Growth and development occurs via a network of signaling factors originating and culminating in accurate gene expression. A number of specific regulators including in plants, non-coding RNAs species regulate key genes involved in differentiation processes and have direct implication in normal growth and development (Carrington and Ambros 2003; Saleh et al. 2011; Sakaguchi and Watanabe 2012). Therefore, identification and characterization of microRNA is inescapable to understand growth and development in plants.

In the present study, \textit{miR165a} and its target member \textit{REVOLUTA (REV)} were isolated from \textit{Brassica} species and characterization was done in \textit{B. juncea}. Comparative genomics study was performed between \textit{Brassica} and \textit{Arabidopsis} to identify the various homologs (orthologs) of \textit{miR165a} and \textit{REV}. Complete genome sequence of \textit{Brassica rapa} (Yang et al. 2005; Hong et al. 2008; Wang et al. 2011c) and \textit{A. thaliana} (Salanoubat et al. 2000; Tabata et al. 2000) helped in the process. In order to ascertain the orthologous nature of AC232510 (\textit{miR165a}) and AC189324 (\textit{REV}) retrieved from the \textit{Brassica} database, global alignment was performed against the whole genome of \textit{A. thaliana} using AVID (Bray et al. 2003). The alignment was visualized through VISTA (Dubchak and Ryaboy 2006). Evidences support that comparative sequence analysis of specific chromosomal region not only provides sequence information but also gives information on gene density, synten and conservation of gene collinearity along the segments (Gao et al. 2006; Town et al. 2006; Panjabi et al. 2008; Trick et al. 2009; Sehgal et al. 2012). The global alignment of 117.8 kb AC232510 and 130.74 kb AC189324 of \textit{B. rapa} with \textit{A. thaliana} genome revealed the collinearity of gene order and content as well as gene density. The collinearity of the two distinct genomic regions, however, was marked by distinct events of gene loss and rearrangement. The BAC sequence AC189324 of 130.74 kb length harbouring \textit{REV} (AC189324) corresponded to 78 kb region of \textit{A. thaliana} with conserved synteny. In contrast, 117.8 kb AC232510 BAC harbouring \textit{miR165a} was orthologous to the 135 kb region of \textit{A. thaliana} revealing instances of gene deletion and genomic re-organization in \textit{Brassica}. Similar findings have been observed in the comparative sequence analysis of the SALT OVERLY SENSITIVE1 orthologous region in \textit{Thellungiella halophila} and \textit{A. thaliana}. It was emphasized that MuDr transposons, LTR- retrotransposons
and long intergenic sequences are responsible for such differences in the orthologs (Nah et al. 2009). Instances of gene loss and genomic re-organization have also been recorded in A. thaliana and Cleome spinosa (Schranz and Mitchell-Olds 2006). Micro-collinearity in much smaller regions of ca. 30 kb have been detected between A. thaliana- Capsella rubella- tomato (Rossberg et al. 2001). Corresponding analyses on collinearity and genome evolution has been carried out between A. thaliana- rice (Wang et al. 2006), Maize (Fu and Dooner, 2002), A. thaliana- B. rapa (Parkin et al. 2005; Kim et al. 2006; Mun et al. 2009), diploid and allotetraploid coffee species (Yu et al. 2011), B. juncea- A. thaliana (Panjabi et al. 2008), Brachypodium- rice- wheat (Gu et al. 2009; Wei et al. 2009), peach/ almond- apple (Chen et al. 2008) and yeast (Kellis et al. 2004). Synteny between or among the chromosomes of one or more species suggests evolutionary relationship of the genomes that can aid in identifying homologous genes and non-coding functional elements, such as regulatory elements (Frazer et al. 2003). We therefore, drew a composite synteny map of AC232510 and AC189324 of B. rapa against A. thaliana that revealed the conserved orthologous genes shared by both the species. Some species specific insertions/deletion as well as duplication of the locus in Brassica rapa, which may be because of chromosome evolution, genome polyploidization and subsequent diploidization events (Wendel 2000; Guiliano et al. 2002; Bowers et al. 2003; Cannon et al. 2003), were also detected.

The study also analyses the nature and extent of sequence divergence in a miRNA and transcription factor locus from five species of Brassica. Sequence comparison of miR165a among Brassica species, A. thaliana and A. lyrata corroborate that the mature sequence of Brassica microRNA is similar to A. thaliana and conserved in all species except that a transition event replaced a consensus T with a C (BoIBS5) and G->A (BraCC3). The discovery of SNP in a miRNA otherwise thought to be conserved across land plants implies that the full spectrum of natural variation in sequence of such conserved system is yet to be discovered (Floyd and Bowman 2004). The precursor sequence, however, exhibited a Brassica specific divergence within the stem-loop. But, such an alteration does not disrupt the miRNA:miRNA* pairing. That miR165 plays an important role in concert with miR166 in regulating
the expression of *HD-ZIP III* transcription factor and hence, influences the plant growth and development is in accordance with the report of Zhou et al (2007). Studies related to gene expression by Takacs and Giraldez (2011) in mutants lacking components of the miRNA-processing machinery have revealed that miRNAs constitute an evolutionarily conserved tool to provide temporal and spatial control of gene expression post-transcriptionally. Vaucheret (2006) emphasized that in plants, precise pairing with stringent rules govern PTGS. Any mutation that affects the miRNA binding site is likely to cause developmental defects according to Mallory et al (2004b). Indeed, a dominant mutation in PHB (*phb-1d and phb-2d*) caused by mutation in *miR165* binding site led to the lack of abaxial/adaxial identity in leaves (McConnell et al. 2001). The retention of such precise base-pairing between miRNA-mRNA target has not yet been investigated in the context of polyploidy. *Brassica* species, given their well-established polyploidy nature allowed us to identify allelic variation in the miRNA and the cognate mRNA target at the locus of pairing as well as in the flanking sequences. Within the *Brassica* species, allelic variation can be observed both in the precursor region of *miR165a* and the target *REV*. Even though the *miR165* binding site in *REV* is split between exons 4 and 5, the splice junctions are conserved such that the binding site is reconstituted in the mRNA. The lack of mutation in the *miR165* binding site in *REV* observed in our study indicates that the miRNA binding site is maintained under purifying selection as has earlier been observed in rice by Guo et al (2008). The important role of members of *HD-ZIP III* family especially *PHV/PHB/REV* in maintaining polarity, root and vasculature patterning, functions that are necessary and conserved in land plant evolution, have been recorded by Prigge et al (2005) and Miyashima et al (2011). It is conceivable that any mutation in its regulation may be highly deleterious and not maintainable in the population (see also Prigge and Clark 2006). In contrast mutations in *miR319a* (Warthmann et al. 2008) and *CUC1*, target of *miR164c* (Kusumanjali et al. 2012) have been observed.

The clustering output of both *miR165a* and *REV* makes obvious the allelic variation that exists within the species as well as clones and the origin of the alleles from diploid into allopolyploid species. Allelic variation in a gene within the species is
supported by the study of Lee et al (2002b) where phylogenetic analysis of the 
*PISTILLATA* intron from 43 species of *Brassicaceae* correlated with floral reduction 
in *Lepidium* and suggested that many species in the new world have originated from 
allopolyplidization. In addition the retention of a few clones of *miR165a* from *B. rapa* var. R-O-18 (A genome) into clusters containing C genome is reminiscent of 
incomplete lineage sorting (Comes and Abbott 2001; Maddison and Knowles 2006).

Tissue specific expression study of *miR165a* was carried out using Northern blot of 
small RNAs (Pall and Hamilton 2008; Bologna et al. 2009) and semi-quantitative 
stem-loop RT-PCR (Chen et al. 2005) in *B. juncea* and *B. rapa*. Previous reports by 
mirRNAs individually regulate spatio-temporal gene expression. In fact, in a wide 
range of organisms, microRNAs are differentially expressed in different tissues at 
different developmental stages as also observed by Rhoades and Bartel (2004) and 
Sunkar et al (2005). In the present study, dynamic spatio-temporal expression pattern 
of *miR165a* was analysed that indeed showed that the level of expression differed 
among the tissues in both the species of *Brassica*. The difference in the expression 
level may be because of apparent variation in the regulation of target genes by 
*miR165a*. In *B. juncea*, abundant expression of *miR165a* was recorded in SAM 
followed by bud, flower and young silique though, in mature leaf the expression was 
negligible and no hybridization signal was detected in 9- and 15-day leaves. Such a 
differential gene expression was earlier shown in *Arabidopsis* by Zhou et al (2007) 
where *miR165* is localized in the shoot apical meristem, leaf primordia and vascular 
tissues and regulates the expression of *HD-ZIP III* transcription factor for normal 
development of plants. The expression of *miR165a* in *B. rapa* and *B. juncea* re-
established the differential expression at interspecies level as well as being tissue 
specific- bud, flower and siliqua. In *B. rapa* transcript level appeared to be increased 
during bud to silique stages. However, in *B. juncea* silique showed higher expression 
followed by flower and bud. Polyloidization and morphological variation could be 
implicated for the difference in expression pattern of *miR165a* at species level. The 
mirRNAs which are conserved in nature do not necessarily exhibit the same expression 
levels or patterns in different species or at different stages within a species. It implies
that, sequence and expression divergence in miRNAs between species may affect miRNA accumulation and target regulation in interspecific hybrids and allopolyploids that contain two or more divergent genomes, leading to developmental changes and phenotypic variation (Ha et al. 2009). This strengthens the fact that differential expression of miR165a at interspecies level may be due to genome reorganization and cause morphological variability.

Correlation of transcript level of REV and PHV with the miR165a expression profile confirmed that miR165a regulates its target members through down regulation. Earlier studies of Emery et al (2003), Tang et al (2003) and Zhou et al (2007) do support the findings that state that miR165/166 regulates HD-ZIP III by down-regulating their transcript. Expression of miR165a in bud, flower and young silique and corresponding reduction in steady state levels of target genes implicates this miRNA-transcription factor module in regulating flower and fruit development. Jung and Park (2007) have reported that the expression pattern of miR165/166 is confined to shoot apical meristem and flower development in A. thaliana. On the other hand Ji et al (2011) state that over-expression of miR165/166 and concomitant reduction in the expression of HD-ZIP III leads to defects in floral stem cells. The comparative expression profile of miR165a and its target members in the present investigation reveal that the levels of target gene transcripts are low in tissues that have higher levels of miR165a and vice-versa. In contrast, REV and miR165a appear to be co-expressed in 15-day-old leaves and flowers of B. juncea as well as bud of B. rapa, as exhibited by near equal accumulation of steady state level of targets and miR165a transcripts. Similar reports of overlapping expression pattern is known between the transcription factors and miRNA in ARF/miR167 (Wu et al. 2006), MYB/miR159 (Allen et al. 2007), CUC/miR164 (Raman et al. 2008) and SPL/miR156 (Wang et al. 2009). The mechanism of regulation of co-expression mediated via respective cis-element and upstream trans-factors needs to be further investigated.

Generation of transgenic plants has been used as reverse genetics tool for functional genomics (Schell 1987; Pitzschke and Hirt 2010). Plants transformed with the same gene construct have been shown to vary considerably in gene expression pattern within and between species (Benfey and Chua 1989). The role of miR165 in plant
development is already established in *A. thaliana*. To understand its function in economically important crop, *Brassica*, *B. juncea* var. Pusa Bold was selected for functional study. The binary vector harboring miR165a was transformed into the plant through *Agrobacterium*-mediated transformation. Various factors such as co-cultivation period, pre-culture of explants, bacterial density, infection time, post cultivation in darkness, and the use of inducers such as acetosyringone affected *Agrobacterium*-mediated transformation efficiency and regeneration of plants thereafter (Barik et al. 2005). Babic et al (1997) tried *Brassica* explants such as hypocotyl and cotyledons but they proved necrotic when directly exposed to *Agrobacterium* suspension without pre-culture on regeneration medium. To avoid necrosis and achieve efficient transformation, explants were pre-cultured for 24 hrs on regeneration medium (MS + BA-1.0 mg/L + 2,4-D-0.05 mg/L + AgNO3-20 µM) before co-cultivation. Such a successful strategy was also employed by Babic et al (1997) for generation of transgenic lines in *B. carinata*. There are other convincing examples of plant systems in which *Agrobacterium*-mediated transformation was established by standardizing the duration of explant preculture, e.g ginger (Suma et al. 2008), *Dendrobium* (Subramaniam et al. 2009), cucumber (Rajagopalan and Perl-Treves 2005), groundnut (Venkatachalam et al. 2000), *Lotus corniculatus* (Jian et al. 2009) and so on. In our study, we added silver nitrate (AgNO3) to the media, considered to be essential for high frequency shoot regeneration. Earlier reports by Barfield and Pua (1991), Mehra et al (2000), Zhang et al (2006), and Bhalla and Singh (2008) do support our use of AgNO3. Since silver nitrate in co-cultivation medium caused blackening of the explants, we removed it from the medium during co-cultivation. High concentrations of silver nitrate becomes toxic during co-cultivation, a fact recorded by Subramaniam et al (2009) in *Dendrobium*. In maize and Fuji apple however, addition of silver nitrate in the co-culture medium facilitated enhanced gene transfer and also recovery by suppressing growth of *Agrobacterium* on target explants (Armstrong et al. 2001; Zhao et al. 2001; Seong et al. 2005). Orlikowska (1997) had earlier reported that silver nitrate does stimulate direct shoot regeneration from rose (*Rosa indica*) after transformation with *Agrobacterium tumefaciens*. Silver nitrate indeed has been shown to have other important effects in plant tissue culture such as,
improving somatic embryogenesis, organogenesis and micropropagation including in *Cassava* species (Zhang et al. 2001).

*MiR165a* overexpressing lines with modifications in vegetative as well as reproductive organs were isolated in the present investigations. In all the independent lines aberrant formation of leaves along with deformed SAM or apical region of plants and vascular patterning of leaves was observed consistently. In addition, the regenerated plants were stunted. A similar observation was made by Zhou et al. (2007) in *Arabidopsis*. The recorded various developmental defects in *miR165* overexpressing plants include aberrant formation of SAM or apical region. According to earlier reports, auxin is responsible for setting up the position of leaf primordia and allowing outgrowth. In very young organ primordia expression of the PIN1 auxin efflux carrier marks the abaxial boundary of *REV* expression (Heisler et al. 2005). Zhou et al. (2007) noticed a link between *miR165* overexpression and alteration in the expression of genes involved in auxin signalling in *Arabidopsis*. Several other reports also (Nagasaki et al. 2007; Chitwood et al. 2009; Schwab et al. 2009) describe the regulation of leaf polarity by the *miR390/ARF* pathway. It is however, not clear whether the developmental processes in apical region of *Brassica* could be affected by an alteration in *miR165a* expression, though the transcription factor class III homeodomain leucine zipper (*HD-ZIP III*) members i.e., *PHAVOLUTA (PHV)*, *PHABULOSA (PHB)* and *REVOLUTA (REV)* are regulated by *miR165/166* and play a significant role in normal development of leaf polarity. Also, higher accumulation of *miR165/166* results in altered adaxial and abaxial surface of leaves (Williams et al. 2005; Kidner 2010; Rubio-Somoza and Weigel 2011). Our studies also demonstrate that overexpression of *miR165a* in *Brassica* leads to severe reduction of *REV* and *PHV* transcript level which results in altered leaf surfaces and deformed apical region (SAM). Correct accumulation of *miR165/166* is also regulated by some other factors like, *AGO1*, *PNHZ/LL* or *AGO10* and *HYPONASTIC LEAVES1 (HYL1)* (Kidner and Martienssen 2004; Liu 2008; Montgomery et al. 2008; Dong et al. 2008). The *AGO1* and *PNHZ/LL* or *AGO10* regulate meristem function but the exact mechanisms are yet not known. However, it has been proposed that *AGO1* regulates meristem function via *HD-ZIP III* genes (Kidner and Martienssen 2005b). Mutation in *AGO1* caused mislocalization of *miR165* in the meristem, which might result in abnormal cleavage of *HD-ZIP III* transcripts and hence in deformed SAM (Kidner and Martienssen 2004). *HYL1* monitors the roles of *miR165/166*, *miR319a*, and *miR160* in leaf flattening through the relative activities of adaxial and abaxial identity responsive
genes (Dong et al. 2008; Liu et al. 2011). YABBY, KANADI and AS are known to be the other factors responsible for polarity formation (Bowman et al. 2002) but not affected by miR165a overexpression (Zhou et al. 2007). The relationship among miR165a, HYL1 and different AGO genes need to be studied in Brassica. Our findings corroborate the fact that expression of miR165a at normal level is important for meristem function in Brassica; overexpression in leaves leads to defects in vein formation. It is already reported that regulation of ATHB8 through miR165/miR166 endorses progression in vasculature differentiation (Donner et al. 2009). Our results also confirm the role of miR165a in flower development. Ectopic expression of miR165a in Brassica leads to aberrant floral development including failed anthesis. According to reports of Jung and Park (2007) and Ji et al (2011) HD-ZIP III and miR165/166 are crucial factors in flower development. Reduction in HD-ZIP III expression by over-expression of miR165/166 or mis-expression renders them resistant to miR165/166 that causes prolonged floral stem cell activity. It indicates that the expression of HD-ZIP III genes need to be precisely controlled to achieve floral stem cell termination (Ji et al. 2011). Since the evolutionarily well-conserved miRNAs are likely to contribute to proper plant growth and morphogenesis by regulating their target (Axtell and Bowman 2008; Todesco et al. 2010), the transcript level of the target members REV and PHV in transgenic lines was estimated by using semi-quantitative RT-PCR and it was highly reduced. The result concurs with the earlier report which demonstrates that overexpression of miR165 causes a drastic reduction in the mRNA levels of all five HD-ZIP III genes (Zhou et al. 2007). In contrast, overexpression of miR166 leads to high levels of accumulation of its transcript in embryo. It mainly down-regulates the expression of ATHB-15, ATHB-9/PHV and ATHB-14/PHB (Kim et al. 2005; Williams et al. 2005), indicating that miR165 and miR166 have differential effects on the regulation of their target genes (Zhou et al. 2007). Therefore, it can be surmised that the phenotypes and pattern of expression obtained in this study is relative to the regulatory processes of miR165a and its target genes. The interactive role of miR166 and HD-ZIP III in Brassica needs to be analyzed for further confirmation.

The present study has also demonstrated the function of Brassica-miR165a in A. thaliana (Columbia) and the phenotypes obtained in Arabidopsis and Brassica establish that miR165a is one of the functionally conserved microRNA in plant genera.