

Paternal arbuscular mycorrhizal fungal status affects DNA methylation in seeds

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Running title: Paternal AMF-mediated effects

Abstract

Most land plants grow in association with arbuscular mycorrhizal fungi (AMF) in their roots and these fungi can cause transgenerational effect on plants' offspring. These may be caused by changes in DNA methylation of the offspring. In this study, we compared the amount of global DNA methylation in seeds of the gynodioecious plant *Geranium sylvaticum* in relation to the gender and the AMF status of the parents producing the seeds. The amount of DNA methylated was positively related to seed mass. Seeds produced by females had a similar proportion of methylated DNA regardless of the AMF status of the father siring the seed. In contrast, seeds from hermaphrodites had higher DNA methylation when sired by AMF fathers. We show for the first time that AMF status of fathers can affect DNA methylation in seeds and that these changes are further dependent on the gender of the mother producing the seeds.

Key words: gynodioecy, maternal and paternal effects, transgenerational effects.

Introduction

The symbiotic association between plants and arbuscular mycorrhizal fungi (AMF) in their roots is both one of the oldest known mutualistic associations and one of the most widespread [1-2]. AMF are tightly linked to plant life history evolution as they mediate resource acquisition and allocation patterns. In AMF symbioses, the plant contributes photosynthates to the biotrophic fungus, while the fungus provides nutrients and possibly water absorbed from the soil in return. The result is a net transfer of phosphorus, nitrogen and other minerals to the plant, usually enhancing plant growth and reproduction [3].

The impact of mycorrhizal association is known to occur at all life stages of plants [3], including having transgenerational effects on offspring performance [4-6].

Transgenerational effects may arise from better seed provisioning (e.g. [7-8]), but the presence of paternally-mediated effects suggests that other epigenetic mechanisms may be also responsible [6]. The most studied epigenetic mechanism is DNA methylation. DNA methylation is a process that occurs dynamically throughout an organism's lifetime, in response to developmental changes and external stressors [9-10]. Crucially in plants, epigenetic information is retained during the production of gametes [11]. This means for plants, epigenetic memory may allow the transmission of information about the environment across generations (e.g. [12-13]). In fact, epigenetic processes are at the core of several types of phenotypic plasticity, and they may mediate some types of maternal environmental effects [14].

There is recent evidence that the relationship with AMF can cause changes in DNA methylation of plants [15]. What is not known is whether AMF can in fact impact DNA methylation transgenerationally. We tested this using a dataset generated as part of a previous study of transgenerational effects of AMF [6]. In this earlier study, we observed for the first time that the AMF status of both parents had transgenerational effects on offspring

performance. The presence of paternal effects was suggestive of some epigenetic transgenerational mechanism. Therefore, the aim of this study was to corroborate the existence of changes in the amount of DNA methylated in seeds in relation to the gender and the AMF status of the parents producing these seeds.

Materials and methods

Study species

Geranium sylvaticum L. (Geraniaceae) is a gynodioecious protandrous self-compatible perennial plant, producing several hundred seeds per flowering season. In the wild, plants are usually colonised by AMF [16]. In this study, we used two different AMF, *Claroideoglomus claroideum* (CLA) and *Glomus hoi* (HOI), which have been reported to differ in the beneficial effects to the study plant and are commonly found in sites where *G. sylvaticum* is the dominant plant [17].

Seed origin

Full experimental set-up is detailed in [6]. Briefly, 306 cloned individuals from nine hermaphrodites and five females were raised and inoculated with CLA or HOI or left noninoculated as non-mycorrhizal controls (NM). The plants were grown under greenhouse conditions during 2005 whereupon we performed a hand-pollination experiment [6]. Plants were transferred outdoors in 2006 and some were destructively harvested to investigate how AMF affected resource allocation [18]. Plants were grown under these conditions until September 2008, when the surviving plants (N = 123) were planted in an experimental field established near Konnevesi, Central Finland (62°38'N, 26°17'E) [19].

Controlled hand-pollinations between- and within-sexes (hermaphrodites, females) and AMF treatments (CLA, HOI, no-AMF: NM) were performed in summers 2010, 2012 and

2013. Developing flower buds were individually bagged and hand-pollinations were carried out in an arbitrary order when the stigma became receptive by rubbing one mature anther of another available flower. Seeds were collected when they turned brown and stored in dry, cold conditions (4°C) until 2016. In this study, on average 5.6 seeds per mother gender–mother AMF treatment–father AMF treatment were used (range: 0-14 seeds).

DNA extraction and global DNA methylation quantification

DNA was extracted using Power Plant Pro DNA isolation kit (Mo Bio Laboratories) following manufacturer's instructions from whole seeds (i.e. seed coat + embryo + endosperm). We chose an ELISA-based approach to quantify global DNA methylation because target gene information was lacking [20]. We used Methyl Flash Methylated DNA Quantification Kit Colorimetric (Epigentek) using 125 ng of DNA per sample with a 260/280 above 1.6, which was estimated by spectrophotometer (Nanodrop Technologies). Global % 5-methyl-cytosine (mC) was estimated by measuring absorbance at 450 nm in a SpectraMax Plus 384 Microplate Reader (Molecular Devices). Samples were run in duplicate and the readings averaged. The average difference between duplicates was $0.19 \pm 0.22\%$ (range = 0-1.26%), with an overall repeatability of 0.87 (95% CI = 0.83-0.91).

Statistical analyses

The repeatability (intraclass correlation coefficient) of the proportion of 5-mC of seeds from the same mother and father pairings was calculated using the package 'ICC' [21]. A linear mixed-effects model was then used to test whether logit-transformed proportion of 5-mC was related to gender of the mother (Female, Hermaphrodite), and the original AMF status of the mother (NM, CLA, HOI) and father (NM, CLA, HOI) that sired the seeds as fixed factors, alongside the two-way interactions between these factors. Pollination year and seed mass

were included as covariates. Maternal and paternal identities were treated as random factors. *Post hoc* pairwise analyses were carried firstly between the AMF status of the pollen donors (i.e. fathers), and then between the same fungal treatment but between gender of the mother (female/hermaphrodite). All statistical analyses were conducted in R v.3.1.2 [22], using the packages ‘car’ [23] ‘lme4’ [24], and ‘lsmeans’ [25]. Full model and partial R^2 were calculated using the ‘r2glmm’ package [26] using the Nakagawa and Schielzeth approach [27].

Results

The proportion of 5-mC ranged between 0.1% and 7.1% (average $1.4\% \pm 0.1$ SE; $N=102$) and it was positively related with seed mass (Table 1; Fig. 1). Repeatability of the proportion of 5-mC in seeds from the same clonal pairs was significant ($r = 0.31$, 95% CI = 0.07-0.53).

Year was unrelated to proportion of 5-mC (Table 1). The original AMF status of the mother did not affect the proportion 5-mC in the seeds, but the AMF status of the father did (Table 1). However, this significant effect of AMF status of the father was dependent on the gender of the mother producing the seeds (Fig. 2). Seeds produced by females had a similar proportion of methylated DNA regardless of the AMF status of the father that sired the seeds (Fig. 2, all pairwise comparisons $P \geq 0.079$). However, seeds produced by hermaphrodites and CLA-inoculated fathers had significantly higher DNA methylation compared to NM ones ($t_{39.10} = -3.997$, $P < 0.001$) and a trend for the same pattern in HOI-inoculated fathers ($t_{33.42} = -2.237$, $P = 0.079$). There was no difference between the two AMF treatments ($t_{52.86} = 1.951$, $P = 0.13$; Fig. 2). When comparing between mother genders, the proportion of DNA methylated was higher in seeds from hermaphrodite in both fungal treatments (CLA: $t_{84.64} = -2.232$, $P = 0.028$; HOI: $t_{85} = -1.984$, $P = 0.05$; Fig. 2), whereas it was lower in NM seeds in comparison to females ($t_{81.28} = 1.904$, $P = 0.060$; Fig. 2).

Discussion

Even though AMF are inherent components of most plant species, only one study has investigated whether AMF may affect DNA methylation [15]. Here we show for the first time that AMF can affect DNA methylation of plants' offspring, that these changes are persistent over several years, and that are further dependent on the gender of the mother producing the seeds.

DNA methylation regulates plant development [28-29] and can influence both maternal and paternal effects [30]. DNA methylation may be particularly important during seed development [31]. Reduced DNA methylation is associated with reduced seed viability, germination and seedling establishment [31-32], suggesting that the greater DNA methylation we found in the seeds was beneficial.

We observed that only the AMF status of the father but not the mother producing the seeds, affected the total amount of methylated DNA. The exact reasons for this are unknown, but paternal methylation effects seem to occur in the endosperm [33-34]. Since AMF are known to impact the nutritional component of the endosperm, but can also impact the seed proteome by up-regulating enzymes involved in energetic metabolism, embryo development, nucleotide metabolism, seed storage and stress responses [35], it is perhaps unsurprising that the DNA methylation in the seeds is a paternal effect. A key next step in our work is to test whether AMF impacts the embryo, integument or endosperm, and particularly to identify the genes that are being methylated and their function. Our results show correlations between AMF with seed size and DNA methylation and demonstrating causal effects will require identifying the genes involved.

What was noteworthy was that the significant effects on DNA methylation were dependent on the AMF status of fathers in relation to the mother's gender. For both AMF

species, global DNA methylation was higher in seeds from hermaphrodite mothers. The reason for this is unknown. Most DNA methylation studies concentrate on hermaphroditic species (e.g. *Arabidopsis*). In gynodioecious species such as *G. sylvaticum*, there is nucleocytoplasmic inheritance of male sterility [36] and theoretical work has shown how intragenomic conflict between maternally and biparentally inherited genes can lead to the maintenance of such gender polymorphism [37]. However, it remains to be tested how paternal effects interplay with complex genomic processes link to the expression of gender polymorphism and how paternal effects interact to create hypo- or hypermethylation.

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Data accessibility

The dataset supporting this article has been uploaded as electronic supplementary material.

Authors' contributions

Data were collected by SV. CDS & SV conceived the study, carried out the data analysis and wrote the manuscript. All authors agree to be held accountable for the content therein and approve the final version of the manuscript.

Competing interests

We have no competing interests.

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Ethical statement

This study was ethically approved by the College of Science Research Ethics Committee (CoSREC130).

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1 **TABLE 1.** Statistical results from the linear mixed effects model in the amount of DNA
 2 methylated (logit-transformed) in seeds of *Geranium sylvaticum*. Full model $R^2=0.255$.
 3

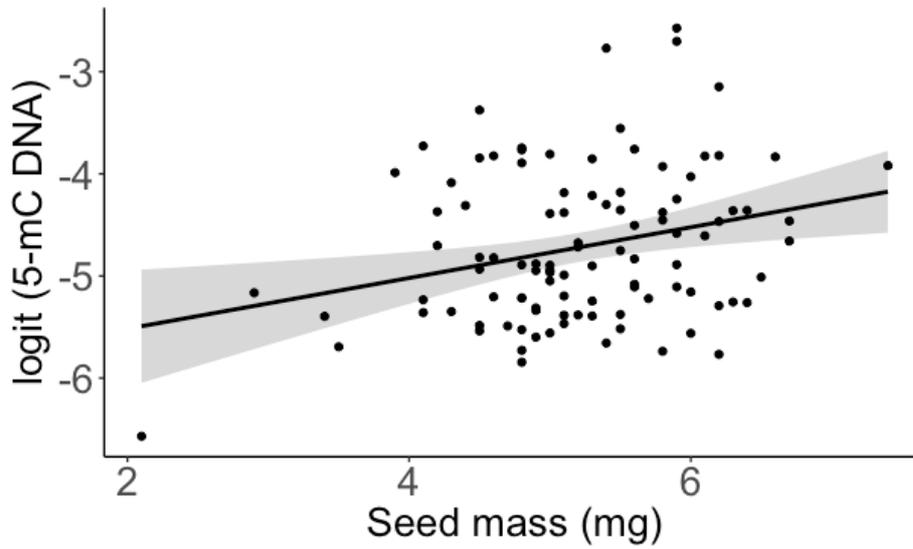
Parameter	d.f.	F	P	Partial R²
Year	2, 66.32	0.88	0.418	0.017
Seed mass	1, 29.50	5.33	0.028	0.052
Mother sex (Sex)	1, 8.71	2.09	0.183	0.058
Mother AM fungal treatment (Mofun)	2, 56.31	0.91	0.407	0.006
Father AM fungal treatment (Fafun)	2, 61.40	7.32	0.001	0.003
Sex x Mofun	2, 56.024	1.39	0.257	0.039
Sex x Fafun	2, 83.65	5.59	0.005	0.147
Mofun x Fafun	4, 83.53	1.45	0.224	0.038

4

5 **FIGURE CAPTIONS**

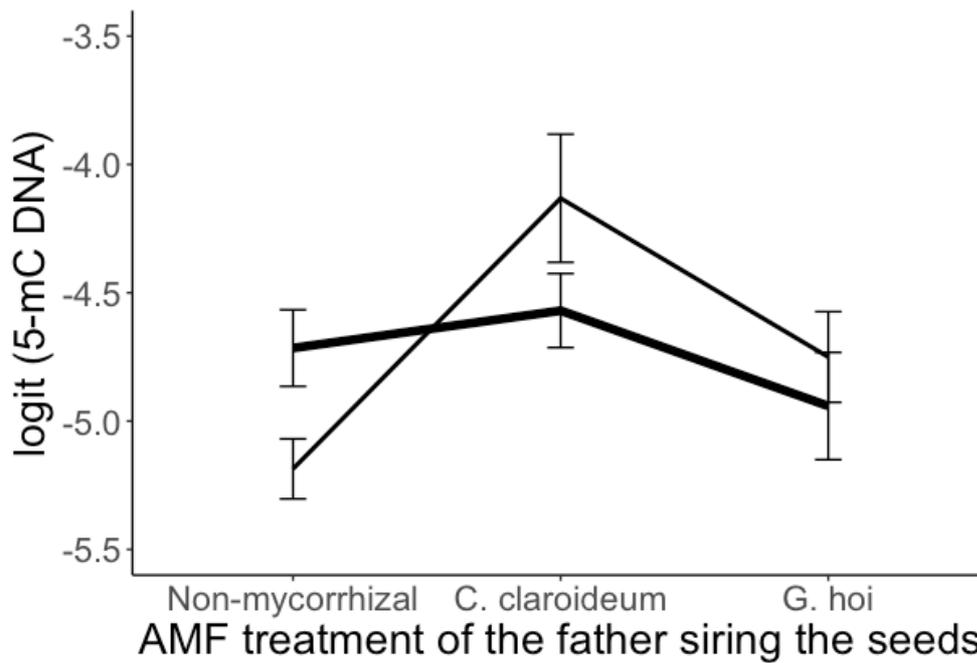
6

7 **Fig. 1.** Relationship between seed mass (mg) and logit-transformed proportion of methylated
8 DNA. Line indicates regression line \pm 95 CI from the linear mixed effect model.



9

10 **Fig. 2.** Mean \pm SE logit-transformed proportion of methylated DNA in seeds produced by
11 female (thin lines) or hermaphrodite (thick lines), in relation to paternal AMF status.



12