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ECTOPIC EXPRESSION OF MPF2-LIKE PROTEIN WSA206 LEADS TO ARREST IN SILIQUE AND SEED DEVELOPMENT IN HETEROLOGOUS HOST

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Abstract

MPF2-like genes belonging to *STMADS11* clade of MADS-box transcription factors are mostly involved in calyx inflation, floral reversion and fertility. However their role in fertility remained enigmatic. In this study we transformed *WSA206* gene paralog - originated through genome duplication in a Solanaceous plant *Withaniasomnifera* - ectopically in a heterologous host *Arabidopsis thaliana.* Interesting phenotypes in floral organs and fruits were observed. Overexpression of WSA206 leads to arrest in silique development. The siliques were compressed and size was drastically reduced from 34mm to 3mm. Along with siliques, the seed development was also suppressed as revealed by shriveling of seeds and reduction in seed number. In extreme cases the siliques were devoid of any seeds. In cases where seeds developed, these were impaired in viability. Besides, the transgenic *Arabidopsis* also exhibited exorbitant growth of calyx to an extent that it resembled inflated calyx in Solanaceae. The calyx remained persistent and encapsulated the under-developed siliques containing non-viable seeds inside. Thus, fertility and sepal development are tightly coupled traits that are controlled by *WSA206*paralog in heterologous system.

Key words: MPF2-like, MADS-box, Expression, *Arabidopsis*, Fertility, Calyx, *Withania*

Introduction

In plants the genetic dissection of fertility function is of utmost importance for the potential use of responsible genes in getting higher yield in crop plants. Male fertility is the most prevalent phenomenon in flowering plants that entails the production of viable pollen grains in anther (Chaudhury*et al*., 1992). It consists of series of developmental events. Initially vegetative meristem generates stamens and anthers. Viable pollen grains are developed inside the inner issues of anther. At maturity these pollen grains are released and deposited on the stigma. Finally there is reciprocity of interactions between the male and female gametes to accomplish self-fertilization. Previous studies have revealed that male fertility is the interplay of large number of genes as indicated by the phenotypic diversity and many loci identified. Perturbation of any of these genes lead to male sterility (McConn, 1996; Luo *et al*., 2000; Goetz *et al*., 2001; Jung *et al*., 2006; Luo *et al*., 2006; Hu *et al*., 2011; Li *et al*., 2015). In spite of fundamental importance of these genes in fertility trait the knowledge about molecular details remained fragmented. Hence deciphering of molecular mechanisms underlying male fertility is inevitable. Therefore, isolation and functional characterization of the genes involved is essential to unwind the hidden strings of gene net-work for dissection of fertility function. The MADS-box transcription factors (TFs) constitute majority of the genes involved in fertility function in plants. MADS-box comprises one of the large families of TFs in land plants with 109 members in *Arabidopsis* which are differentiated into 17 clades on the basis of phylogeny, structural and functional characterization (Becker and Theissen, 2003; Gramzow & Theissen, 2014).

Best known for their implication in calyx inflation, *MPF2-like* genes pertinent to the *STMADS11* clade of the MADS-box family also exert their functions in leaf development, flowering time, inflorescence architecture and floral reversion (He & Saedler, 2005; Zhang *et al*., 2012; Khan *et al*., 2013). However, our knowledge about their involvement in molecular processes related to fertility has remained obscure. Recently, WSA206, a MADS-box family member is found to be recruited in fertility function in *Withaniasomnifera* from where this gene is originally isolated. This paralog is originated through genome duplication of MPF2-like proteins into WSA206 and WSB206 in the genus *Withania*. These *MPF2-like* genes are the close orthologs of *MPF2* of *Physalisfloridana* which is recruited in calyx inflation and fertility traits through interaction with other proteins (He & Saedler, 2005; Zhang *et al*., 2012). This gene also interacts with WsMAGO2, a duplicated MAGO NASHI protein which is involved in fertility function (Ihsan*et al*., 2015). Therefore the major thrust of this study was to functionally characterize the WSA206 i.e. MPF2-like protein by overexpressing it in a heterologous host *Arabidopsis*.

Materials and Methods

The *Arabidopsis thaliana* ecotype "Columbia" was grown in the glasshouse of National Institute for Genomics and Advanced Biotechnology (NIGAB), National Agricultural Research Centre (NARC), Islamabad, Pakistan. The *Arabidopsis* seedlings were grown in the glasshouse under long day conditions with temperature 25-28°C and illumination period of 16h. Overexpressing gateway construct containing *WSA206* gene driven under 35S promoter was transformed into *Arabidopsis* by inflorescence dip method of Clough and Bent (1998). Design of construct and procedure adopted for transformations and genotyping

conditions for *35S::WSA206-mYFP A. thaliana* plants were according to Khan *et al*. (2009). For overexpression analysis of *WSA206* in *Arabidopsis*, the T4 transgenic seeds of homozygous lines were grown in pots. When mature siliques developed, the plants were analyzed for various phenotypic parameters. Different lines of T4 transgenic plants of *Arabidopsis* bearing siliques were analyzed for fertility trait comparison with wild type counterparts. The length of the mature siliques was matched with wild type plants. In certain lines the siliques were cut open under microscope and number of healthy seeds was counted. Crossing of defective and wild type plants was done to confirm the type of infertility. Beside fertility, the development of calyx inflation was also assessed. The observations were recorded with respect to calyx appearance; shape of the "Inflated Calyx Syndrome" (ICS) and degree of inflation. The images were taken with a Nikon camera fixed in a microscope.

Results and Discussion

Fertility is a fundamental and evolutionary selected trait for the production of viable seeds in plants but it remained orphan in terms of unveiling molecular mechanism behind different events embedded in this traits. Furthermore, the significance of this trait in the production of hybrids can hardly be exaggerated. This study was undertaken to decipher the role of a MADS-box protein MPF2-like (WSA206) in heterologous host *Arabidopsis thaliana*. *WSA206* gene was ectopically expressed in *Arabidopsis*. A total of 10 lines of T4 transgenic plants were analyzed in this study. Fig. 1AB demonstrates that silique formation in transgenic *Arabidopsis* lines OeT4-02, OeT4-09, OeT4-11 and OeT4-12 is completely impaired. In extreme cases the siliques do not develop at all. In instances where siliques developed, the seed set was aborted. If seeds were present these were infertile. The size of siliques was 3mm in severely affected OeT4#26 against 34mm in wild type plants. Accordingly, the number of seeds was drastically reduced. When siliques were cut open to observe and count the seeds under microscope, the seeds in small siliques of WSA206 overexpression lines were shriveled (Fig. 1Cab). Most of the seeds aborted and trace of rudimentary seeds remained persistent. In the short siliques as in the case of OeT4#10 line only two (Fig. 1C left) seeds developed normally against 34 (Fig. 1C right) in the wild type counterpart. In severely affected OeT4#26 plants the siliques were completely devoid of any seeds. In order to detect the type of infertility, the wild type pollen grains were crossed with WSA206 overexpression mutant. The normal wild type healthy phenotype with normal siliques length and proper seed set could be rescued suggesting a male fertility issue in *Arabidopsis* (Fig. 2A). Other MADS-box proteins have also been found associated with fertility issues in *Arabidopsis*. These include *SVP* and *AGL24* overexpression lines and *ap1* mutants that exhibit alterations in siliques morphology and seed set (He *et al*., 2004; Gregis *et al*., 2006). Interestingly, some of these genes also belong to *STMADS16* clade of MADS-box transcription factors.

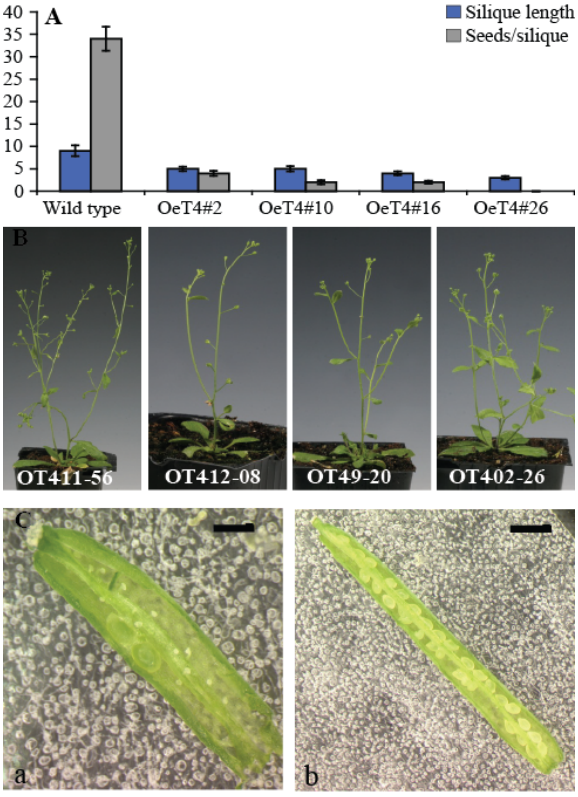

Fig. 1. Ectopic expression of *WSA206* in heterologous host *A. thaliana* induces male infertility.
A- Graph showing the length of the siliques and seed number inside each siliques for four transgenic lines with 20 plants in each line. The transgenic lines are indicated on X-axis. Vernier scale was used to measure the siliques length and seeds were counted under microscope after disrupting the siliques longitudinally. Error bars indicate the standard deviations.
B- Phenotypes of transgenic *Arabidopsis* plants. Four lines of mature transgenic plants of *Arabidopsis* at T4 generation are shown.
C-Siliques of transgenic and wild type plants were cut open under microscope to observe and count the number of viable seeds. Scale bar is equal to 40nm.

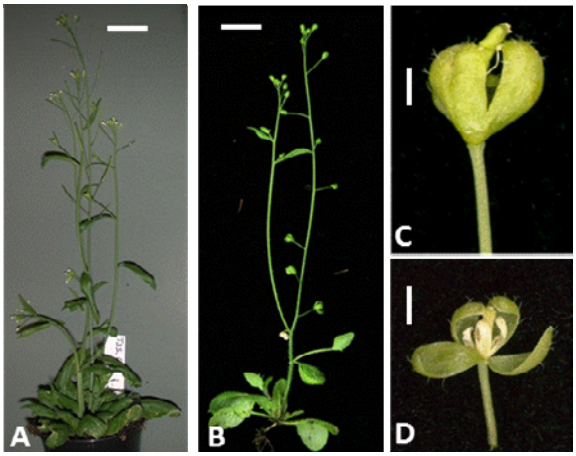

Fig. 2. Overexpression of WSA206 in *Arabidopsis* leads to arrest in siliques development and induces secondary sepal growth.
A- Recovery of normal wild type phenotype from fertility defective plant after crossing with normal wild type. Scale bar represents 5 mm.
B- The silique development is suppressed. Scale bar cover 1 mm length.
CD- Calyx inflation and suppression of silique formation. Scale bar = 1mm.

In transgenic plant lines where the male fertility problem was obvious, surprisingly, the calyces of transgenic plants grew exorbitantly large and resembled the “Inflated Calyx Syndrome” of *Withania* and *Physalis* (He and Saedler, 2005; Hu and Saedler, 2007; Khan *et al*., 2009)(Fig. 2BCD). In wild type plants, no secondary growth of calyx was observed and calyx shed off earlier than in transgenic plants. Furthermore, in the transgenic plants the calyx remained persistent and grew to make a balloon like capsule of the ICS (Fig. 2C). These results demonstrated that fertility and calyx inflation are tightly coupled traits controlled by the WSA206 (MPF2-like) protein.

The above results allow us to infer that unequivocally WSA206 is recruited in fertility trait which is evident from disrupted phenotypes of siliques and seeds. Interestingly, overexpression of WSA206 in two different hosts leads to two opposite phenotypes. This discrepancy compelled to contemplate that how ectopic expression of the same gene i.e. *WSA206* in native plant (*Withania*) promoted the fertility (data not shown) while in heterologous host (*Arabidopsis*) affected the fertility. It is difficult to reconcile the differences in the overexpression of *WSA206* in *Arabidopsisvs.* loss of function in *Withania* phenotypes as both lead to male sterility (data not shown). Moreover overexpression of WSA206 in native host i.e., *Withania* promotes berry development, seeding set, pollen viability and calyx inflation. But interpretation in heterologous host needs extreme care since observed phenotypes in cells or tissues deprived of gene expression say little about the normal function of the gene. However, it can be put forwarded that broader phenotypes like "male fertility defects" can be caused *via* many different molecular mechanisms. It is not absolutely clear in this case if the male sterility defect in the two cases is due to the same mechanism. But the extraordinary growth of the calyx phenotypes due to overexpression of same gene *WSA206* is supportive of the normal function of this gene in heterologous host. Discrepancies in expression divergence have already been documented for the *STMADS16* gene. Heterologous expression of this gene is concentrated in the floral organs of *A. thaliana* though original expression of this gene is accrued in vegetative tissue only (He *et al*., 2010). Another evidence in the favor of this study comes from the research work on deciphering the genetic mechanism underlying the evolution of leaf form between *A. thaliana* and its closely related wild plant *Cardamine hirsute*. Hay and Tsiantis (2006) demonstrated that *Cardamine* loci of both *SHOOTMERISTEMLESS*(*STM*) and *BREVIPEDICELLUS* (*BP*) are active in *Arabidopsis* shoot apical meristem and leaves whereas the *Arabidopsis* homologs are restricted to the shoot apical meristem.

In essence, our results demonstrate that WSA206 affects silique development, seed set and viability in *Arabidopsis* thereby revealing a critical role of this gene in fertility function. Furthermore, ICS and fertility are tightly coupled traits which are controlled by this paralog i.e., WSA206. Since a number of cytotoxic genes are available, a potential application of *WSA206* lies in engineering male sterility for hybrid production for enhancement of crop yield.

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