MADS-Box Transcription Factor *FRUITFULL* Orthologs i Nicotiana Promote Transition to Flower, but Did Not Significantly Alter Capsule Development

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ABSTRACT

Understanding the genetics mechanisms of flower and fruit development is very crucial for efficient bre many agronomical important crops. MADS-box group of transcription factors has been known for its sig contribution of controlling fruit development. *FRUITFULL* (*FUL*) is one of the genes that plays important role regard. For more understanding of *FUL* gene function in capsule dry-fruit development, this study used virus Gene Silencing method to knockdown *FUL* gene expression in the newly developed model plant, *Nicotiana obt* Treated plants exhibited delayed flowering, shorter inflorescence stems and longer fused sepal when compared type and control group. However, no altered fruit phenotypes were observed among the treated *N. obtusifoli* Downregulation of FUL gene in *Nicotiana obtusifolia*, allows us to assess a better understanding of its functio fruit species of the family *Solanaceae*. It also suggests that FUL protein plays different roles in the developme versus fleshy fruits in Solanaceae.

Key words: Nicotiana obtusifolia, *FRUITFULL* gene, Flower development.

INTRODUCTION

Fruit is a very important organ for plant as it mediates maturation and dispersal of the seeds. Understanding the genetics mechanisms of flower and so fruit development is very crucial for efficient breeding of many agronomical important crops. One gene heavily implicated in fruit development is AGAMOUSE-LIKE 8 (AGL8) or *FRUITFULL* (*FUL*). This gene is one of the MADS-box group of transcription factors. *FUL* gene function has been studied intensively in *Arabidosis thaliana* in which *ful* mutants show improper fruit development (Gu et al, 1998; Ferrándiz et al, 2000a; Ferrándiz, 2002). Although floral organ identity is not affected in *Arabidopsis ful* mutant, *FUL* gene found to act redundantly with *APETALA1* (*AP1*) and *CAULIFLOWER* (*CAL*) genes to promote transition to floral meristems (Ferrándiz et al, 2000b). *Arabidopsis FUL* with *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1)* gene have been shown to regulate flowering time, affect determinacy of all meristems and plant longevity (Smykal et al, 2007; Melzer et al, 2008). Overexpression of *Arabidopsis* FUL in *Brassica juncea* (Østergaard et al, 2006) and *Antirrhinum FUL ortholog*, *DEFICIENS-homolog28* (*DEFH28*), in *Arabidopsis* (Müller et al, 2001) affect lignification process and carpel wall development. *FUL* tomato orthologs, *SIFUL2*, regulate the expression of ripening-related genes (Bemer et al, 2012; Wang et al, 2014). *FUL* gene also has assigned a role in tomato leaf development et al, 2013). As with many crops produce dry fruit, examining the role of FUL in capsu bearing species and studying whether *FUL* g similar role in capsule fruit species *Arabidopsis* (silique fruit) should increa understanding of *FUL* gene function i development. This study aims at studyi function of a *FUL* orthologs in capsule dr bearing species *Nicotiana obtusifolia*, whi chosen for many reasons including compatib ability to flower and grow faster compared *Nicotiana* varieties. *N. obtusifolia* is a mer the *Solanaceae* family which become vital to in multiple ways. Genera such as *Datura* and have been shown to have anticancer an asthmatic properties (Chiarini, 2009; Soni 2012). In addition, Solanaceae species s tomato and eggplant are increasingly in component in human diet (Chiarini, Determining the roles of *FUL* genes in dry fleshy fruits contributes to the understandin gene regulatory network behind ripening. Induced Gene Silencing (VIGS) method is this study to specifically silence or redu expression of *FUL*2 gene in *N. obt NobFUL2*, through Post-Transcriptional Silencing mechanisms (PTGS) (Dinesh-Kum 2003). *Tobacco Rattle Virus* (TRV) vector s is one of the widely used in VIGS (Bach Dinesh-Kumar, 2012). It composed of two components (vectors): TRV1, which

movement and coat proteins and is not altered in the VIGS procedure, and TRV2, from which the virulence genes replaced by a fragment of the target gene to initiate an immune response. Upon infection of virus to the plant, synthesis of viral double-stranded RNA (dsRNAs) lead to the activation of the antiviral RNA silencing pathway and the subsequent knockdown of the endogenous host gene (Ding and Voinnet, 2007). The silencing signal spreads systemically through the phloem system of the plants (Kalantidis et al, 2008). Compared to other techniques to knock down genes, VIGS has the advantages of being rapid tool that does not need stable plant transformation. It can be used in wide range of plant system (dicot or monocot) (Burch-Smith et al, 2004; Robertson, 2004; Aly et al, 2009) with relatively low cost.

MATERIALS AND METHODS

Plant Materials and growth conditions:

Nicotiana obtusifolia seeds were obtained from third generation of accession TW143, US *Nicotiana* Germplasm collection. Seeds were germinated and continue to grow on soil in 2.5 inch pots under 12 hours light regime.

RT-PCR

The expression of *Nicotiana obtusifolia FRUITFULL 2* (*NobFUL2*) gene was assayed in cauline and rosette leaves, young bud, pre-anthesis bud, 4dpa fruit and 7 d pre-dehiscence. Total RNA from tissue samples was prepared using Trizol reagent (Invitrogen) and then was subsequently treated with DNaseI (NEB). 1µg of RNA was used for cDNA synthesis with SuperScript III (Invitrogen). Forward Primer 5'-GGTGAAAGAAAGGGAGAAAGA-3' and reverse primer 5'-TATCCAAGGCGAGGATGATA-3' used to amplify 425bp from *NobFUL2*. Reactions were run for 34 cycles at an annealing temperature of 58°C. ACTIN was used as endogenous control.

Quantitative RT-PCR (qRT-PCR)

To test the down-regulation of *NobFUL2* in VIGS-treated plants, RNA was extracted from rosette leaves and pre-anthesis bud tissue. Total RNA was prepared using RNeasy kit (Qiagen) and subsequently treated with DNase (NEB). Total RNA (1µg) was used for cDNA synthesis with SuperScript III (Invitrogen). Leaf cDNA was diluted 1:5 and bud cDNA samples were diluted 1:20. PCR product was amplified using locus-specific primers designed using Primer ExpressTM version 3.0 (Applied Biosystems) Forward primer 5'TCGTCTACGGTTAGGAAGAATATG 3' and reverse primer 5' GGCGAGGATGATATGGTACTACATTA 3' Down-regulated samples were analyzed relative to Independent wild-type samples using ΔΔCt method of analysis.

TRV-VIGS

A 425 bp fragment of *NobFUL2* which the K and C domains of the protein was ar from inflorescence cDNA using primer,5'CGACGACAAGACCCTtactgctactg reverse 5'GAGGAGAAGAGCCCTcatattcttcct 3' introduce compatible ends (uppercase letters) facilitate cloning of *NobFUL2* fragment into LIC vector as described in Dong, et al, Followed by *Agrobacterium tume* transformation and cultivation on selecti medium. The cultures were then mixed ir ratio, Infiltration of seedling with only three was performed after 19-22 days of seed saw the time of infiltration, some seedling left ur (labeled WT), and the rest infiltrated with TRV2-empty (labeled empty) or TRV2-N (labeled FUL). Physiological observations a collection started from the day of infiltrati extended until fruit dehiscence. A calculations were performed on measu between WT, empty vector, and VIGS-*Nc* plants.

RESULTS AND DISCUSSION

NobFUL2 spatial gene expression is sim Tomato *FUL* genes:

With the exception of the intense stud *Arabidopsis* fruit development, most developmental studies have primarily focu fleshy species Tomato for their essential human diet (Giovannoni, 2004). As a conse few studies have investigated the *FUI* expression in dry-fruits species. In *Ni sylvestris* only one ortholog of the *FUL* ge identified (Jang and An; 1999) and anoth ortholog, *NtMADSII*, was described in *Ni Tabuccum* (Jang et al, 2002). On the othe four *FUL* paralogs in tomato were studied (I et al, 2006; Bemer et al, 2012; Burko et al, NobFUL2 protein contains Lys[51] and characteristic of the MADS domain of the family and does not have CaaX-box, but enc the polypeptide "MPQWMLR", simi *Arabidopsis* FUL gene (data not shown). Nc protein share 97% identity to *Nicotianc* NtMADS11 (accession No. AAO12211. NsMADS1 (accession no. NP_001289 NobFUL2 also share 92% identity to Tomatc SlFUL2 (accession no. NP_001294867.1) ar identity to SlFUL1 (TDR4; accessio AAM33098.1). Therefore it was given th NobFUL2. In the current study, *NobFUL* found to be expressed in vegetative organ, leaves. The transcript was present at much level at the young floral bud and mature stages compare to young fruit (Figure1). Thi indicates that it functions in both reproduct vegetative organs. Similar spatial expression

have been reported with NsMADS2, NtMADS11 and predicted their role in floral induction process and the maintaining of the reproductive phase (Jang and An, 1999; Jang et al, 2002). Similar results have been reported with the expression of the Tomato orthologs, *SlFUL1* and *2*, as high in flowers and throughout fruit development, as well as low level transcript in leaves, indicating role in leave development, floral induction and fruit development, particularly in shape, pigmentation, and, most notably, pericarp thickness, which is a feature that differentiates dry and fleshy fruits (Busi et el, 2003; Bemer et el, 2012; Burko et el, 1013; Wang et el, 2014).

VIGS treatment causes *NobFUL2* downregulation in leaves and floral bud tissue:

After using VIGS to knockdown *NobFUL2* gene, it was very important to determine the level of downregulation if any in all treated plants. Therefore RT-PCR was conducted for all 70 *NobFUL2* infiltrated plants. All showed successful down-regulation in comparison with wild type (WT) and the TRV2 empty vector-treated plants (data not shown). As more quantified step, qRT-PCR were performed on seven individuals selected randomly from the VIGS-treated plants (Figure 2-A). The samples were taken two weeks after treatment to give sometime for the virus to spread across all leaves and stem tissue. Compare to the WT, *NobFUL2* expression level in treated-plants leave tissue was 70% less (Figure2-A). Considering the effect of *NobFUL2* downregulation on flowering time and development, re-checking of *NobFUL2* expression level in the bud tissue was necessary step to evaluate and discuss the outcome of VIGS treatments. As expected, the level of *NobFUL2* gene expression in bud tissue was not the same as in leaves tissue of the same plant (Figure2-A, B). In two samples of bud tissue (1A and 6A; Figure 2-B), *NobFUL2* expression was 85% less compare to WT and was even less than *NobFUL2* transcript in leaves. Three samples, 5A, 57A and 79C, have shown expression equal to 70% less than WT and similar to the gene expression level in leaves. Two samples show higher level of *NobFUL2* transcript than its counterpart in leaves, but the expression level was still less than WT by 50%. QRT-PCR data show down regulation of *NobFUL2* gene expression in both leaves and bud tissue which confirm that the phenotype we are getting is correlated with the *NobFUL2*-VIGS treatment is due to the downregulation of *NobFUL2* and that the downregulation was exist in the bud tissue as well as in leaves. Yet is mosaic, but mostly the expression was 70% less than untreated plants (Figure 2-A, B).

Downregulation of *NobFUL2* causes flowering and shorter inflorescence stem:

Nicotiana obtusifolia plant grows and d as a ring of rosette leaves, followed by elongi inflorescence stem (bolting), and then flo The timing of these developmental ev response to down-regulation of *NoFUL* observed and compared with observations type and empty vector-treated control Flowering time, is one of the well-known f of the *FUL* gene (Jang et al, 1999; Pabón-l al, 2012; Ferrándiz and Fourquin 2014). Th flowering time was the first observation recorded. In order to study and quant differences in flowering time, many obs have been recorded on treated, control a plants, including the time of bolting, meas the time from germination to elongation inflorescence when reach 1cm long. significant phenotypes were the number of leaves at bolting, the number of days to (opening) of the first flower. Down-regula *NobFUL2* resulted in delayed bolting compared to wild type and empty vector plants ($p<0.001$) (Figure 3). As shown in F VIGS- *NobFUL2* treated plants bolts at 5 days with more than 10days delay compare which bolt at 41.5±1.7 days and control gr which bolts at 43 days. Another indication in flowering time was shown by the delay time of anthesis of the first flower. VIGS-*No* treated plants have their first flower to anthesis at 71±3 days with more than 13 day compare to WT which start anthesis at 56±3 and empty-group plants to start anthesis at days (Figure 3). The number of rosette le bolting (Figure 4) was also counted as ad measures of flowering time. *NobFUL2* regulated plants had significantly more leaves, 15.8±1 leaves, when compared to wild type, 10.7±0.9 leaves, and empty vecto with 12±1 leaves (Figure 4). According present finding, *NobFUL2* gene plays a promoting floral transition and so affecting time. This finding has been indicated before *Nicotiana* species and *Arabidopsis* (Jang et a Smykal et al, 2007; Ferrándiz et al, 200 variety of other phenotypic abnormalities re including significantly shorter inflorescenc of VIGS-treated plants, 30±3.3 cm, compar WT and empty vector plants which reach and 35.9±2.9 cm long respectively ($p<.001$; 5). This results were correlated with the find average internode lengths of the VIGS-*No* treated plants were significantly shorter th and empty vector plants (data not shown) Ad abnormal phenotypes were recorded and h been indicated in any previous study;

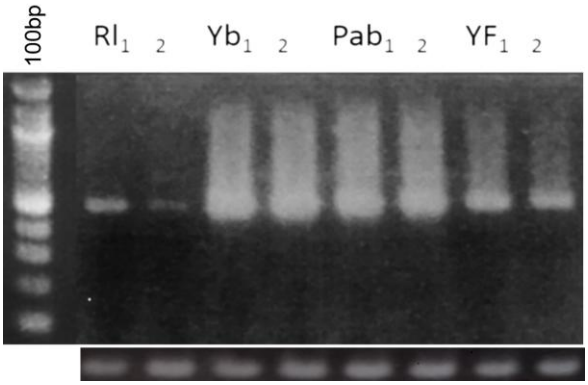

Figure 1: Expression profile of *NobFUL* gene in different type of tissue.
From a wildtype (WT) *N.Obtisofolia*, different tissue samples were collected from two individual Rosette leaves (Rl), Young bud (Yb), Post-anthesis bud (Pab), Young fruit (YF). The level of *N* gene expression is shown. Actin expression was used as an endogenous control (bands at the bott

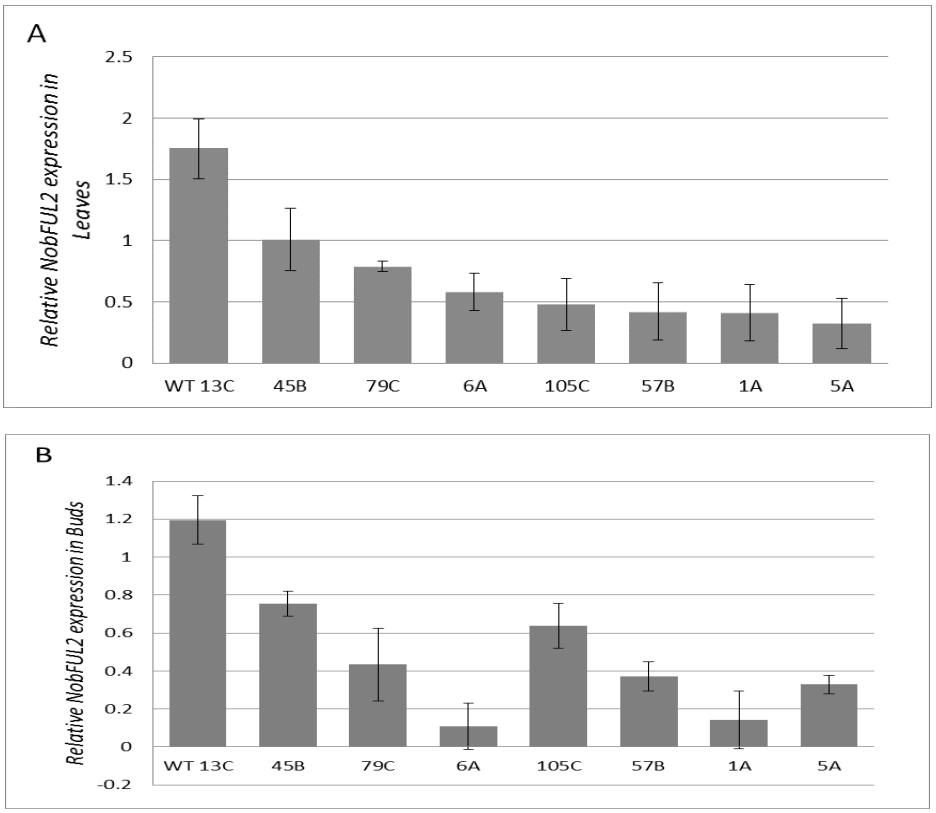

Figure 2: Down-regulation of NobFUL2 in Rosette leaves and Buds of VIGS-treated plants.
(A) Quantitative RT-PCR using cDNA prepared from leaves of VIGS-treated plants showing t change in *NobFUL2* expression relative to wild-type leaves in seven plants (numbered). (B)Quan RT-PCR using cDNA prepared from pre-anthesis buds of VIGS-treated plants that showed regulation of nobful2 in leaves. Values are means. SD for three technical replicates. *N. Obtusifoli* was used as endogenous control.

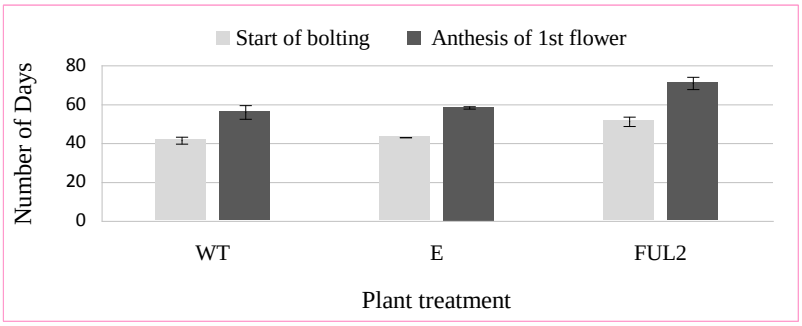

Figure 3: Time for bolting and anthesis of first flower.
Number of days was counted from germination until bolting and until anthesis of first flower seedlings were kept without treatment (WT), some were infiltrated with empty TRV2 vector (some were infiltrated with TRV2 vector containing 425bp *of NobFUL2* gene (FUL2). The dif between controls and *NobFUL2* plants is significant (p<.001) as measured by ANOVA ana significant differences.

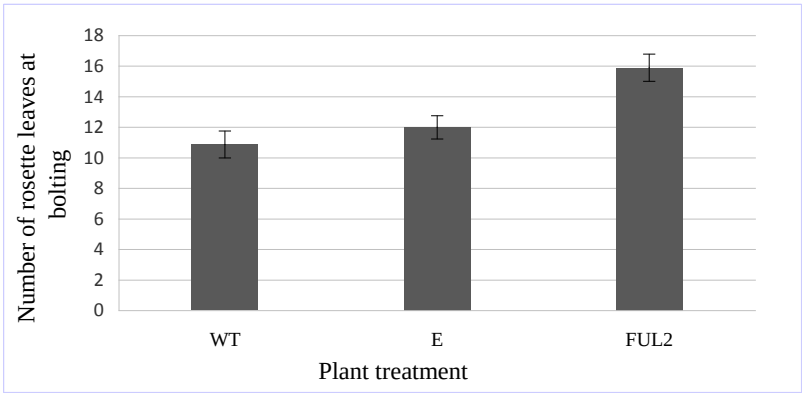

Figure 4: Variation in the number of rosette leaves at the time of bolting.
The number of rosette leaves at the time of bolting for wild type (WT), empty vector (E), and *N* VIGS-treated plants. The difference between controls and *NoFUL2* plants is significant (p<.001).

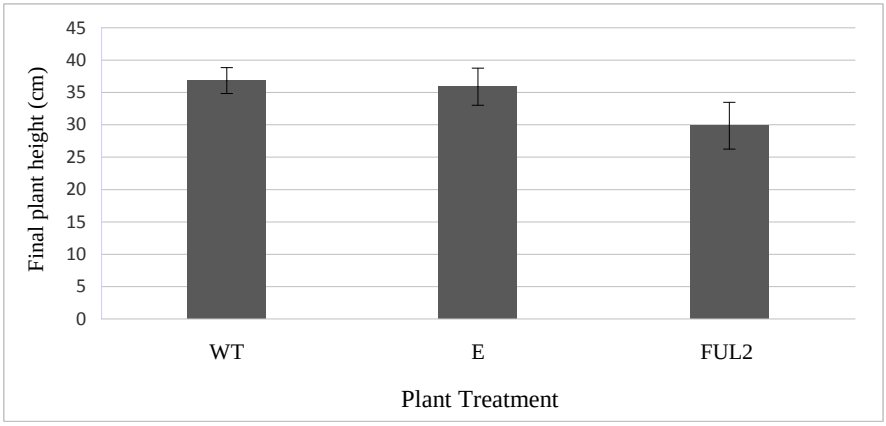

Figure 5: Final inflorescence height of VIGS-NobFUL2 treated plant compared to WT and E Inflorescence stem height is significantly shorter upon *NobFUL2* down-regulation when comp WT and empty-vector (E) plants.

Figure 6: Flower sepal morphology.
(A) Showing a flower at anthesis of the wild type *N. obtusifolia* with normal unfused sepal showing a flower at anthesis of VIGS-NobFUL2 treated plant with longer fused sepal.

increased branching (data not shown) and longer fused sepals (Figure 6) were observed in treated plants. No abnormal developmental or morphological phenotypes were observed in the fruit structures and the timing of fruit dehiscence. That is in contrast to what was expected. Previous studies indicate that the *FUL* genes in *Nicotiana* and *Arabidopsis* have equivalent roles inhibiting fruit dehiscence and so affecting fruit development (Smykal et al, 2007; Ferrándiz and Fourquin, 2014). In Tomato fleshy fruit, upon down-regulation of *SlFUL1* and *SlFUL2* fruit coloring and ripening were impaired including early ripening, discoloration of the fruit, and a thinner pericarp. (Bemer et al, 2012; Wang et al, 2014). These findings suggest that the differences in *FUL2* function play a significant role in distinguishing dry and fleshy fruit development in Solanaceae. Taking in consideration that three *NobFUL* genes were isolated from *N. Obtisfulia* (data not shown). Functional study of all three genes would reveal additional information about floral transition and fruit development. At the end it may lead to novel genetic technologies for modification of *FUL* to develop more economically desirable fruits.

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الملخص العربى

FRUITF هو احد جينات عوامل النسخ التابعة لمجموعة MADS-box وهو عامل مؤثر نمو نبات النيكوتين من حيث تعزيز الانتقال الى مرحلة الازهار ولكنه لم يغير تطور الثمار الكابسولية بشكل ملحوظ فى هذا النبات

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فهم آليات الوراثة التى تؤثر على نمو وتطور الازهار والثمار هو أمر بالغ الأهمية للوصول الى برامج تربية ير من المحاصيل الزراعيةالهامة. مجموعة MADS من جينات عوامل النسخ المعروفة بمساهمتها الكبيرة في على نمو الثمار. الجين *FRUITFULL* هو واحد من تلك الجينات التي تلعب دورا هاما في هذا الصدد. لمزيد وظيفة هذا الجين في تطور الثمار الكبسولية الجافة، فقد استخدامنا فى دراستنا هذه طريقة الناقل الفيروسى ، لوقف او اضعاف التعبير الجينى للجين *FRUITFULL* وذلك على نبات *Nicotiana obtusifolia* المستخدم بات نموذجى.

اتات المعالجة أظهرت تأخر فى الازهار وقصر طول الساق بالاضافة الى التحام السبلات، بالمقارنة مع النوع مجموعة الضابطة. ومن ناحية اخرى لم يلاحظ أي تغير فى نمو و شكل الثمار بين النباتات المعالجة. وقف ، التعبير الجينى للجين *FRUITFULL* فى نبات *Nicotiana obtusifolia* اضاف فهما اعمق لوظيفة هذا الجين ت العائلة الباذنجانية ذات الثمار الجافة .نتائج هذه الدراسةتقترح ان الجين *FRUITFULL* يلعب أدوارا مختلفة ر الثمارالجافة بالمقارنة مع الثمار اللحمية داخل هذه العائلة.

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