12-2011

MSH1 INFLUENCE ON PLANT MITOCHONDRIAL GENOME RECOMBINATION AND PHENOTYPE IN TOBACCO

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MSH1 INFLUENCE ON PLANT MITOCHONDRIAL GENOME RECOMBINATION AND PHENOTYPE IN TOBACCO

by

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A THESIS

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Master of Sciences

Major: Biological Sciences

Under the Supervision of Professor Sally Mackenzie

Lincoln, Nebraska

December, 2011

MSH1 INFLUENCE ON PLANT MITOCHONDRIAL GENOME RECOMBINATION AND PHENOTYPE IN TOBACCO

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University of Nebraska, 2012

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Plant mitochondrial genomes are composed of unusually complex structures, due to active recombination at numerous repeated sequences in the genome. The maintenance of mitochondrial genome stability is under the control of identifiable nuclear genes. In plants, three nuclear genes (*MSH1*, *RECA3* and *OSB1*) have been shown to participate in recombination surveillance and the suppression of illegitimate recombination in mitochondria. Disruption of these loci in Arabidopsis results in reproducible mitochondrial genome rearrangements. We demonstrated that repeat-mediated *de novo* recombination was also enhanced in both Arabidopsis and tobacco during passage through *in vitro* culture. Furthermore, *in vitro* conditions led to suppression of *MSH1* and *RECA3* expression. Subsequent regeneration processes restored normal *MSH1* transcript levels and mitochondrial DNA configuration in tobacco. Our results show the utility of *in vitro* culture as an effective means to study the dynamic features of plant mitochondrial genomes and to facilitate more complete mitochondrial sequence assembly in plants.

Disruption of the nuclear gene *MSH1*, which functions in maintaining mitochondrial and chloroplast genome stability, produces an array of unusual plant growth phenotypes and imparts stress tolerance in Arabidopsis. Similarly, transgenic suppression of *MSH1* by RNA interference in crop plants (tobacco, tomato and soybean)

produces mitochondrial genome alterations and the associated phenotype of cytoplasmic male sterility. We have observed other phenotypes in *MSH1*-RNAi tobacco, including dwarfism, enhanced branching, altered leaf morphology, and delayed flowering. The dwarfed growth phenotype was partially reversed by application of gibberellic acid (GA). We have characterized these novel phenotypes, and shown them to be heritable in lines lacking the RNAi transgene. We have also investigated their behavior in crossing.

ACKNOWLEDGEMENTS

First, I express my sincere and deepest gratitude to my advisor Dr. Sally Mackenzie, who has been supporting and encouraging me by all means during the time of my research and study. I also thank my committee members Dr. Tom Elthon, Dr. Alan Christensen, Dr. Jeffrey Mower and Dr. Dong Wang, for critically analyzing and taking great interest in my work.

I would like to extend my gratitude to my current and former colleagues from the lab, Dr.Ying-Zhi Xu, Dr. Yashitola Wamboldt, Dr. Maria Arrieta-Montiel, Dr.Jaime Davila, Dr.Vikas Shedge, Roberto de la Rosa Santamaria, Kamaldeep Virdi and Geetanjali Kandari, for their great support and helpful suggestions on my research. I also thank Dr. Tom Clemente and Shirley Sato for the assistance in my research project.

Finally, I would like to thank my parents for their love, support and cheer all the time. I wish to share my happiness of degree achievement with them.

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CHAPTER ONE

Literature Review

Heteroplasmy and stoichiometric complexity of plant mitochondrial genomes

Early evidence about the origin of mitochondria has suggested that mitochondria arose from an endosymbiotic event involving the encapsulation of α-proteobacteria in an ancestor common to all extant eukaryotes a billion years ago (Gray et al., 1999). A subsequent endosymbiotic event gave rise to the present day chloroplast, during which a cynobacteria-like organism is in association with a eukaryote which has already contained mitochondria. Following this important event, the divergence between the plant and animal mitochondrial genomes is established. Contrary to animal mitochondrial genomes, which are characterized by a highly homogenous population of DNA molecules of small size (usually between 16-20 kb; Boore et al., 1999), often circular structures with dense gene organization, plant mitochondrial genomes have several distinctive features that include large size (379-2,900kb in the single plant family Cucurbitacea; Alverson et al., 2010), variable organization and multipartite DNA structures combining large linear and small circular or branched forms (Oldenburg and Bendich 1996; Backert et al.,1997).

The plant mitochondrial DNA population is commonly referred to as heteroplasmic, which refers to the fact that a predominant main genome is accompanied by substoichiometric mitochondrial DNA molecules, termed "sublimons". In plants, these sublimons are found in 10- to100- fold lower abundance compared with the main genome (Laser et al., 1997; Woleoszynska and Trojanowski, 2009). In one case, the copy number of sublimons was estimated at one copy per 100-200 cells (Arrieta-Montiel et al., 2001).

Heteroplasmy is currently believed to be the natural physiological state of plant mitochondria (Arrieta-Montiel et al., 2001; Welch et al., 2006), although substoichiometric forms were initially described in mitochondrial mutants (Small et al., 1987), breeding lines (Bellaoui et al., 1998) and tissue cultures (Kanazawa et al,1994).

Heteroplasmy imparts the evolutionary benefit of genetic diversity through a process called substoichiometric shifting - the transition in the concentration of mitochondrial DNA configuration relative to the prevalent form. The ratio of prevalent mitochondrial forms to substoichiometric forms is variable, subject to the control of nuclear and/or environment factors (Arrieta-Montiel and Mackenzie, 2011), which is validated in experiments in Chapter Two. Substoichiometric shifting can be detected by DNA gel blot analysis and standard PCR techniques, during which mitochondrial forms existing at a substoichiometric level are shifted to the approximately the same copy number of the principal genome.

Recent evidence indicates that asymmetric recombination activity at intermediate-size genomic repeats in plant mitochondria contributes to substoichiometric shifting. (Arrieta-Montiel et al., 2009; Davila et al. 2011). Another suggested mechanism for substoichimetric shifting is the favored replication of the subgenomic mitochondrial DNA molecules (Kanazawa et al., 1994). It was postulated that tobacco mitochondrial genome alterations from plant to callus stage were probably the consequence of DNA recombination while genome configuration reversion occurring during tobacco regeneration could only result from the preferential amplification of the mitochondrial DNA molecules (Kanazawa et al., 1994). Whether this favored replication operates by its own or is facilitated in any way by recombination remains unclear.

Plant mitochondrial recombination and nuclear gene control

Plant mitochondrial genomes are typically composed of direct and inverted repeat sequences of up to several kilobases in length (Allen et al., 2007; Unseld et al., 1997). Homologous recombination at the large (>1,000 bp) repeats in plant mitochondrial genomes are of high frequency, resulting in the formation of multiple forms of genomic and subgenomic molecules (Mackenzie and McIntosh, 1999). Apart from the homologous recombination that is prevalent at large repeats within plant mitochondria, a low frequency ectopic recombination activity at intermediate (50 to 550 bp) repeats involves asymmetric DNA exchange, which leads to the accumulation of only one of the predicted recombinant products (Shedge et al., 2007). Intermediate repeats can be large in number in most plant mitochondrial genomes. In Arabidopsis, approximately 47 intermediate repeat pairs (Arrieta-Montiel et al., 2009; Davila et al., 2011) account for the de novo asymmetric recombination that is detected. In some other plant species like tobacco, 26 repeats from 200 to 405 bp are predicted (Arrieta-Montiel et al., 2009), and Chapter Two shows evidence of recombination among these repeats.

Nuclear genes have been identified that influence plant mitochondrial genome recombination. Introduction of the nuclear fertility restorer (Fr) gene to a cytoplasmic male sterile (CMS) common bean line restores pollen fertility accompanied by mitochondrial DNA rearrangements within restored plants. These rearrangements are also identical to those observed upon spontaneous cytoplasmic reversion to fertility, and result in copy number suppression of the mitochondrial male sterility sequence *pvs-orf239* (Mackenzie and Chase, 1990). At least three nuclear genes have been cloned and reported to participate in the control of plant mitochondrial recombination in Arabidopsis: *MSH1*,

RECA3 and *OSB1*. The nuclear gene *MSH1*, originally designated *CHM1-1* (Redei, 1973), was shown to encode the homolog of *E.coli* MutS protein that participates in mismatch repair and suppression of illegitimate DNA recombination (Abdelnoor et al., 2003). Mutation of *MSH1* results in accelerated mitochondrial DNA exchange activity at 47 repeat sites in Arabidopsis (Arrieta-Montiel et al., 2009; Davila et al., 2011). Disruption of *RECA3,* which resembles bacterial recombination component *RecA*, leads to recombination at a subset of the repeated sites active in the *msh1* mutant, while mitochondrial rearrangement patterns in *recA3* and *msh1* double mutants suggested *MSH1* and *RECA3* function in distinct but overlapping pathways (Shedge et al., 2007). *OSB1* (organellar single-stranded DNA binding protein 1) is also believed to play a role in controlling stoichiometry of alternative mtDNA forms generated by recombination, and *OSB1* T-DNA mutants appear to display a more gradual accumulation of mitochondrial rearrangements (Zaegel et al., 2006). Among these nuclear genes, *MSH1* appears to have the most profound effect on plant mitochondrial recombination surveillance and inherited plant phenotype-associated recombination activities (Shedge et al., 2007; Sandhu et al., 2007). In plants, while mitochondrial substoichiometic shifting can occur spontaneously at low frequency, disruption of nuclear genes *MSH1*, *RECA3* or *OSB1* appears to be an effective way to enhance DNA exchange activity.

An application of *in vitro* tissue culture to the study of the plant mitochondrial genome

Earlier evidence has shown mitochondrial genome polymorphism in numerous plant species, such as maize, wheat, cucumber, Brassicas and tobacco when passaged through tissue culture. These mitochondrial alterations include disappearance or decrease in the

relative stoichiometry of particular molecules in wheat callus culture (Rode et al., 1987), and the amplification of pre-existing substoichiometric mitochondrial DNA molecules in cultured cells of *Brassica campestris* (Shirzadegan et al., 1989). In T-cytoplasm maize, reversion to fertility via substoichiometric shifting occurs by passaging through culture and regeneration of whole plants. Under *in vitro* culture condition, it has been suggested that the mitochondrial alterations are associated with repeat-mediated recombination (Vitart et al., 1992; Kanazawa et al., 1994). The derived recombinant molecules are found to be amplified during the regeneration process in *Nicotiana sylvestris* (Vitart et al., 1992), or alternatively, can be reversed to substoichiometric levels as reported in plants regenerated from *Nicotiana tabacum* callus (Kanazawa et al., 1994).The involvement of nuclear genes responsible for the control of mitochondrial rearrangements under tissue culture condition has also been reported (Hartmann et al., 2000).

Speculation has been made of association of the nuclear gene *MSH1* with enhanced mitochondrial recombination under cell suspension culture conditions, suggesting that *MSH1* might be suppressed in its expression or activity (Arrieta-Montiel et al., 2009). Chapter Two will test this model, showing that when Arabidopsis and tobacco were passaged through *in vitro* culture, the function of nuclear genes *MSH1* and *RECA3* in maintaining mitochondrial genome stability was relaxed, resulting in repeat-mediated *de novo* mitochondrial recombination. This feature of *in vitro* culture can be exploited to solve the problem of incomplete mitochondrial genome information for physical mapping that arises due to difficulties in accessing substoichiometric molecules. It is providing a useful system for the assembly of mitochondrial genome sequences into a more accurate physical map of the genome.

Influence of the nuclear gene *MSH1* on plant phenotype, development and responses to the environment

Although the extensive mitochondrial genome polymorphism in higher plants has been well documented over the past two decades, the genetic control and phenotypic consequences are only recently emerging. Among the aberrant plant phenotypes originating from mitochondrial genome dysfunction in higher plants, the most prevalent phenotype was cytoplasmic male sterility (CMS), which refers to a maternally inherited inability to produce viable pollen in an otherwise phenotypically normal plant. CMS has been observed in over 150 plant species (Laser and Lersten, 1972). In most of the cases, CMS is caused by the expression of novel mitochondrial open reading frames (ORFs) associated with mitochondrial genome rearrangements (Hanson and Bentolila, 2004). Suppression of *MSH1* in tobacco, tomato and soybean by RNAi confirmed a condition of heritable male sterility accompanied by reproducible mitochondrial DNA rearrangements and transcriptome responses, implying that *MSH1*-associated mitochondrial genome changes can give rise to CMS (Sandhu et al., 2007; Sandhu, unpublished).

Early publications documented other phenotypes including alterations in plastid development to produce leaf variegation and mosaic phenotypes (Sandhu et al., 2007; Bartoszewski et al., 2007), altered GA-mediated pathways for growth and flowering (Pellny et al., 2008), changes in stem height and flowering time (Albert et al., 2003), and changes in leaf morphology (Mackenzie unpublished). In *msh1* Arabidopsis, the phenotypes including reduced growth rate, delayed flowering (Shedge et al., 2007), altered leaf morphology and enhanced thermotolerance (Shedge et al., 2010). Recent results suggested these phenotypes are the consequence of chloroplasts changes in

response to *MSH1* disruption (Xu et al., 2011; 2012). A wide range of emerging phenotypes and physiological changes were observed upon *MSH1* disruption, facilitating an understanding of the role chloroplasts play in plant cellular and developmental processes.

The nuclear gene *MSH1* is a *MutS* homolog that suppresses homologous mitochondrial DNA exchange in plants. Fused fluorescence reporter gene assays validated the localization of *MSH1* to both mitochondrial and plastid nucleoids in Arabidopsis (Xu et al., 2011). Genetic hemicomplementation experiments further demonstrated *MSH1* functions within the mitochondrion and plastid to influence organellar genome behavior and plant growth patterns in Arabidopsis (Xu et al., 2011). Dual targeting of *MSH1* in Arabidopsis also enhances the plant's stress responses to the environment. *MSH1* regulation at a cellular metabolic and developmental level in crop plants such as tobacco, sorghum and tomato is a subject under future investigation.

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CHAPTER TWO

Utility of *in vitro* culture to the study of plant mitochondrial genome configuration and its dynamic features

Abstract

Recombination activity plays an important role in the heteroplasmic and stoichiometric variation of plant mitochondrial genomes. Recent studies show that the nuclear gene *MSH1* functions to suppress asymmetric recombination at 47 repeat pairs within the Arabidopsis mitochondrial genome. Two additional nuclear genes, *RECA3* and *OSB1*, have also been shown to participate in the control of mitochondrial DNA exchange in Arabidopsis. Here, we demonstrate that repeat-mediated *de novo* recombination is enhanced in Arabidopsis and tobacco mitochondrial genomes following passage through tissue culture, which conditions *MSH1* and *RECA3* suppression. The mitochondrial DNA changes arising through *in vitro* culture in tobacco were reversible by plant regeneration, with correspondingly restored *MSH1* transcript levels. For a growing number of plant species, mitochondrial genome sequence assembly has been complicated by insufficient information about recombinationally -active repeat content. Our data suggest that passage through cell culture provides a rapid and effective means to decipher the dynamic features of a mitochondrial genome by comparative analysis of passaged and non-passaged mitochondrial DNA samples following next-generation sequencing and assembly.

Introduction

The plant mitochondrial genome is organized into an unusual multipartite structure derived from high and low frequency DNA recombination between repeated sequences in the genome (Fauron et al. 1995). Large sized (>1,000 bp) repeats participate in high-frequency reciprocal DNA exchange to subdivide the genome and facilitate inter-conversions between DNA molecules (Mackenzie and McIntosh 1999). Intermediate-sized (50 to 550 bp) repeats mediate low frequency asymmetric DNA exchange that results in accumulation of only one of the expected recombinant products (Shedge et al. 2007). Frequency of DNA exchange at these intermediate repeats appears to control the relative copy number of the recombinant forms within the genome.

In plants, the mitochondrial DNA population in vegetative tissues is organized into predominant and substoichiometric DNA configurations. Changes in relative abundance of these mitochondrial genomic forms, often occurring within a single plant generation, is referred to as substoichiometric shifting (SSS) (Arrieta-Montiel and Mackenzie 2011), a phenomenon first reported in maize (Small et al. 1987). The SSS process participates in the rapid generation of mitochondrial genome variation within a plant species (Davila et al. 2011), and appears to underlie reversible phenotypic transitions between cytoplasmic male sterile to male fertile plants within a population (Mackenzie 2011). Asymmetric recombination at intermediate-size repeats in mitochondria accounts for SSS activity (Arrieta-Montiel et al. 2009).

Three nuclear genes have been cloned and reported to participate in the control of plant mitochondrial recombination in Arabidopsis: *MSH1, RECA3* and *OSB1* (Abdelnoor

et al. 2003; Shedge et al. 2007; Zaegel et al. 2006). Among these nuclear genes, *MSH1* appears to have the most profound effect on plant mitochondrial recombination surveillance and inherited plant phenotypic effects (Shedge et al. 2007; Sandhu et al. 2007). In Arabidopsis, mutation of *MSH1* elevates the mitochondrial DNA exchange activity at 47 repeat pairs ranging from 50-550bp in size (Arrieta-Montiel et al. 2009; Davila et al. 2011). Although mitochondrial recombination and SSS activity can occur spontaneously at low frequency, disruption of nuclear genes *MSH1*, *RECA3* or *OSB1* enhances SSS frequency.

Earlier evidence of mitochondrial DNA polymorphisms arising in cultured cells is plentiful (Rode et al. 1987; Shirzadegan et al. 1989; Vitart et al. 1992; Hartmann et al. 2000). Most provocative among these reports is the observation of recombination events in tobacco that are reversible with plant regeneration, implying SSS activity under culture conditions (Kanazawa et al. 1994). We investigated the nature of mitochondrial genome changes under tissue culture conditions to assess the feasibility of capitalizing on this process for mitochondrial genome mapping. Here we present evidence that tissue culture results in reduced expression of both *MSH1* and *RECA3*, together with enhanced recombination at intermediate repeats. This effect is reversible with plant regeneration, providing a useful system for the assembly of mitochondrial genome sequences into a more accurate physical map of the genome.

Materials and methods

Sterilization and plating of Arabidopsis and tobacco seeds

Seeds were measured in an Eppendorf tube to 100 ul. 1 ml 100% ethanol was added into tube. The tube was vortexed or turned upside down for 1 minute. After the seeds settled, ethanol was taken out with a pipet.1 ml 50% (v/v) Clorox with a drop of Tween 20 was added to tube. Seeds were kept shaking in Clorox for 20 minutes (Arabidopsis) or 30 minutes (tobacco). Five washes with sterile water were performed. Each time after 1 ml water added, the tube was flicked upside down to make sure all the seeds get rinsed. After seeds settled, water was taken out and then fresh water was added to get the seeds in suspension. 1 ml seeds solution was added to a Petri dish with 0.5X Murashige and Skoog media and then 1ml sterilized 0.4% agar (w/v) was added on top. The plate was tilted to get the seeds spread more or less evenly over the surface. Antibiotic selection was performed by adding cefotaxime at 100 mg/ml to the germination plates. The seed plates were placed in a growth chamber at 12-h daylength and 24 ℃ for germination.

Arabidopsis and tobacco callus induction

Callus-inducing medium for Arabidopsis consists of basic Murashige & Skoog (1962) inorganics and MS vitamins supplemented with 1 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg L^{-1} benzylaminopurine (BAP), 1 mg L^{-1} 1-naphthyl acetic acid (NAA), and 1 mg L^{-1} Indole-3-acetic acid (IAA). Callus-inducing medium for tobacco includes basic Murashige & Skoog inorganics and Gamborg's B5 medium vitamins, supplemented with 0.25 mg L^{-1} benzylaminopurine (BAP), 1 mg L^{-1} 1-naphthyl acetic acid (NAA). Basic Murashige & Skoog inorganics media with 3% (w/v) sucrose were adjusted to pH 5.7 $\pm$0.1 before the addition of 0.8% (w/v) agar and then sterilized by autoclaving. MS medium and Gamborg's B5 medium vitamins supplemented with plant hormones were

adjusted to pH 5.0 ±0.1 and filter sterilized before being added into the basic Murashige & Skoog inorganics media.

In a sterile hood environment, leaf segments of Arabidopsis (8-10 days old) and tobacco (14-16 days old) were detached and lightly abraded on surface with a sterilized scalpel and transferred to callus-inducing medium for callus initiation, following the protocol of Encina et al. (2001). Callus cultures were maintained in the dark at 24 ℃.

Tobacco regeneration

Tobacco shooting medium consisted of basic Murashige & Skoog inorganics and Gamborg's B5 vitamins, supplemented with 1 mg L^{-1} benzylaminopurine (BAP), 0.1 mg L^{-1} 1-naphthyl acetic acid (NAA) and 3% (w/v) sucrose. Tobacco rooting medium included basic Murashige & Skoog inorganics, Gamborg's B5 vitamins, 0.1 mg L^{-1} 1-naphthyl acetic acid (NAA) and 1% (w/v) sucrose. All media were solidified with 0.8% (w/v) agar (pH 5.7 ±0.1). To induce regeneration of tobacco shoots, 3- to 4- week-old tobacco callus was cultured on tobacco shooting medium. After formation of shoots, the newly grown shoots were cut and transferred to new shooting medium. After the 1st subculture, regenerated young seedlings were transferred to tobacco rooting medium in culture cups and later transferred to potting mix later when enough roots were emerging. Shooting and rooting processes were maintained in a growth chamber at 12- h daylength and 24 ℃.

Arabidopsis cell suspension culture from callus (optional)

Cell suspension culture medium is the same as callus-inducing medium for Arabidopsis without the addition of agar. In a sterile hood environment, approximately 3 g of 3- to 4-week-old calli were weighed and transferred in 80 mL of liquid cell suspension medium in a 100 ml Erlenmeyer flask and incubated in a rotary shaker set at 110 rpm, at 24 ℃ in dark. Cell suspension was subcultured every 3 days by settling the cells down in the bottom of the flask and replacing the entire supernatant with 80 ml of fresh suspension medium using a pipet. Cell clumps were sieved through 850 µm sieves (Sigma) to obtain a homogenous material for suspension culture. Dark yellow dead tissue was removed.

Usually the initiation of 3 g (fresh weight) callus suspended in 80 ml of medium is subcultured and sieved every 3 days, and a double increase in fresh weight is obtained during a growth period of 10 days. At this stage, the suspensions can be divided into two portions. To achieve this, approximately 80 ml of fresh suspension medium was added to the 80 ml cell suspension in the flask. After swirling the flask, 80 ml aliquots were dispensed into new flasks (Mathur and Koncz 1998). Alternatively, instead of dividing, the entire approximate 160 ml cell suspension can be transferred into new 500 ml flask for expansion culture. With this technique, a fine stable cell suspension can be obtained after approximate 2 months of incubation; with regular subculturing it can be maintained for a much longer period.

Total genomic DNA isolation, gel blot and PCR assays

1-, 2-, 3- and 4-week-old Arabidopsis and tobacco calli were sampled. Young leaves from Arabidopsis Col-0 and *msh1* mutant plants (Abdelnoor et al. 2003) and tobacco wild-type (Xanthi) plants served as control samples. Total genomic DNA was isolated

from calli and plants, according to Li and Chory (1998). Total genomic DNA was digested with *Bam*HI (Arabidopsis) and *Cla*I (tobacco), and analyzed by DNA gel blot hybridization (Hybond-N, Amersham). Mitochondrial DNA repeats in Arabidopsis and tobacco mitochondrial genomes were PCR amplified, labeled with [α-^{32}P] dCTP by random priming (Stratagene), and used as probes. Primers for PCR amplification of Arabidopsis and tobacco mitochondrial repeats are listed in Table 2.1.

Total genomic DNA from tobacco regenerants at three different growth stages was assayed by three-primer competitive PCR, which is to detect parental molecules and recombinant molecules amplified by three primers flanking a pair of repeat sequences in one reaction. Primers (Table 1) were designed to assay each environment flanking Repeat A. Actin was used as an internal control.

RNA isolation and Real-Time quantitative PCR analysis

Total RNA was extracted from Arabidopsis and tobacco callus, and wild-type and regenerated plants, with TRIzol (Invitrogen) and purified with the plant RNeasy kit (Qiagen). RNA was purified and normalized to equal initial amount used to synthesize first-strand cDNA (SuperScript III First-Strand Synthesis SuperMix for qRT-PCR; Invitrogen). cDNA then were used for quantitative PCR with SYBR GreenER for iCycler (Invitrogen). Quantitative PCR analysis used iCycler iQ software (version 3.1, Bio-Rad). The ubiquitin gene was used as an internal standard in gene expression analyses. Fold change for *MSH1, RECA3* or *OSB1* expression in each sample, compared to wild-type, was calculated as $2^{-\Delta\Delta Ct}$ ($\Delta\Delta C_t = \Delta C_{tS} - \Delta C_{tWT}$, $\Delta C_{tS} = C_{t\mathit{MSH1}} - C_{tUbq}$, $\Delta C_{tWT} = C_{t\mathit{MSH1}}$ -

C_{tUbq}). Primers for RT-PCR analysis of ubiquitin, *MSH1*, *RECA3* and *OSB1* in Arabidopsis and ubiquitin and *MSH1* in tobacco are included in Table 1.

Results

Mitochondrial recombination increased markedly at very early stages of Arabidopsis and tobacco callus culture

Initiation of callus formation could be detected one-week following placement of Arabidopsis and tobacco leaf segments on callus-inducing medium, with mature friable callus formed after 4 weeks (Fig. 2.1). Total genomic DNA extracted from 1-, 2-, 3- and 4-week-old calli was digested with *Bam*HI (Arabidopsis) or *Cla*I (tobacco) for mitochondrial genome analysis. In Arabidopsis, repeat-mediated recombination was detected in 1-week-old cultures, with the level of recombination increasing over time. The 4-week-old cultured tissues displayed the same 4.1-kb recombinant form at Repeat F that is observed in the *msh1* mutant (Arrieta-Montiel et al. 2009), (Fig. 2.2a). Recombination at Repeat D gave the predicted 2.2-kb recombinant molecule in callus culture, again increasing with time (Fig. 2.2a). In the recombination events at Repeat F and D, both parental forms were also retained. In tobacco callus, recombination at Repeat A was evident in the predicted recombinant 6-kb form (Fig. 2.2c), which was observed only at very low levels in the wild-type plant.

MSH1 and *RECA3*, but not *OSB1*, expression levels are modulated under tissue culture conditions

Real-time PCR analysis in Arabidopsis and tobacco callus showed down-regulation of *MSH1* expression relative to wild-type (Col-0 and Xanthi) plant samples (Fig. 2.3). In both Arabidopsis and tobacco, the decrease in expression was more pronounced with age

of the callus. *RECA3* expression was also down- regulated in Arabidopsis, but *OSB1* transcript levels did not change under tissue culture conditions. We suggest that the mitochondrial genomic rearrangements observed are the likely consequence of altered *MSH1* and *RECA3* expression.

***MSH1* expression is responsible for SSS activity during tobacco plant regeneration**

During tobacco regeneration from 4-week-old callus, three stages were investigated (Fig. 2.4a): Stage 1, one month following transfer of 4-week-old callus to shooting medium; Stage 2, two months following callus transfer, when roots emerge in rooting medium; and Stage 3, one week following transfer of the young seedling to potting mix. Genomic DNA was extracted from the different growth stages, and three-primer competitive PCR analysis (Fig. 2.4b) allowed resolution of changes in relative stoichiometries for parental and recombinant forms during the regeneration process. Recombinant forms were predominant in callus, and Stage 1 reversed the trend back toward substoichoimetric levels in the subsequent regeneration stages. Recombinant and parental forms were confirmed by DNA sequencing. Similarly, down-regulation of *MSH1* expression in Stage 1 was gradually reversed during regeneration, reaching normal *MSH1* transcript levels in the regenerated plant (Fig. 2.4c).

Discussion

Over the past twenty years, mitochondrial genome rearrangements were often reported to occur in plant cells grown *in vitro* (Cloutier et al. 1994). It was generally thought that these rearrangements were the consequence of extended culture periods. Evidence presented here of mitochondrial SSS activity within the first week of callus culture

suggests that the rearrangement activity is non-random and initiates immediately. Previous studies of the *msh1* mutant in Arabidopsis, involving 47 mitochondrial repeats that are enhanced in DNA exchange activity (Arrieta-Montiel et al. 2009; Davila et al. 2011), permitted assay of SSS activity under cell culture conditions. These earlier studies showed that an "early" generation *msh1* mutant produces mitochondrial genome rearrangements at lower frequency that are reversible following re-introduction of the *MSH1* gene (Davila et al. 2011). The "advanced" generation *msh1* mutants produced more extensive rearrangements that were less readily reversible and could become fixed in the mitochondrial population. Combining the *msh1* and *recA3* mutations resulted in the most extensive mitochondrial genome rearrangements (Shedge et al. 2007). In this study, we have shown that down-regulation of both *MSH1* and *RECA3* occurs under cell culture conditions, suggesting that conditions are appropriate for rapid and extensive mitochondrial genome changes. The effects of *in vitro* culture on the nuclear background giving rise to the mitochondrial DNA rearrangements were reported previously (Hartmann et al., 2000). However, the mechanism underlying the down-regulation of nuclear genes under *in vitro* culture conditions in this study is still unknown.

A question raised by this study is whether plant regeneration would be facilitated if the mitochondrial genome were stably maintained during *in vitro* culture. Tobacco, where plants can be readily regenerated from callus, has been reported to display a highly reversible mitochondrial rearrangement process (Kanazawa et al. 1994). We also observed reversibility of mitochondrial genome rearrangement in tobacco upon regeneration from callus. Might this reversibility be a factor in the plant's amenability to regeneration? There appear to be several plant developmental implications of the *msh1*

recA3 double mutation (Shedge et al. 2007; 2010), suggesting that this type of gene expression change can have dramatic implications for plant growth.

Because the plant mitochondrial genome is characterized by numerous recombinational repeats, assembly of an intact genome sequence, particularly using deep sequence reads of relatively small size, can be extremely difficult. Sequence assembly without the availability of information from substoichiometric forms provides an incomplete picture of the genome, so that intra-specific mitochondrial comparative studies can imply far more extensive genomic variation than is actually present. Information about substoichiometric forms can allow one to deduce the inter-convertibility of related mitochondrial configurations. In many plant species, particularly those being investigated for ecological studies, little mitochondrial genome information is currently available and details of intra-specific mitochondrial relationships may be of crucial importance. In these cases, implementing callus culture for mitochondrial genome analysis may be valuable. Comparative assembly of the callus culture-derived mitochondrial genome sequence and intact plant-derived form would reveal recombinational repeats and substoichiometric forms. This type of information is valuable for subsequent ecotype comparisons and for developing an understanding of evolutionary trends within a species (Davila et al. 2011).

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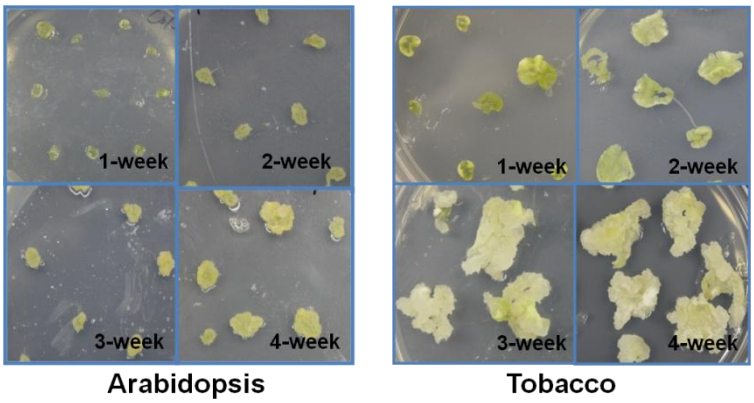

Fig. 2.1 1-, 2-, 3- and 4-week-old Arabidopsis and tobacco calli on callus-inducing medium.

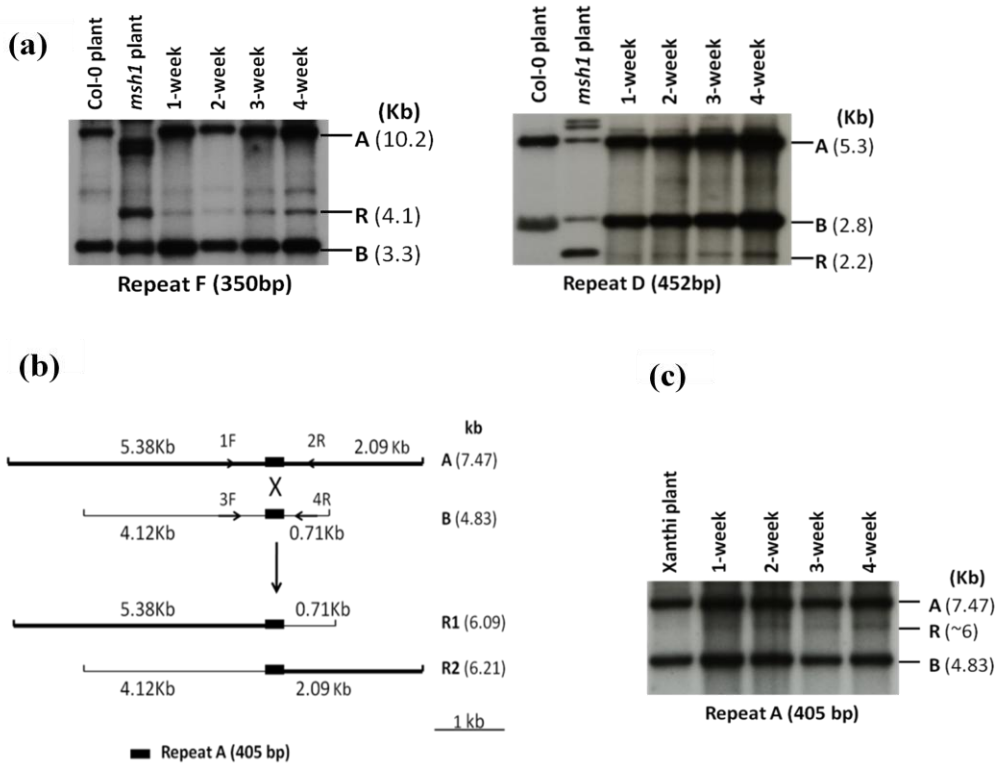

Fig. 2.2 Repeat-mediated recombination in Arabidopsis and tobacco callus cultures. DNA gel blot analysis shows changes in mitochondrial DNA configurations, with A and B designating parental configurations and R the recombinant form. The indicated repeats were used as probes.

(a) Analysis of recombination at Repeats F and D in1-, 2-, 3- and 4-week-old Arabidopsis callus. Col-0 and *msh1* mutant plant tissues served as controls, and DNA was digested with *BamH*1.

(b) *Cla*I restriction map of the parental forms (A and B) and the predicted recombinant forms (R1 and R2) in tobacco mitochondria. 1F, 2R and 4R are primers designed for three-primer competitive PCR.

(c) Evidence of mitochondrial DNA recombination at Repeat A in 1-, 2-, 3- and 4-week-old tobacco callus. Xanthi plant tissue serves as a control, and DNA was digested with *Cla*I.

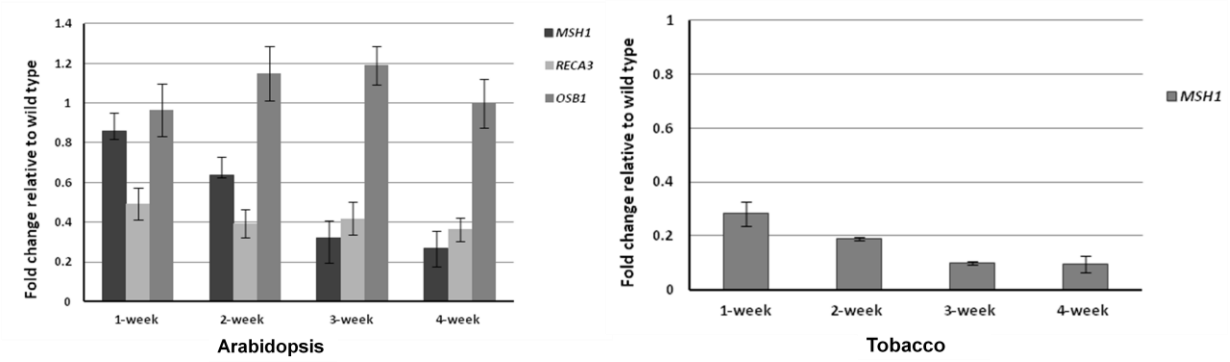

Fig. 2.3 *MSH1*, *RECA3* and *OSB1* gene expression changes in 1, 2, 3 and 4-week-old Arabidopsis and tobacco calli. Gene expression is interpreted as the threshold cycle number (Ct) and normalized as fold change, compared with wild-type control plants set at 100%. Ubiquitin serves as an internal standard. The final results were the average of three biological replicates.

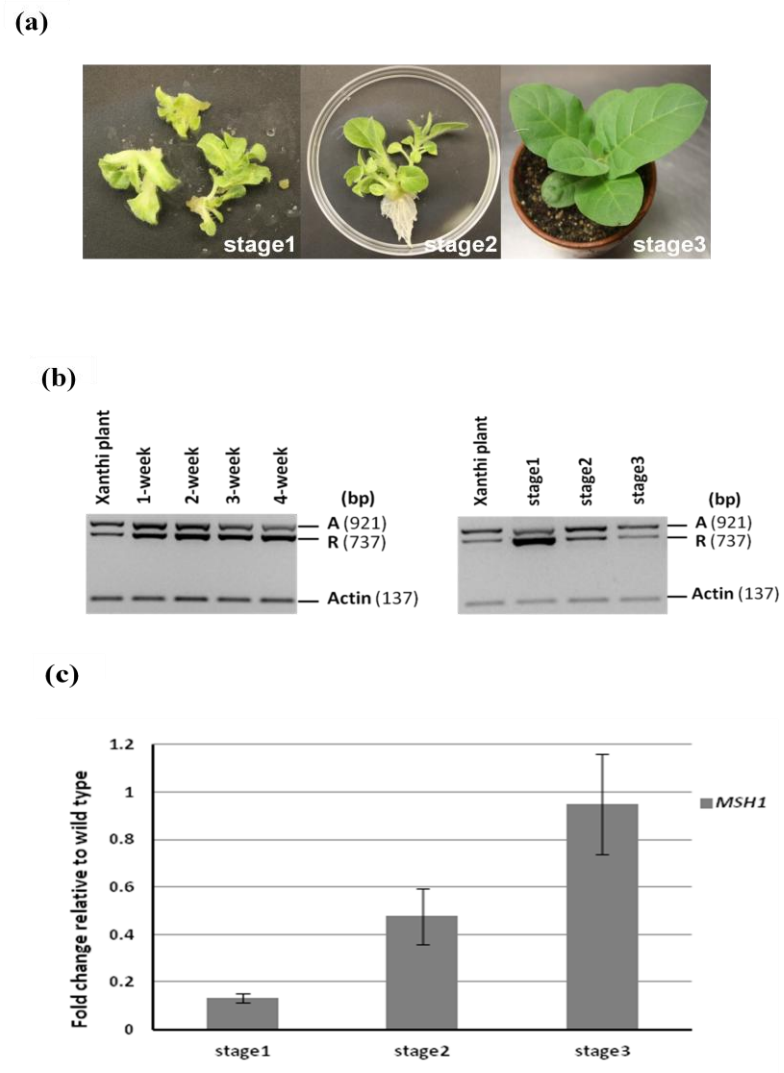

Fig. 2.4 Mitochondrial genome configuration and associated *MSH1* expression changes during tobacco regeneration.

(a) Tobacco regenerants at three different growth stages from 4-week-old callus.

(b) Substoichiometic shifting detected by PCR in experiments involving regeneration from 1-, 2-, 3- and 4-week-old tobacco calli at three stages. Actin is used as an internal standard. A designates the parental band, while R designates the amplified recombinant band. 1F, 2R and 4R are the primers used in three-primer competitive PCR.

(c) *MSH1* gene expression fold change by real-time PCR in regenerated tobacco at three stages. Xanthi plants serve as a control and ubiquitin as an internal standard. Results shown are the average of three biological replicates.

Table 2.1 Primers used in the study

Arabidopsis Repeat D-F	AGTGATCTGTTCATCTAACTCA
Arabidopsis Repeat D-R	TACTACTACCTCGTCCATTG
Arabidopsis Repeat F-F	CACGAGGAATGGAAAGAAACAT
Arabidopsis Repeat F-R	GCGCACAAACCACTCTAAAG
Tobacco Repeat A-F	TGGTAGTCGTGGTTGATTCGAGGAT
Tobacco Repeat A-R	TTAGGGGCGGAATCGAATGATTACG
Tobacco PCR-1F	GCGGCTACGAAGCAGTCAAG
Tobacco PCR-2R	TGAACACTGCTCTGCTGCATG
Tobacco PCR-3F	AGCGAAGAAAGCGGGCTTTG
Tobacco PCR-4R	ATTTCCCTCTATCAGGAACCCGCT
Tobacco Actin-F	GAACGGGAAATTGTCCGCGATGTT
Tobacco Actin-R	ATGGTAATGACCTGCCCATCTGGT
Arabidopsis Real-Ubquitin-F	CACCATTGACAACGTCAAGGCCAA
Arabidopsis Real-Ubquitin-R	CACGCAGACGCAAGACCAAATGAA
Arabidopsis Real-*MSH1*-F	TCATGCGTGTATGTGATGCGGAGA
Arabidopsis Real-*MSH1*-R	ACTTGACCCTTGCAGTCCTTCCTT
Arabidopsis Real-*RECA3*-F	ATCTAACATGCATTTCCCGCACGC
Arabidopsis Real-*RECA3*-R	TGGACGCAGACATTGAGACCACTT
Arabidopsis Real-*OSB1*-F	ACGATTGGTGGGACAACAGGAGAA
Arabidopsis Real-*OSB1*-R	TCTGAGCAAAGCCAGAGAGCTTCA
Tobacco Real-Ubquitin-F	TTTGCACCTTGTGCTTCGTCTTCG
Tobacco Real-Ubquitin-R	CCATCTTCCAATTGCTTTCCCGCA
Tobacco Real-*MSH1*-F	TGATGGATCCTACTTGGGTGGCAA
Tobacco Real-*MSH1*-R	ACCTTTCCATGGCGACTCCATATC

CHAPTER THREE

Phenotypic consequences of RNA interference (RNAi)-mediated suppression of *MSH1* in tobacco

Abstract

Earlier evidence showed that RNAi suppression of *MSH1* in tobacco generated heritable male sterility. This transgenically induced phenotype is associated with mitochondrial DNA rearrangements and shows maternal inheritance in subsequent generations. Here, various phenotypes besides male sterility, including dwarfism, delayed flowering, altered leaf morphology and altered growth branching were documented. Preliminary selection of each heritable phenotype was carried out and additional generations of testing are needed. Plants displaying altered leaf morphology were used as explants for *in vitro* regeneration and genetic crossing; results from these experiments were consistent with a possible epigenetic trait, with no evidence of vegetative cytoplasmic sorting or maternal inheritance. Partial reversal of the dwarf phenotype was also observed with gibberellic acid (GA) application, similar to earlier observations in *MSH1*-RNAi lines of sorghum.

Introduction

Environmentally-induced shifts in phenotype (phenotypic plasticity) play an important role in plant performance in response to environment change (Nicotra et al. 2010). Accumulating evidence suggests that transgenerational phenotype maintenance may involve non-genetic contributions under the control of epigenetic mechanisms. (Kappeler and Meaney 2010; Danchin et al. 2011).

MSH1 is a nuclear gene product that has been shown to be dual targeted to both mitochondria and plastids to maintain genome stability in Arabidopsis. Suppression of *MSH1* expression produces cytoplasmic male sterility and variegation through direct DNA rearrangement of the chloroplast and mitochondrial genomes (Sandhu et al.,2007; Xu et al., 2011). RNAi suppression of *MSH1* also gives rise to additional phenotypic plasticity including dwarfed growth and reduced internode elongation, enhanced branching, reduced stomatal density, altered leaf morphology, and delayed flowering (Xu et al., 2012). Genetic hemi-complementation experiments showed that this phenotypic plasticity derives from changes in chloroplast state (Xu et al., 2011; 2012). The phenotypic consequences occur in response to organellar disruption but there is little or no direct evidence of organellar genome instability contributing to those developmental changes. The altered phenotypes in Arabidopsis and sorghum were partially reversed with gibberelic acid application, which suggests perturbation of the chloroplast-associated GA biosynthesis process (Xu et al., 2012). In the monocot *Sorghum bicolor*, this developmental reprogramming, once established in response to *MSH1* suppression by RNAi, is stably heritable independent of the RNAi transgene in subsequent generations (Xu et al., 2012). Complete reversal of phenotype in *MSH1*-RNAi lines lacking the transgene occurs with pollination by wild-type plants, suggesting epigenetic influences that arise through an organellar signal following *MSH1* suppression (Xu et al., 2012).

In the dicot tobacco, we have documented a wide range of phenotypes emerging with RNAi suppression of *MSH1*. These transgenically-induced phenotypes resemble those observed in Arabidopsis and sorghum, including male sterility, dwarfism, altered flowering time and flower morphology, enhanced branching and altered leaf morphology;

these phenotypes show variable inheritance in subsequent generations, based on preliminary data. Transmission of altered plant phenotypes through tissue culture differed from seed transmission patterns. This study provides evidence to suggest that *MSH1*-RNAi in tobacco produces similar developmental reprogramming as has been observed previously, and provides an alternative model for studies of species differences in phenotypic outcomes.

Materials and Methods

Plant materials

The RNAi construction and transformation procedure for transgenic tobacco plants (*Nicotiana tabacum* cv. Xanthi) is described in a previous study (Sandhu et al.,2007)**.** Seed progeny of T_2 plant 23-5, derived from transformant T_0 (Sandhu et al., 2007) tobacco plant #23, was investigated for phenotype segregation through multiple generations in this study. Tobacco plants were grown under a 14-h light-10-h dark cycle at 25 ℃ in greenhouse mix (40% Canadian Peat; 40% Coarse Vermiculite; 15% Masonry Sand; 5% Screened Topsoil).

Tobacco regeneration from leaves

Thick texture leaves of tobacco plants were collected and rinsed. Leaf sections of uniform size were immersed into a beaker with 10% (v/v) Clorox for 8 min, then rinsed in sterile water. A sterilized hole-punch was used to obtain uniform sized leaf segments. These segments were transferred to tobacco shooting medium and then rooting medium as described in Chapter Two.

Application of exogenous GA_3

The effect of GA_3 on height of tobacco plants (wild-type, transgenic and non-transgenic plants) were tested by spraying the entire plant with a 250-ppm GA_3 solution containing 0.1% (v/v) Tween 80 (Vidal et al., 2001). Control plants were sprayed with a solution replacing GA_3 with 70% (v/v) ethanol. The first treatment was made when plants were 1 month-old and the second was performed 20 days later. Tobacco plant height was measured before each treatment and again 20 days after each treatment.

Results

Variant phenotypes were observed in *MSH1*-RNAi tobacco plants

Altered phenotypes in transgenic *MSH1*-RNAi tobacco lines included dwarfism, delayed flowering, enhanced branching, and wrinkled or thick leaf growth patterns (Fig. 3.1).

Male sterile T_2 plant 23-5 was derived from the confirmed transformant *MSH1*-RNAi tobacco plants from Sandhu et al. (2007). Segregation of variable phenotypes through multiple generations is shown in Table 3.1. All the plants listed in the table were confirmed to be *MSH1*-RNAi transgenic lines by PCR. Male sterility seems to have low heritability through generations (Table 3.1 a). Dwarfism, branching and wrinkled leaf phenotypes were detected in an increasing proportion of the population for each generation (preliminary data in Table 3.1 b, d, e). Altered flowering time in tobacco was classified as 10 days or more later/ earlier than wild-type (Table 3.1 c). Plants observed were generally a maximum of 10 days earlier than normal flowering time, while late flowering plants could be up to several months later than normal. Severely delayed

flowering plant 23-5-3-4 had a larger proportion of late flowering progeny than did the less severe later flowering plant 23-5-3-4-1. Interestingly, two early flowering plant lines generated a larger proportion of late flowering than early flowering plants. Similarly, late flowering plants segregated a small proportion of early flowering plants. Both late and early flowering plants could be fertile, semi- sterile or male sterile. These results are evidence of the complexity of the *MSH1* suppression phenotype.

T_3 plant 23-5-10 (*MSH1*-RNAi transgenic) displayed much thicker leaf texture (Fig. 3.1 b right) compared with normal wild-type leaves. The thick leaf segments from 23-5-10 were used as explants subjected to tissue culture and nine tobacco plants were regenerated. All regenerated plants (*MSH1*-RNAi transgenic) from plant 23-5-10 through tissue culture maintained the thick leaf texture; however, seed progenies (*MSH1*-RNAi transgenic) obtained by crossing plant 23-5-10 with wild-type pollen all displayed the normal leaf morphology (Fig. 3.2 a; Table 3.2). To confirm this difference in phenotype transmission, regenerated plant 23-5-10-L2(3) was subjected to a 2^{nd} round of tissue culture and regeneration, and no phenotype sorting was observed. However, when the leaf variant was crossed as female to wild-type, progeny no longer showed the thick leaf phenotype (Fig. 3.2 b; Table 3.2). This observation suggests that thick leaf phenotype is the consequence of epigenetic changes in response to *MSH1* disruption in tobacco. Likewise with other phenotypes of the plant 23-5-10, such as male sterility, dwarfism and branching, plants passed through tissue culture seem to have a good propensity to maintain the variant parental plant phenotype (data not shown).

The dwarf phenotype is influenced by application of gibberellic acid (GA_3)

Experiments have demonstrated that gibberellic acid (GA) could partially reverse the dwarfism in *MSH1*-RNAi sorghum (Xu et al. 2012). Here, GA_3 was used in tobacco to investigate the effects on transgenic (RNAi+) and non-transgenic (RNAi-) plants derived from population segregation. Wild-type, transgenic and non-transgenic tobacco plants all showed an increase in height with GA_3 treatment (Fig. 3.3). After the 2nd treatment, medium height transgenic plants were promoted to a height greater than normal wild-type. Likewise, dwarf non-transgenic plants were restored to approximately normal wild-type height.

Discussion

Although the frequency of some variant phenotypes increased with selection in our preliminary studies, additional generations are likely needed to complete the heritability test. All current tobacco lines contain the *MSH1*-RNAi transgene; we now intend to carry out selection for stable heritable phenotypes in lines without the transgene to test for evidence of epigenetic and/ or organellar factors in the transmission of phenotypic variation across generations.

In tobacco, multiple traits emerged from *MSH1* suppression, including dwarfed growth, branching and delay flowering, similar to those described for sorghum (Xu et al., 2012). This suggests that similar developmental pathways are programmed to respond to *MSH1* down-regulation in both monocot and dicot species. In sorghum, the dwarf phenotype is consistently co-inherited with enhanced tillering and delayed flowering (Xu

et al., 2012). Based on data to date, however, the altered tobacco phenotypes seemed to segregate independently with no evidence of stable co-inheritance across generations.

Although dual targeting of *MSH1* has not been confirmed in tobacco, similarity of derived phenotypes suggests that both plastid and mitochondrial properties are altered in tobacco *MSH1*-RNAi lines as well. Leaf variegation of the *msh1* mutant in Arabidopsis was shown to be associated with plastid genome instability as a consequence of *MSH1* mutation (Xu et al., 2011). Likewise, altered developmental phenotypes, including dwarf growth, enhanced branching, and delayed flowering were shown to be plastid-associated changes in Arabidopsis (Xu et al., 2012). Disruption of the plastid *accD* gene in tobacco gives rise to leaf morphology changes (Kode et al., 2005). Similarly, mutation of the nuclear gene *CND41*, encoding a plastid nucleoid protein in tobacco, causes a dwarf phenotype and altered leaf morphology, which is partially reversed by application of GA (Nakano et al., 2003). GA treatment partially restores the dwarf phenotype in *MSH1*-RNAi lines of sorghum (Xu et al. 2012) and tobacco. These results are not completely surprising, since GA biosynthesis is carried out as a plastid function. Similarly, altered flower time, a light responsive process, is also controlled by chloroplast functions. Consequently, we assume that disruption of *MSH1*, a protein localized within the plastid nucleoid (Xu et al. 2011), perturbs the organelle and gives rise to similarly altered phenotypes.

In summary, the results presented in this study document various trangenically induced phenotypes, and provides the opportunity to investigate *MSH1* behavior in plant development and adaptive phenotypic plasticity responding to environment in the future.

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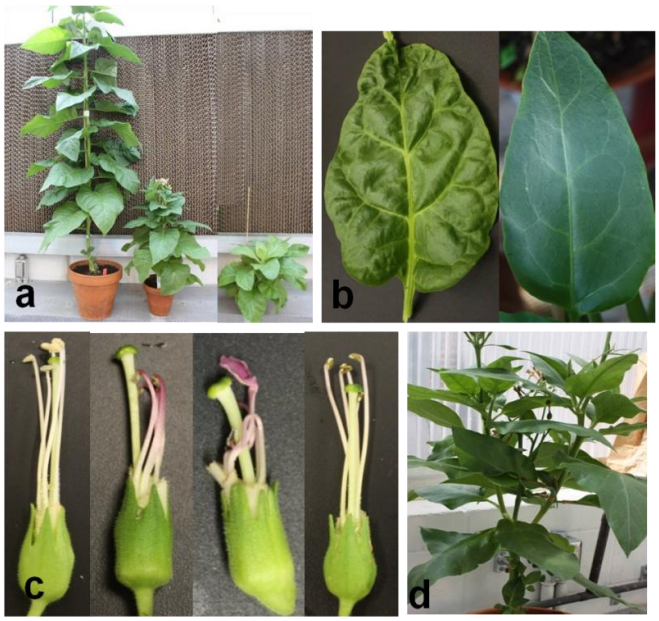

Fig. 3.1 RNAi suppression of *MSH1* expression results in various phenotypes in tobacco.

(a) dwarfism (left: wild-type)

(b) left: wrinkled leaf surface; right: thick leaf texture

(c) male sterility, absence of visible pollen accompanied by short filament or petaloid anthers (left: wild-type)

(d) branching

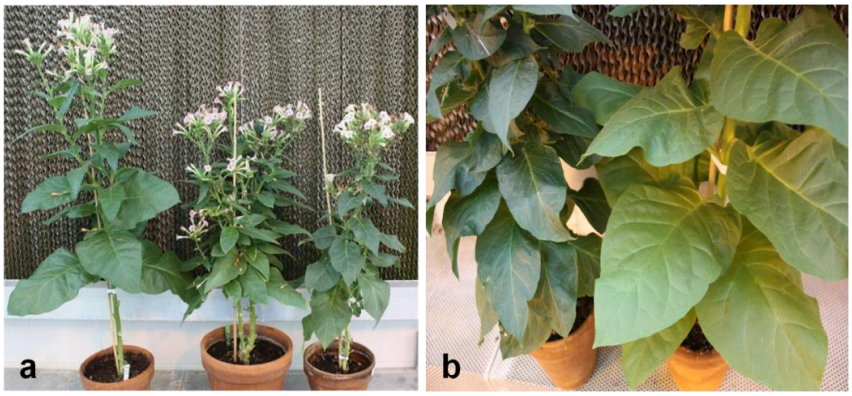

Fig. 3.2 The thick leaf phenotype was maintained in regenerated plants but not in plants from seed.

(a) Left: seed progeny from plant 23-5-10; middle: plant 23-5-10; right: regenerated plant 23-5-10-L2(3) from plant 23-5-10

(b) Left: regenerated plant from 23-5-10-L2(3); right: seed progeny from 23-5-10-L2(3)

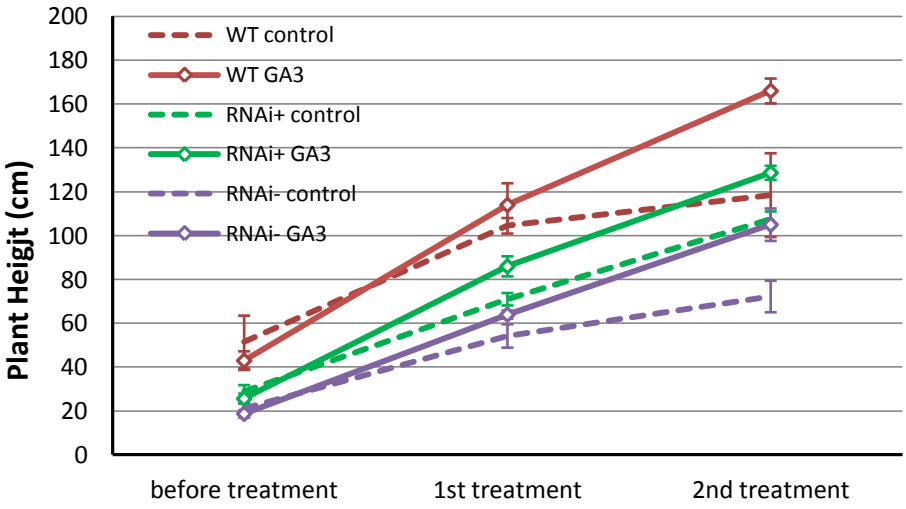

Fig. 3.3 Effect of GA3 on transgenic plants (RNAi+) and non-transgenic plants (RNAi-) height. The entire plants were untreated (control) or treated with GA_3 (250ppm) 1 month following seed germination. The 2nd treatment was applied 20 days after the 1st treatment. Values are averages of three biological replicates.

Table 3.1 Evaluation of transgenic tobacco plant populations for male sterility, dwarfism, delayed or advanced flowering, branching and wrinkled leave phenotype.

(a) male sterility segregation through generations

Generation	Self Progeny				Testcross Results[d]			
	No. of plants	No. (%)			No. of plants	No. (%)		
		Fertile	Semi-sterile[b]	Male Sterile[c]		Fertile	Semi-sterile	Male Sterile
T2[a] 23-5					20	2 (10%)	15 (75%)	3 (15%)
T3 23-5-3	10	5 (50%)	4 (40%)	1 (10%)				
23-5-18					5	4 (80%)	0 (0)	1 (20%)
T4 23-5-3-4	4	0 (0)	4 (100%)	0 (0)				
23-5-3-2					4	1 (25%)	2 (50%)	1 (25%)
23-5-18x WT-4					4	3 (75%)	1 (25%)	0 (0)
T5 23-5-3-4-1	5	2 (40%)	1 (20%)	2 (40%)				

[a] T_2 plant 23-5 is the selected progeny from confirmed transformants T_0 plants with RNAi suppressed *MSH1* expression in tobacco (Sandhu, et al., 2007)

[b] Semi-sterility in tobacco is defined as dramatic reduction or absence of visible pollen on the anthers of some plants, greatly reduced capsule size and reduced seed set.

[c] Full male sterility is absence of visible pollen on some plants and fully collapsed seed capsules with no seed set.

[d] Testcross progeny derive from pollination with wild-type pollen.

(b) dwarfism segregation through generations

Generation (Phenotype)	Self Progeny				Testcross Results			
	No. of plants	No. (%)			No. of plants	No. (%)		
		Normal	Medium	Dwarf		Normal	Medium	Dwarf
T_2 23-5					20	3 (15%)	15 (75%)	2 (10%)
T_3 23-5-3 (dwarf+)[a]	10	2 (20%)	4 (40%)	4 (40%)				
T_4 23-5-3-4 (dwarf++)	4	0 (0)	1 (25%)	3 (75%)				
T_5 23-5-3-4-1 (dwarf+)	5	0 (0)	0 (0)	5 (100%)				

[a] The wild-type plant height is subject to the range of 112-170 cm. Dwarfism in tobacco is defined as the half or less of wild-type height. Among all the dwarf plants, the "+"is assigned according to the following categories: dwarf: 80-60 (cm); dwarf +:60-50 (cm); dwarf++:50-40 (cm). Medium height is defined as about two thirds of the wild-type height.

(c) flowering time segregation through generations

Generation (Phenotype)	Self Progeny				Testcross Results			
	No. of plants	No. (%)			No. of plants	No. (%)		
		Normal	Late flowering[a]	Early flowering[b]		Normal	Late Flowering	Early Flowering
T_2 23-5					20	9 (45%)	9 (45%)	2 (10%)
T_3 23-5-3 (9 days late) 23-5-17 (10 days early)	10 15	4(60%) 6 (40%)	6 (60%) 8 (53%)	0 (0) 1 (7%)				
T_4 23-5-3-4 (49 days late) 23-5-17-2 (6 days early)	4 4	0 (0) 2 (50%)	4 (100%) 0 (0)	0 (0) 2 (50%)				
T_5 23-5-3-4-1 (30 days late) 23-5-17-2-1 (7 days early)	5 5	0 (0) 1 (20%)	2 (40%) 3 (60%)	3 (60%) 1 (20%)				

[a] Normal flowering time of wild-type tobacco plant is observed as about 2 months after seed germination. Late flowering is defined as 10 days or more days later than normal flowering time;

[b] Early flowering is defined as 10 days or more days earlier than normal flowering time;

(d) branching segregation through generations

Generation (Phenotype)	No. of Plants	Self Progeny No. (%)		No. of plants	Testcross Results No. (%)	
		Normal	Branching		Normal	Branching
T_2 23-5				20	13 (65%)	7 (35%)
T_3 23-5-10 (branching) 23-5-17 (branching)	5 15	4 (80%) 6 (40%)	1 (20%) 9 (60%)			
T_4 23-5-10-4 (branching) 23-5-17-1 (branching) 23-5-17-5 (branching)	4 4 4	2 (50%) 0 (0) 1 (25%)	2 (50%) 4 (100%) 3 (75%)			

(e) wrinkled leaf phenotype segregation through generations

Generation (Phenotype)	Self Progeny			Testcross Results		
	No. of plants	No. (%)		No. of plants	No. (%)	
		Normal	Wrinkled		Normal	Wrinkled
T_2 23-5				20	13 (65%)	7 (35%)
T_3 23-5-15 wrinkled	15	10 (67%)	5 (33%)			
T_4 23-5-15-4 slightly wrinkled 23-5-15-13 wrinkled	4 4	1(25%) 1(25%)	3 (75%) 3 (75%)			

Table 3.2 The thick leaf phenotype's segregation through regeneration and seed progeny

Generation (Phenotype)	Progeny from	No. of plants	Phenotype No. (%)	
			Normal	Thick
T_3 23-5-10 (thick leaf)	Cross (xWT)	10	10 (100%)	0 (0)
	Regeneration	9	0 (0)	9 (100%)
Regenerated 23-5-10-L2(3) (thick leaf)	Cross (xWT)	4	4[a] (100%)	0 (0)
	Regeneration	5	0 (0)	5 (100%)

[a] In the crossed progenies of regenerated plant 23-5-10-L2(3), two plants have slightly thick leaves on the top and normal leaves going down from the top