome revealed its complete length of 4794 nucleotides consisting of non-overlapping open reading frames (ORFs) of MP and Gn/Gc in ambisense orientation separated by 402 nt AU-rich intergenic region. The 5’ and 3’ untranslated regions (UTR) of the M RNA were 55 and 47 nt long, respectively. The first ORF (924 nt) located at 5’ encoded a protein of 307 amino acids with a predicted molecular mass of 34.22 kDa and was similar to NSm gene of the other tospoviruses. The second was ORF was larger (3360 nt) and located towards 3’ end containing 3366 nt in viral complementary strand of the genome, encoded the precursor of the glycoproteins, Gn/Gc containing 1121 amino acids with a predicted molecular mass of 127.15 kDa.

Multiple sequence alignment of M segment of WBNV-wDel isolates with the reported M segments of Tospovirus strains revealed that WBNV wDel isolate shared common features of genus tospoviruses. The M RNA genome sequence of WBNV-wDel was compared with that of WBNV-Wm-Som isolates and nine different tospoviruses. The 5’ UTR and NSm of Wm-Som isolate were near identical to wDel isolates; interestingly, Gn/Gc and 3’ UTR were significantly different sharing only 74.6% and 80.8% identity, respectively. Comparison of terminal sequence revealed that the eight nucleotides in both the termini of M RNA of WBNV were identical to all the tospoviruses compared. Wide variations in the UTRs of other tospoviruses were observed that ranged from 20.1 to 98.1%. A contrasting difference was observed with Gloxinia tospovirus-HT1 (GTV-HT1), which was highly different (37.5% identity) at 5’ UTR, whereas quite similar (85.1% identity) at 3’ UTR. The IGR of showed a least similarities (29.3-52.7%) compared to the other coding and non-coding regions in the M RNA of all the other tospoviruses.

Phylogenetic analysis based on complete M RNA sequence revealed three distinct groups of relatives of WBNV, among these tospoviruses, GBNV was closest to WBNV sharing 79.1% sequence identity. The melon infecting tospoviruses, MYSV and WSMoV known to occur in Japan and Taiwan (Yeh et al., 1992, Kato et al., 2000., Chu et al., 2001) shared only 63.3% and 75.2% identity, respectively with WBNV. The WBNV isolates, wDel and Wm-Som, were closely clustering together based on complete nucleotide sequence of M RNA and amino
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acid sequence of NSm protein. Interestingly, Wm-Som showed greater divergence from wDel in the Gn/Gc protein, suggesting intraspecies incongruence between the two isolates of WBNV within a genome segment. Intraspecies incongruence in phylogenetic trees has been observed in Cucumber mosaic virus (White et al., 1995, Roossinck, 2002) and has been attributed to recombination, an indication of modular evolution a virus (Roossinck, 2005). Recombination analysis of M RNA of the tospoviruses known to occur in India revealed potential recombination in the Gn/Gc. Interestingly, WBNV-Wm-Som isolate showed greater number of recombination events throughout the genome with the Indian tospoviruses and possessed a recombination hot spot in Gn/Gc ORF (breakpoints in the genome, 3839-4027 nt) as indicated by all the six recombination analysis methods. Co-infection of viruses/isolates in an ecological niche provides opportunities for recombination and evolution. Since, all the Indian tospoviruses were originally reported from central and southern part of India (Reddy et al., 1992, Jain et al., 1998, Satyanarayana et al., 1998, Krishnareddy et al 2006., Krishnareddy et al., 2008) and WBNV-Wm-Som isolate was also reported from central India, it might have differentially evolved due to recombination with the available relatives as compared to WBNV-wDel isolate originating from northern India.

WBNV causes severe epidemics in watermelon crops resulting economic losses in India. Resistant cultivar of watermelon is not available and other management options are not suitable for containing WBNV in watermelon. Transgenic cultivars resistant to virus have been developed for other cucurbits. Transgenic watermelon resistant to tobamovirus (CGMMV) (Park et al., 2005) and Potyvirus (Zucchini yellow mosaic virus and Papaya ringspot virus type W) (Yu et al., 2010) has been reported. Transformation of watermelon is difficult due to its recalcitrant nature to in vitro regeneration (Gaba et al, 1996). A few reports are available for regeneration and transformation of watermelon (Gielen et al., 1991; MacKenzie and Ellis 1992; Pang et al., 1992; Jan et al., 2000). Regeneration of an Indian watermelon cv. Sugar Baby has been reported (Chaturvedi and Bhatanagar, 2001). In the present study, efforts have been made to transform watermelon cv. SugarBaby with transgene constructs derived from NP gene of WBNV. Two types of constructs were prepared one was full NP gene in sense orientation in
pBI121 and another was partial NP gene (~300nt) in antisense orientation in pBINAR from WBNV. The constructs were mobilized into A. tumefaciens strain LBA4404 and transformation was conducted in watermelon cv. Sugar Baby.

Chaturvedi and Bhatnagar (2001) have reported the regeneration of watermelon cv. Sugar Baby by using 0.7 mg/l (3 mM) and 0.6 mg/l (3 mM) IAA, they obtained 55% of the tissue culture raised plants. Therefore in the present study a highly efficient regeneration system from cotyledon explants of citrullus lanatus cv. Sugar Baby has been developed. We have found that 2 mg/l BAP and 0.1 mg/l IAA induces shoot organogenesis (76%) from the calli. The similar results (60-92 % frequency) were obtained for ten different cultivar, when the combination of 5.0 mg/l BA and 0.5 mg/l IAA were used (Dong and Jia, 1991). Different BAP concentration alone induced low frequency of shoot with the other present study where as other worker reported (Rose, 1985; Srivastava et al., 1989; Compton and Gray, 1993; Choi et al., 1994) higher frequency of shoot at same treatment of BAP. This differential response may be due to different genotypes of watermelon. This indicates that type and concentration of phytohormones have been reported to be very crucial factor for regeneration process (Bakery et al., 2002).

Successful optimization of regeneration protocol from cotyledon explants of watermelon cv. Sugar Baby was an important step for the development of transgenic plants. A number of factors involved in improvement of transformation efficiency are co-cultivation duration, type of explants, role of phenolic compounds, bacterial density, temperature and medium composition (McCormick, 1991; Hamza and Chupeau, 1993; Hu and Phillips, 2001). During transformation process, antibiotics are the common selective agents in almost each plant species and the most commonly used antibiotics are kanamycin and hygromycin. Kanamycin was more effective than hygromycin as the selective agent for watermelon transformation (Akashi et al., 2005). However selection of transformants was a problem as the watermelon tissues are moderately resistance to kanamycin (Gaba et al., 2004). The antibiotics such as carbenicillin, cefotaxime or augmentin have also been used to eliminate Agrobacterium from co-cultivated explants. These antibiotics have been said to have minimum phytotoxicity effect and caused less mortality of explants (Hu et al., 2001). In the present study,
augmentin at 250 mg/l concentration drastically suppressed Agrobacterium growth and kanamycin at 100 mg/l concentration inhibited regeneration of shoots from non-transformed (control) explants. Green shoots recovered on kanamycin showed complete resistance even at higher doses of Kanamycin. The presence of kanamycin during rooting of shoots was found to eliminate escapants (Hu and Phillip, 2001). The higher dose of kanamycin concentration had adverse effect on the regeneration of transformants, while, the low concentration of kanamycin increased the number of false transformants, therefore, optimization of appropriate concentration of kanamycin was essential for the selection of transformants. Our results also indicated that there is a reduction in number of false transformants by increasing kanamycin concentration up to 100 mg/l. Augmentin was found to be the best for elimination of Agrobacterium. Our results showed that augmentin at concentration of 300 mg/l and above completely inhibited A. tumefaciens.

The effect of bacterial concentrations on watermelon transformation efficiency was determined. Agrobacterium at 0.6 OD was found to be the optimal for induction of the highest number of kanmycin resistant transformants. Inoculation duration of 20 min with 48 hours co-cultivation period showed the maximum transformation efficiency on selection medium. Acetosyringone was reported to enhance transformation efficiency dramatically by many researchers (Davis et al., 1991; Murray et al., 1998; Drori and Altaman, 2001). During present study, use of acetosyringone at concentration of 200 µM was found to be the best for transformation efficiency which supported the earlier findings. After several experiments for optimizing the different parameters of transformation, it was concluded that the transformation efficiency was optimum with the following parameters: 0.6 optical density of A. tumefaciens (LBA 4404 strain) at OD600; inoculation duration of 20 min and co-cultivation duration for 48 hours and 200 µM acetosyringone.

The established protocol for Agrobacterium-mediated transformation in watermelon cv. Sugar Baby using GUS gene (Table 5.2) showed 7.8% transformation frequency as evident by the GUS gene expression in the cotyledon explants using GUS histochemical assay and PCR. However previous worker reported transformation frequencies were 1.6% (Cho et al.,
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2008), 14.2% (Dabauza et al., 1997), 6% (Choi et al., 1994), to 96% (Ibrahim et al., 2009) in other cultivars of watermelon. The standardized transformation protocol used for the transfer of NP gene into watermelon cv. Sugar Baby resulted in 0.9% transformation efficiency as verified by PCR and PCR Southern blot analysis. However, lower transformation efficiency (0.1-0.3%) was reported earlier when CGMMV viral gene were introduced into watermelon (Park et al., 2005). This may be due the different cultivars used in these studies. Transgenic plants of groundnut resistant to a Tospovirus PBNV were developed for the first time by Yang et al. (2005). The present study is another effort for Agrobacterium-mediated transformation of watermelon against Tospovirus. The procedure developed by this study will be helpful to produce transgenic watermelon resistant to tospoviruses.