Epigenetic inheritance in apomictic dandelions:

Stress-induced and heritable modifications in DNA methylation and small RNA

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This research was conducted under the auspices of the Graduate School of
Experimental Plant Sciences (EPS)

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Thesis
submitted in fulfillment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Wednesday 7 September 2016
at 4 p.m. in the Aula

Veronica Preite
Epigenetic inheritance in apomictic dandelions: Stress-induced and heritable modifications in DNA methylation and small RNA,
152 pages.

PhD thesis, Wageningen University, Wageningen, NL (2016)
With references, with summary in English

ISBN 978-94-6257-871-5
DOI http://dx.doi.org/10.18174/386851

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Abstract

Epigenetic variation, such as changes in DNA methylations, regulatory small RNAs (sRNAs) and chromatin modifications can be induced by environmental stress. There is increasing information that such induced epigenetic modifications can be transmitted to offspring, potentially mediating adaptive transgenerational responses to environmental changes. However, it is unclear if this phenomenon is common and relevant for adaptation under natural conditions. My thesis study aimed to examine epigenetic inheritance in common and widespread apomictic dandelions (*Taraxacum officinale* Wig.). Due to their asexual reproduction mode by producing clonal seeds offspring from seeds are genetically uniform and thus suitable to investigate epigenetic effects that are not confounded with genetic variation.

I exposed apomictic dandelion lineages to drought and salicylic acid (SA) stress, which induces plant defense responses following pathogen attack, and found effects on patterns of DNA methylation up to two stress-free offspring generations after exposure. However, a heritable stress signal was not present in all tests and was stress- and lineage-dependent. Drought stress triggered a weak and lineage-dependent signal that was lost again in the second offspring generation. SA treatment revealed a stress-related increased rate of DNA methylation changes in the two offspring generations, but no stress signal was found in the stressed generation itself. I also observed changes in small RNA production due the drought and SA stress experienced two generations ago. These transgenerational sRNA effects showed association with gene functions related to grandparental drought and SA stress, which suggests functional relevance of the transgenerational effects.

I used a reciprocal transplantation field experiment to investigate whether exposing dandelions to natural field stresses also triggers DNA methylation changes. The experiment revealed evidence of adaptive divergence between the populations, suggesting that non-native habitats are experienced as more stressful. However, under these field conditions no induction-based DNA methylation changes were found that persisted into offspring.

By using AFLP and MS-AFLP screening of natural apomictic dandelion populations across a north-south transect in Europe I examined if natural, heritable DNA methylation variation reflects underlying genetic variation, or if it shows patterns that are not predictable from underlying genetics. I found that a large part of heritable DNA methylation differentiation along the north-south transect was correlated with genetic differentiation. However, a fraction of differentiation in heritable DNA methylation was independent from genetic variation. This suggests a potential of epigenetics to play an evolutionary role independently, at least to some extent, from underlying genetics. Overall, I found indications of epigenetic inheritance in apomictic dandelions. Whether epigenetic variation would result in adaptive phenotypic variation in nature and whether it would persist long enough to play a relevant role in adaptation remains unclear and requires further study.

1

General Introduction

Organisms are subjected constantly to changing environmental conditions, for instance to diurnal and seasonal changes. In the past ten thousand years regional climatic conditions changed dramatically and with it whole ecosystems changed as well. Species of each kingdom, Monera, Protista, Fungi, Plantae and Animalia evolved in these changing abiotic and biotic conditions. Species managed to survive and reproduce due to their variety of morphological, physiological and behavioural traits that have been continuously shaped by natural selection. The species that were most adapted to changes, for instance through phenotypic plasticity and evolution of adaptive traits, survived.

The phenotypic variety that one genotype can express depending on its environment is known as phenotypic plasticity. However, plastic responses alone may not suffice to cope with rapid climate change, and evolution of mean trait values is needed (Nicotra et al. 2010). Besides adjusting their phenotype to environmental changes, organisms that evolved locomotion can also respond with behavioural changes, such as for instance escaping from extreme conditions. Interestingly, also plant species can escape unsuitable conditions by migration over evolutionary timescales towards more suitable habitats. The processes of migration towards suitable conditions and adaptation to changing conditions are intermingled; for example, at the leading edge of the migration front during climate change-induced range expansion, plants have to adapt to novel biotic and abiotic conditions (Davis & Shaw 2001). For organisms to successfully adapt to environmental changes it needs natural selection acting on variation in fitness-related traits that are at least partially heritable (Houle 1992).

In recent years it became more and more clear that non-genetic mechanisms can have major effects on heritable trait variation and they might play a role in adaptation in addition to, or in interaction with, the classical genetic adaptation. Non-genetic mechanisms include molecular changes to DNA that affect gene expression, referred to as epigenetics, which means literally: epi- (greek for "on top of") genetics. Epigenetic variation can result in altered phenotypes without changing the underlying genetic code. This means that epigenetic effects can generate additional variation on top of genetic variation that could be subjected to natural selection. Variation in epigenetic information can derive through spontaneous changes, just as random genetic mutations do, but also through environmental induction. One source of phenotypic plasticity, the range of phenotypes that one genetic code can produce in different environments, is thought to be epigenetic variation that is sensitive to environmental cues (Latzel & Klimešová 2010; Massicotte & Angers 2011; Richards et al. 2010). In addition to mediating phenotypic plasticity, epigenetic modifications can also arise stochastically (Van der Graaf 2015). Both spontaneous and environmentally induced epigenetic variation could become especially important for organisms to cope with changing conditions when genetic variation is limited. This could be true for, for instance, asexual plant species which are thought to be evolutionary dead-ends because of their limited genetic variation.

Classically, heritable epigenetics is defined as gene regulatory effects that persist through cell divisions. Heritable epigenetics can be understood as epigenetic-mediated gene regulation that is induced by environmental changes that persists even if the initial stimulus is transient. Furthermore, epigenetic effects can also persist across generations

and this so called transgenerational epigenetic inheritance is the focus of my thesis. Environmentally sensitive epigenetic inheritance could facilitate and fine-tune the short-term adaptation to fast-changing conditions such as may occur under climate change. One important aim of my thesis is to investigate this suggested role of epigenetic variation in nature by screening standing epigenetic variation in plant populations and by performing multi-generational stress experiments.

Epigenetic mechanisms

There are a variety of epigenetic mechanisms which regulate gene expression and they are widespread in different taxa. Epigenetic mechanisms include DNA methylations, histone modifications, and small RNAs (sRNA). Spontaneous and environmentally induced changes and reversions add a dynamic feature to these mechanisms. This dynamics generates variation in gene regulation within generations during different developmental stages and across generations. Epigenetic variation has therefore been proposed to influence a large percentage of phenotypic variation (Manolio et al. 2009; Becker & Weigel 2012).

DNA methylations, histone modifications and sRNAs are often interlinked and together regulate gene expression through regulation of promoter regions, transcription factors, chromatin accessibility, chromosome organisation or DNA repair (Cedar & Bergman 2009; Jablonka & Raz 2009). DNA methylation studies in plants showed that cytosines are methylated mostly in the di-nucleotide combination of CG, but also in CHG and CHH context, where H can stand for a C, A or T nucleotide (Law & Jacobsen 2010). Experimentally removed DNA methylations through chemicals resulted in an increase of phenotypic variation, indicating the epigenetic importance for gene regulation (Bossdorf et al. 2010). Studying the late flowering mutant in *Arabidopsis* revealed that DNA methylations at the 5' flanking region of a homeodomain gene regulated this mutant phenotype (Soppe et al. 2000). The mechanistic idea behind the regulatory feature of DNA methylation is that highly methylated gene promoters or transcription factors impede the binding to the transcriptomic protein complex. Alternatively, DNA methylation might attract proteins that alter histone modifications, e.g. specific histone tail modifications can change how densely the DNA strand is packed around the nucleosomes. Thus a more loosened DNA structure would show more accessibility to the transcriptional enzymes. One important function of DNA methylations in plant genomes is to suppress the transcriptional activity of transposable elements (TEs) and thus preventing the jumping of TEs which can cause deleterious consequences to the DNA (Fedoroff 2012). A demethylated, activated TE can thus jump and insert its genomic region into other parts of the genome where it can disrupt gene activity (Richards 2006; Zilberman et al. 2007; Bucher et al. 2012). Gene regulation involving sRNAs is thought be mainly based on sRNAs that degrade target mRNA molecules (posttranscriptional gene silencing) and on sRNAs that guide other epigenetic marks, such as methylation, towards specific loci in the genome and thereby silencing such loci (transcriptional gene silencing).

The methylome is usually demethylated and reprogrammed during gametogenesis and/or embryogenesis in order to transmit only the important DNA methylations to the next generation. In plants not all DNA methylations are reset during this process, and in particular DNA methylation in CG context is thought to be relatively resistant to re-setting (Saze et al 2003). Thus, DNA methylation patterns can be shared between parents and offspring either because they escape re-setting during gametogenesis/embryogenesis, or because they are re-set and subsequently rebuilt in offspring in the same way as in parents. Which, why and how DNA methylations are chosen to be transmitted is, however, unclear. Studies revealed that mostly TE-silencing DNA methylations are faithfully transmitted (Becker et al. 2011; Schmitz et al. 2011). Moreover, also transgenerational stress-induced DNA methylation changes (Verhoeven et al. 2010b) and transgenerationally stable stochastic DNA methylation changes (Van der Graaf 2015) have been observed.

Epigenetic phenotypes

Several plant studies investigated heritable phenotypic consequences of DNA methylation variation and discovered naturally occurring epialleles. For instance, Cubas et al. (1999) revealed cytosine methylations on a floral morphology gene which were correlated with a naturally occurring floral mutant of *Linaria vulgaris*. No genetic mutations were found that correlated with the phenotype and furthermore, both floral types were found growing on different branches but together on same individuals. Flowers that developed on cuttings from these branches kept their original flower type. Genetic mutation was excluded and the floral mutants were therefore explained by pure, genetically un-confounded, epialleles. These epialleles and the floral mutant phenotypes showed occasional reversion to the wild type by demethylation. Similar short-term heritable DNA methylations have been found in the flowering genes of *Arabidopsis thaliana* (Jacobsen & Meyerowitz 1997) and in the fruit ripening genes of tomato (Manning et al. 2006).

The most convincing experimental evidence for epigenetic inheritance comes from studies on epigenetic Recombinant Inbred Lines in *Arabidopsis* (epiRILs). These isogenic lines differ in their DNA methylation patterns, since they derive from crosses between genetically very similar parents, but one parent is a mutant that leads to a lack of DNA methylations. After backcrossing the F1 to the wildtype parent, a panel of epiRILs was derived, that did not carry the mutation themselves. These epiRILs showed heritable phenotypic variation and DNA methylations that were stably transmitted for at least eight generations (Johannes et al. 2009; Zhang et al. 2013; Cortijo et al. 2014). EpiRIL studies provide a possibility to investigate phenotypic variation under almost uniform genomes, and heritable phenotypic differences between lines could be convincingly attributed to heritable DNA methylation differences (Cortijo 2014). The only downside is that the epiRILs shed little light on the relevance of natural epigenetic variation since they derive from an artificial DNA methylation mutant. Indications for functionally important epigenetic variation in nature are based on the fully sequenced epigenome of *Arabidopsis* mutation accumulation lines which showed heritable epimutations that accumulate over time (van der Graaf et al. 2015; Becker et al. 2011; Schmitz et al. 2011). However, a large

proportion of such stochastic epimutations may be heritable but not for a large number of generations (Becker 2011; Schmitz 2011).

Heritable DNA methylations changes and phenotypic diversity in plants can arise through environmental induction or through spontaneous epigenetic mutations. In few plant studies indications for epigenetic and transgenerational responses to stress were found, for instance in the form of heritable sRNA signals (Bilichak et al. 2015) or as heritable DNA methylation changes (Verhoeven et al. 2010b; Luna et al. 2012). However, also stochastic epimutations can have important influences: a modeling study based on empirically observed epimutation rates in the *Arabidopsis* mutation accumulation lines suggests that they arise fast enough to uncouple epigenetic variation from genetic variation but revert slow enough to sustain long-term selection responses (van der Graaf et al. 2015). Both spontaneous and environmentally-induced epimutations can have effects on evolutionary processes, since natural selection could target next to heritable genetic variation also heritable epigenetic variation (Richards et al. 2010).

Relation of epigenetic variation with underlying genetic variation

One difficulty in studying the importance of epigenetic variation in nature is to disentangle effects that are deriving from genetic variation versus effects that are autonomous from genetic variation. Genetic and epigenetic variation can both provide a target for natural selection and therefore play a role in adaptation. Only epigenetic variation that is not fully determined by underlying genetic variation has the potential to explain any adaptation or phenotype that is not explained already from genetics. However, many epigenetic effects were so far found to be associated with genetic variation (Dubin et al. 2015; Becker & Weigel 2012). Recent studies and discussions contribute to the understanding of how common and important epigenetic variation is that is autonomous or at least partially unlinked from genetic effects (Richards 2011).

In my thesis I tested the importance of epigenetic inheritance in genetically uniform asexual lineages to minimize the genetic variation. For my transgenerational experiments I used offspring grown from field-collected seeds from apomictic dandelion lineages. Apomictic plants produce asexually through clonal seeds, which develop to genetic copies of the mother plant. Variation generated by epigenetic mechanisms has been proposed to compensate, at least to some extent, for the lack of genetic variation in asexuals (Angers et al. 2010). Asexually reproducing plants are, in theory, constrained in their potential to adapt to changing environments due to their limited genetic variation. However, quite some asexual plants are highly successful invaders and colonized vast areas sometimes with just a single genotype (Hollingsworth & Bailey 2000; Ahmad et al. 2008). The success of asexual plants as biological invaders could be explained by preadaptation of the genotypes to conditions in the new habitat, possibly through high phenotypic plasticity (Baker 1965) which could be mediated by epigenetics. After the last retreat of land ice, approximately 10,000 years ago, obligate apomicts migrated from glacial refugia towards Northern Europe (Comes & Kadereit 1998). Nowadays, apomictic dandelions are numerous and widespread in Europe and their populations consist of many different apomictic lineages.

Adaptive potential of epigenetic variation in nature

Epigenetic inheritance is particularly interesting to study in plant species given their sessile life style and late differentiation of the germline. Plants, which set apart their germline relatively late in their development, could for instance accumulate stress-induced epimutations during their life that subsequently enter the germline which develops from somatic tissue only during flower development. Epigenetic variation has shown to be induced by environmental factors, such as herbivory, pathogen attack or extreme temperature conditions. For instance, *Arabidopsis thaliana* revealed transgenerational fungal resistance (Luna et al. 2012) and in *Mimulus guttatus* increased trichome density was observed upon herbivory stress (Scoville et al. 2011). In both examples the physiological or phenotypic adjustments were suggested to be linked with gene regulation, which are possibly affected by epigenetic modifications. It may be envisioned that also an increase of variation, rather than a shift in trait mean value, could be beneficial for the survival of a plant population (Herman et al. 2014). With unpredictably fluctuating environmental conditions plants could focus on spreading their risk of "maladaptation" through increasing the phenotypic variation in their offspring, also named bet-hedging (Nevoux et al. 2010). A theoretical modeling approach supported the importance of increased epigenetic variation that derives through incomplete resetting of DNA methylations and generates methylation variation in the offspring. They suggested that incomplete resetting may be especially adaptive in fast changing environments, since it prevents mismatched phenotypes (English et al. 2014).

Whether epigenetic variation may or may not play an important role in adaptive processes also depends on the persistence of epigenetic changes (Herman et al. 2014). Only selection on epialleles that persist in the long-term could produce an epigenetically-derived adaptation. It is however questionable to what extent such long-term stable epialleles exist. However, also selection on transient epialleles can strongly affect the dynamics of adaptation, possibly causing rapid initial adaptive responses which are subsequently 'solidified' by genetic mutations (Klironomos et al. 2013). A theoretical model on evolution that combines genetic effects and non-genetic inheritance, even if transient, was explaining scenarios that could not be explained by classical Mendelian genetic evolution alone. These scenarios are describing phenomena where the parental environment had affected the offspring phenotype: such as transgenerational epigenetic inheritance, maternal effects, RNA-mediated inheritance and also cultural inheritance (Bonduriansky et al. 2012). Recently another modelling study came to the same conclusions, but with stochastically derived epimutations instead of environmentally-related epimutations (Kronholm & Collins 2015).

So far, there are indications but no tangible evidence that epigenetic inheritance is important in adaptation of natural plant population. The role of epigenetics in nature should be tested on field-collected material and/or with experiments that are conducted under natural conditions (Bossdorf et al. 2008). Natural populations of *Viola cazorlensis* for example revealed pronounced epigenetic variation, which showed a higher diversity than the populations' genetic variation. Additionally, and more importantly some DNA

methylations showed association with the on-site observed herbivory (Herrera & Bazaga 2011). Natural populations of *Fallopia japonica* clones, an invasive plant species in Europe, also revealed a correlation between DNA methylation variation and the expressed phenotypic plasticity (Richards et al. 2012). It is expected that asexual lineages, such as *F. japonica,* harbor almost no genetic variation. The observed phenotypic polymorphism in *F. japonica* might therefore be attributed to pure epigenetic effects. In both cases an association was observed between ecological factors and DNA methylation variation, suggesting that epigenetics might enable plants to cope better with various habitats. These are important discoveries to help understanding the role of epigenetics in nature, but we are just beginning to comprehend the effects of natural epigenetic variation. Detailed follow-up studies are needed to pinpoint functional epigenetic loci that cause relevant phenotypic effects, and to evaluate the link between this epigenetic variation and underlying genetics.

Apomictic dandelions as model system for natural epigenetic variation

I used apomictic dandelions (*Taraxacum officinale*), a widespread grassland plant species, to investigate heritable epigenetic effects that are not confounded with genetic variation. The common dandelion exists in two variants that differ in their reproductive mechanisms: there are diploids that reproduce sexually and polyploids (mostly triploids) that reproduce asexually via unfertilized seeds, named apomixis (Mogie & Ford 1988). The offspring of these apomicts are genetic clones of the mother plant. New apomictic dandelion lineages are constantly produced through crossings between sexual (diploid) mothers and pollen from apomictic (triploid) fathers in mixed populations. Some of these newly generated apomictic dandelion lineages could migrate away from these mixed populations along latitudinal or altitudinal gradients, for example from Central towards Northern Europe (Richards 1973). Apomictic dandelions provide therefore a variety of clonal lineages and accessions from different geographic and climatic regions. For my transgenerational experiments I used offspring from field-collected apomictic individuals. Barring effects of novel genetic mutations, any phenotypic deviation between the mother and the daughter thus could be attributed to non-genetic effects.

In theory, stress-induced epigenetic inheritance could link gene regulation with the environment and together shape the phenotype. For example a previous study on an apomictic dandelion lineage revealed stress-induced (most notably chemical induction of herbivore and pathogen defenses) DNA methylation changes that were transmitted to the subsequent generation (Verhoeven et al. 2010b). And in *Brassica rapa* a heat-stress induced effect on the small RNAs of the stressed plant and their unstressed progeny was observed (Bilichak et al. 2015). In my thesis I investigated stress-induced epigenetic changes (DNA methylations and small RNAs) in several apomictic dandelion lineages by applying drought stress and salicylic acid, which mimics pathogen attack. Both drought stress and pathogen attack can be devastating for plant populations when reaching an extreme level. Additionally, under the pace and impact of the current climate change drought events are predicted to become more severe and more frequent. The rapidly changing environment will promote that organisms migrate and establish in previously

cooler areas (Walther et al. 2009), which in turn affects the composition of organisms living together (Van der Putten 2012). Consequently, we can predict that range-shifting plant species will also encounter new pathogens or that pathogen attack in synergy with abiotic stresses becomes more severe for the plant.

One drawback of using a non-model species is that, in contrast to *Arabidopsis thaliana*, the dandelion genome is not yet fully known. I therefore used AFLPs to screen the genetic structure of apomictic dandelion populations and methylation-sensitive AFLPs to screen their methylation pattern. Furthermore, since small RNAs are involved in the transmission of epigenetic information, I performed additionally a small RNA screening to test for transgenerational effects.

The aim of my thesis is to further the understanding of the evolutionary role of stress-induced and heritable epigenetic effects. For this purpose I performed greenhouse and field experiments. With multi-generation greenhouse experiments using plants from genetically uniform apomictic dandelion lineages I investigate stress-induced epigenetic effects such as changes in DNA methylation (using MS-AFLPs) and sRNAs. Based on previous findings in apomictic dandelions and other plant species I hypothesize that stress-induced epigenetic effects can be transgenerationally inherited. In addition to the artificially added stresses I also investigated transgenerational effects in response to natural stresses as perceived under natural field conditions. For this purpose I conducted a reciprocal transplantation experiment which tested whether heritable DNA methylations can be induced by transplantation to a novel but natural growing site. And at last I investigated the pattern of epigenetic and genetic variation in natural populations using offspring from seeds that were collected along a south-north transect in Europe. My hypothesis is that epigenetic variation, just like genetic variation, shows geographic differentiations in natural populations. Furthermore, since epigenetic variation could be unlinked from genetic variation I specifically tested if epi-variation simply mirrors genetic variation, or if it shows a deviating pattern. The below thesis outline summarizes each of the four experiments conducted in my work.

Thesis outline

Chapter 2

How much transgenerational DNA methylation variation can be environmentally induced and for how many generations they can persist is to date unclear. In chapter 2, I present results from a multi-generation experiment: drought and salicylic acid (which mimics pathogen attack) were applied to two apomictic dandelion lineages, deriving from three sampling locations. This first generation (G1) was then propagated in an unstressed environment for two generations (Fig. 1.1). With the methylation sensitive restriction enzyme *Hpa*II I screened the DNA for stress-induced DNA methylation changes in G1 and tracked these motifs back to see if they are persisting to the generations G2 and G3.

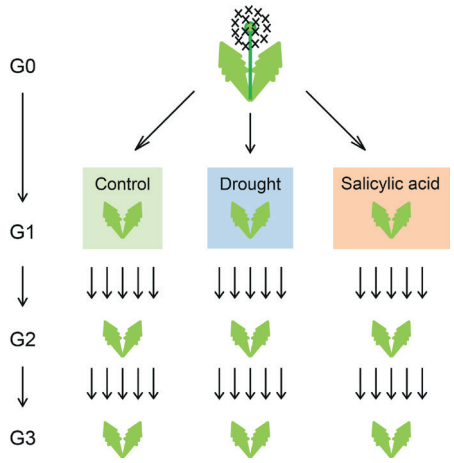

Figure 1.1 Multi-generation experiment: the offspring, (G1), of the mother plant, (G0), is equally distributed over the three treatments, control, drought and salicylic acid. The subsequent two generations were grown under unstressed conditions

Chapter 3

Small RNAs can maintain and regulate epigenetic information, such as DNA methylations. Following from the same multi-generation experiment in chapter 2 I screened for small RNA changes in G3 after stress induction by drought and salicylic acid stress. The here addressed question is whether environmental stress leaves a sRNA footprint that is sustained over two stress-free generations.

Chapter 4

Based on previous greenhouse experiments, which showed that stress can trigger heritable DNA methylation changes, I investigated transgenerational effects in response to natural stresses as perceived under natural field conditions. I reciprocally transplanted several apomictic dandelion lineages, collected in The Netherlands and Czech Republic into their own and each other's natural habitat. The prediction of the reciprocal transplantation experiment is that growing in a novel environment is more stressful than growing in native environment, which will be reflected in more DNA methylation changes (detected with *Hpa*II screening) in plants that are transplanted to non-native sites.

Chapter 5

In this chapter the standing heritable variation of DNA methylations in natural populations of apomictic dandelions along a south-north transect in Europe is investigated (see sampling design Fig. 1.2). Habitat-specific DNA methylations could have evolved if epigenetic information plays an important role for asexually reproducing plants to adapt to environmental changes. This can be either due to heritable, environment-induced epigenetic modifications or due to environment-specific selection of epigenetic variants. To find such an environmental footprint in the DNA methylome, AFLP and MS-AFLPs (*Hpa*II and *Msp*I) were screened to capture the plants' genetic and epigenetic structure. This experiment tests if patterns of heritable DNA methylation variation simply mirror underlying patterns of genetic variation, or if epi-variation shows unique patterns that deviate from genetic patterns.

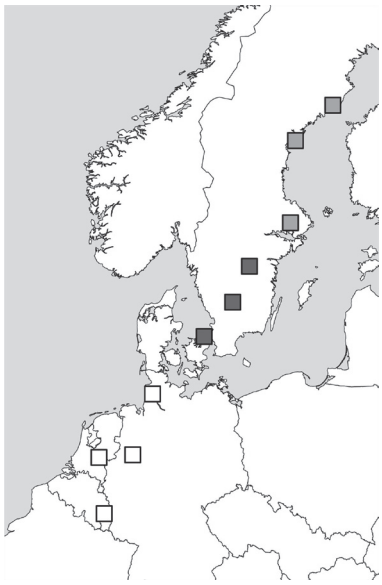

Figure 1.2 Map of North West Europe. Boxes represent the ten areas and the filling represents the regions (North, Center, South) where apomictic dandelions were sampled.

2

Transgenerational epigenetic inheritance in two widespread apomictic dandelion lineages

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Abstract

DNA methylation is one of the mechanisms underlying epigenetic modifications. DNA methylations can be environmentally induced and transmitted to successive generations. However, it remains unclear how common such environmentally-induced transgenerational DNA methylation changes are and for how many generations they can persist. We exposed multiple accessions of two different apomictic dandelion lineages of the *Taraxacum officinale* group (*Taraxacum alatum* and *T. hemicyclum*) to drought and plant pathogen stress, the latter mimicked by adding salicylic acid (SA). Using methylation-sensitive amplified fragment length polymorphism markers (MS-AFLPs) we screened genome-wide methylation changes after stress treatments and assessed the heritability of induced changes for two subsequent unexposed offspring generations. Irrespective of the initial stress treatment, a clear build-up of heritable DNA methylation variation was observed across three generations. Environmental treatments affected DNA methylation patterns in a stress-specific way: drought stress showed some evidence for a directional, accession-specific methylation change, but only in the exposed generation. By contrast, SA stress did not cause a detectable response in the exposed generation but increased the rate of methylation changes in their offspring. While the functional consequences of the MS-AFLP-detected DNA methylation changes remain to be demonstrated, our study shows that environmental stress can affect DNA methylation patterns not only in exposed plants but also in their unexposed progeny. Less than one third of the changes observed in stress-exposed plants were faithfully transmitted to the third generation. We conclude that stress-induced transgenerational DNA methylation modification in dandelions is genotype and context-specific.

Introduction

Epigenetic modifications, such as DNA methylation, can affect gene activity without changing the underlying DNA sequence and are involved in transposable elements (TEs) silencing (Lippman & Martienssen 2004). Exposure to biotic and abiotic stress has been shown to alter DNA methylations (Aina et al. 2004; Choi & Sano 2007), and some stress-induced methylation changes were found to be transmitted to successive generations (Cheng et al. 2004; Boyko et al. 2007; Verhoeven et al. 2010b; Kou et al. 2011). Such a transgenerational 'memory' of stress could play a role in adaptation by generating epigenetic variants that cause specific tolerance to the environmental stress that triggered them (Luna et al. 2012; Rasmann et al. 2012). In addition to genetic variation, DNA methylation could increase the range of variation for selection to act on and is thought to be one of the underlying mechanisms for phenotypic plasticity (Angers et al. 2010; Richards 2011).

To be transgenerationally effective epigenetic information needs to be transmitted through genome resetting and reprogramming during gametogenesis and zygote development. Unlike in mammals, in plants a considerable part of the DNA methylations appear to be meiotically stable (Feng et al. 2010) and may be transmitted between generations via small RNAs that could guide re-establishment of parental DNA methylation patterns in offspring (reviewed in Bond & Baulcombe 2014 and Iwasaki & Paszkowski 2014). For instance, Rasmann et al. (2012) showed that an induced defense against herbivory was transmitted in an *Arabidopsis* wild type but not in small-RNA-deficient mutants. Recent studies are providing first estimates of the rate and transgenerational stability of spontaneous DNA methylation modifications (Becker et al. 2011; van der Graaf et al. 2015). But it remains unclear to what extent the rate of heritable modifications is affected by stress exposure, and for how many generations DNA methylations can persist. It is also unclear what level of persistence is necessary to have an important impact on adaptive processes (Rapp & Wendel 2005; Herman & Sultan 2011; Herman et al. 2014).

Variation in DNA methylation can arise spontaneously (Becker et al. 2011; Schmitz & Ecker 2012) or induced by environmental signals (Dowen et al. 2012). Methylations that adjust gene regulation as a specific response to an environmental stress can be regarded as a directed epigenetic effect. Alternatively, stress can also cause non-directed, random, methylation changes that result in phenotypic differences contributing to natural variation. If ecologically important DNA methylation effects are transgenerationally stable, then natural selection can act on these epigenetic phenotypic differences (Rapp & Wendel 2005). Little is known about the role of spontaneous versus stress-induced DNA methylations, as well as directional versus random response to stress in evolutionary processes of plant populations.

DNA methylation changes can be mediated by nearby (cis) and distant (trans) sequence information, but they can also be autonomous, independent of genetic variation (sensu Richards 2006). It has been proposed that such autonomous heritable methylations could explain evolutionary processes in ways that cannot be explained by sequence variation alone (Richards 2006; Bossdorf et al. 2008). In practice, it is difficult to distinguish autonomous from genetically-mediated epigenetic variation since it is possible that genetic changes that influence a particular epigenotype remain undetected (Richards 2006 and 2011; Johannes et al. 2009). Populations that lack significant genetic variation, such as asexually

propagating lineages, might therefore be well suited to investigate the potential of autonomous epigenetic inheritance (Bossdorf et al. 2008). In addition, for asexually reproducing plants epigenetic variation might be relatively important because their potential to adapt to changing environments is constrained by limited genetic variation. The additional variation generated by epigenetic mechanisms has been proposed to compensate, at least to some extent, for the lack of genetic variation in asexuals (Angers et al. 2010). This might contribute to the ecological success of some asexual invaders that colonize vast areas as a single dominant genotype (Hollingsworth & Bailey 2000; Ahmad et al. 2008; Zhang et al. 2010).

To investigate heritable DNA methylations we used apomictic, i.e. asexually reproducing, dandelions of *Taraxacum* Wigg. sect. *Taraxacum* (commonly also called *Taraxacum officinale* Wigg., see Kirschner & Štěpánek 2011). Dandelions show geographic parthenogenesis where the distribution of apomictic lineages exceeds the distribution of sexually reproducing dandelions towards northern regions. In Europe many different obligate apomictic lineages colonized northern regions after the retreat of land ice, approx. 10,000 years ago (Comes & Kadereit 1998). This particular geographical distribution pattern provides a natural study system of widespread apomictic dandelion lineages, with each lineage harboring limited potential to adapt through genetic variation. Previous research on a newly synthesized apomictic dandelion showed that stress exposure can cause DNA methylation changes and moreover, that these changes could be stably transmitted to the next generation (Verhoeven et al. 2010b). The present study aims to investigate the persistence and the generality of inheritance of stress-induced epigenetic modification in apomictic dandelion lineages.

To study stress-induced heritable DNA methylations we carried out a controlled experiment exposing apomictic dandelions to two different stresses and investigated the persistence of induced methylation changes in two successive unexposed generations. Two apomictic dandelion lineages were used that were collected from three different sites which we hereafter abbreviate as: FI, which stands for a high latitude site in Finland, CZH for a medium altitude site in East Czech Republic and CZL for a low altitude site in Central Czech Republic. Since northern and mountainous regions may represent more stressful (abiotic) environmental conditions we hypothesized that in those habitats plants may have been selected for higher levels of plasticity. Such a higher plasticity might be partly mediated by a higher capacity for stress-induced methylation modifications. By stressing apomictic dandelions at an early vegetative stage and studying methylation changes propagating beyond the first offspring we ensure the detection of epigenetic effects beyond maternal effects.

We used drought as abiotic stress and salicylic acid (SA) to mimic biotic stress of e.g. biotrophic pathogens (Delaney et al. 1994). Drought and SA-induced stress represent important environmental factors for plants in all sampling regions in Central Bohemia, the White Carpathian region and South Finland. Spring droughts occur regularly, although in relatively mild form, in CZ and in continental Finland (Potop et al. 2014). Pathogen pressure is a very common biotic stress and intensifies towards lower latitudes in Europe (Schemske et al. 2009; Verhoeven & Biere 2013). Moreover, these stresses are predicted to become more severe and frequent as the current climate change proceeds (IPCC 2013; Pautasso et al. 2012).

Using asexually reproducing dandelions as a model enables the detection of stress-induced and heritable epigenetic variation that is not likely to be confounded with genetic variation. And SA treatment induced the strongest DNA methylation response among a series of treatment in a previous transgenerational stress experiment in dandelions (Verhoeven et al. 2010b). Based on methylation-sensitive amplification polymorphisms (MS-AFLPs) that detect DNA methylation variation at genome-wide (anonymous) marker loci, we specifically tested three hypotheses: 1) upon stress application DNA methylation patterns change, 2) these methylation modifications are inherited to next generations, and 3) plant accessions originating from the higher latitude and altitude sites show higher levels of methylation variation than plants from low latitude and altitude sites.

Material and methods

Study species

The apomictic common dandelion of sect. *Taraxacum*, the *T. officinale* group, is a common perennial forb in lawns, meadows and pastures that has spread worldwide, especially in temperate zones but also reaching into polar and alpine zones (Richards 1973). Dandelions form taproots with rosettes and produce wind- dispersed seeds. In apomictic dandelions these seeds are produced from unreduced egg cells via embryogenesis without fertilization by male gametes (parthenogenesis). Likewise, the endosperm develops autonomously without fertilization (Koltunow 1993). Most apomicts are polyploid (Mogie & Ford 1988; Asker & Jerling 1992). In the case of *T. officinale* the apomicts are mostly triploid while the sexuals are diploid (Richards 1973, 1989; Riddle & Richards 2002). New apomictic lineages arise in mixed populations of apomictic and sexual dandelions when pollen from apomicts fertilizes sexual dandelions (Richards 1973). This results in offspring of various ploidy levels, some of which are functionally apomicts (Tas & Van Dijk 1999). In the regions without sexual common dandelions, local populations consist of few to numerous distinct apomictic lineages, morphologically and genetically recognizable entities, sometimes referred to as microspecies, under binomials. Hundreds of microspecies within the *T.officinale* group have been described in Europe (Kirschner & Štěpánek 2011).These apomictic dandelion lineages are often widespread with a distribution that extends from western to eastern Europe, and from the southern Central Europe to Northern Europe. The distribution pattern in the sect. *Taraxacum* resembles a classical geographic parthenogenesis, since the distribution of the apomicts extends the one of the sexually reproducing dandelions (Menken et al. 1995; Verduijn et al. 2004).

Plant material and growing conditions

Seeds were collected from two widespread apomictic lineages of apomictic dandelions: *T. alatum* H. Lindb. and *T. hemicyclum* G. E. Haglund. Seed heads were collected in spring 2013 from three locations in North-Eastern Europe: from two locations in Czech Republic, that differed in elevation, and from one location in Finland (Fig. 2.1). Throughout this study we refer to the descendants of a single field-sampled individual as an accession. The collection of seeds in the field was done by taxonomic specialists that recognize these geographically widespread *Taraxacum* microspecies by specific phenotypic traits. After having the seeds propagated for one generation under common greenhouse conditions we confirmed the clonal identity of the *T. alatum* and *T. hemicyclum* plants with eight microsatellite markers which showed nearly identical multilocus genotypes for all accessions within a microspecies (Table 2.S2, supporting information).

Figure 2.1 Map of the sampling sites. Seeds of *T.alatum* and *T.hemicyclum* were collected in the Bohemian lowlands (CZL, circle), the Carpathians (CZH, triangle) and in Finland (FI, rectangle).

Throughout all generations of the experiment we used the same protocol for seed collection and seed sterilization and the same temperature and light conditions for the germination, growth and vernalization stages. Seeds derived from the first produced seed head per plant; seeds were surface-sterilized for 5 minutes with 0.5% sodium hypochlorite including 0.05% Tween20 (Sigma-Aldrich, Zwijndrecht, The Netherlands) and afterwards washed with demineralized water. Sterilized seeds were germinated on 0.8% agar plates for 10 days (14 h light / 10 h dark, 18 °C / 14 °C, 60% relative humidity on average, daylight maintained at a minimum of 30 µmol/m^2/s). Seedlings were individually transplanted to 9 x 9 x 10 cm pots containing a mixture of 80% potting soil and 20% pumice that was equalized to 210 ± 5 g. Nutrients were supplied with 1.5 g of Osmocote granules (15 N + 3.5 P + 9.1 K + 1.2 Mg + trace elements; Osmocote exact Mini, Everris international BV, The Netherlands). Afterwards the seedlings were grown under the same condition as during germination but with a light level of approximately 315 µmol/m²/s and were watered several times per week, depending on the rate of water loss. Prior to vernalization, rosette leaves were clipped back to 4-5 cm and the plants were put in a cold room at 4 °C (16 h daylight) for 5 weeks, with occasional watering depending on moisture loss.

Stress experiment

For each of the six accessions used in this study (2 apomictic lineages x 3 sampling sites) seeds were derived from a single greenhouse-propagated individual. Seeds were germinated and propagated as described before and 36 seedlings per accession were distributed over control, drought stress and salicylic acid (SA) stress (12 replicate plants per treatment). All plants of *T. alatum* were grown together in one climate chamber; the same applies to plants of *T. hemicyclum*. Within each apomictic lineage plants from all three accessions were randomized within treatment. Plants from a treatment group (control, drought, salicylic acid) were placed in rows to ensure a better application of the stress treatments and a non-touching between plants from different treatments, which we think was especially crucial for the application of the SA treatment. After four weeks of growth in the climate chamber the drought stress started: water was withheld from the 'drought' treatment until at least 80% of all 'drought' plants showed wilted leaves, at which moment all 'drought' pots were fully saturated with water. While the other groups were regularly watered, the 'drought' group experienced this deprivation of water ten times within a period of four weeks. After five weeks of growth, a one-time SA treatment was applied: 0.5 ml of a 10 mM SA solution (Sigma S-7401, dissolved in 0.1% Triton X-100 surfactant solution, pH = 2.3) was spread over three medium-sized leaves. The third, control, group received no treatment, also no mock treatment, since these plants were also used as control for the drought treatment. After eight weeks of growth, leaf punches were collected from the third fully developed leaf of each individual plant and put on ice for subsequent DNA isolation. Subsequently the plants were moved to a cold room for vernalization. All plants flowered approximately six weeks after the end of the vernalization period and seeds were collected from each plant. Using single-seed descent the subsequent two generations, G2 and G3, were grown under common control conditions in the greenhouse following the same experimental design and separated per genotype (as described for G1). For the drought experiment we evaluated DNA methylation for all plants in G1 and G3, to specifically address the question whether drought-induced DNA methylation changes exist that persist for two subsequent unexposed generations. For the SA experiment we evaluated DNA methylation in all three generations, but we limited this analysis to the *T. alatum* and *T. hemicyclum* plants from only one accession, the northern accession, FI. DNA was isolated from leaf punches taken after 7 weeks of growth for G2 and taken after 4 weeks of growth in G3.

DNA isolation and MS-AFLP

DNA was isolated following the CTAB procedure by Rogstad (1992) with minor modifications (Vijverberg et al. 2004) using approximately 1 cm^2 of fresh leaf tissue. During sampling, the leaf tissue was kept on ice in microtubes containing two 1/8" steel balls and after grinding, the samples were homogenized in CTAB buffer using a Tissuelyser II (Qiagen, The Netherlands) followed by washing and DNA precipitation steps. The final DNA pellet was dissolved in 50 µl TE and stored at -20°C until DNA was collected for all generations.

For the MS-AFLP analysis the isolated DNA was digested with the methylation sensitive enzymes *Hpa*II as frequent cutter and *Eco*RI as rare cutter following Keyte et al. (2006) with some modifications. *Hpa*II recognizes the tetranucleotide sequence, 5´-CCGG, which can be methylated on one or both DNA strands and at the internal and/or external cytosine. *Hpa*II cuts if the restriction site is free from methylations or if the external cytosine is hemi-methylated (e.g. see Schulz et al. 2013). Usually MS-AFLPs are run with a combination of the methylation-sensitive restriction enzymes *Hpa*II and *Msp*I, which enables the distinction between methylation polymorphisms and DNA sequence polymorphisms. However, in samples where genetic variation can be assumed to be negligible, such as under apomictic reproduction as in our experiment, variation in *Hpa*II and *Msp*I fingerprint profiles can be interpreted directly as methylation polymorphisms (Verhoeven et al. 2010b). We therefore used only *Hpa*II to capture methylation variation. Based on previous testing we selected eight *Eco*RI/*Hpa*II primer combinations (Table 2.S3). The digestion mix contained ten units of each *Eco*RI (100,000 U/ml) and *Hpa*II (50,000 U/ml) and the corresponding buffer (all from New England BioLabs, 180 Bioke, The Netherlands) in a total volume of 20 µl containing 50 ng of DNA. The digestion ran for three hours at 37°C. Afterwards adapters were ligated in a total reaction volume of 30 µl containing: 1 Unit of T4 DNA ligase and ligase buffer (ThermoFisher scientific, The Netherlands), 3.75 pmol of *Eco*RI adapter and 37.5 pmol of *Hpa*II adapter for 18 hours at 22 °C followed by 10 minutes at 65°C. The ligation product was diluted to 15% in water (Sigma Aldrich, the Netherlands). Pre-amplification was performed in a total volume of 50 µl using: 1 x buffer, 125 nmol $MgCl_2$, 2.5 U Taq DNA polymerase (all from GC biotech BV, The Netherlands), 10 nmol dNTPs (ThemoFisher scientific), 15 pmol of each pre-selective primer and 10 µl of diluted ligation product. The reaction started with 2 minutes hold at 72°C followed by 20 cycles of 30 sec at 94°C, 30 sec at 56°C, 2 min at 72°C and finished with 10 min incubation at 60°C and hold at 10°C. These pre-amplified products were diluted to 5% and proceeded to the selective amplifications in a total volume of 25 µl containing: 1 x buffer, 37.5 nmol $MgCl_2$, 1.25 U Taq DNA polymerase (all from GC biotech B.V., the Netherlands), 7.5 nmol dNTPs (ThermoFisher scientific, the Netherlands), 10µg BSA, 5 pmol labelled selective *Eco*RI primer, 20 pmol selective *Hpa*II primer and 5 µl diluted pre-amplified product. The selective amplification was started with 2 min hold at 94°C, followed by 10 cycles of 30 sec at 94°C, 30 sec at 65°C, 2 min at 72°C and 25 cycles with 30 sec at 94°C, 30 sec at 56°C, 2 min at 72°C and ended with 10 min at 60°C before hold at 10°C. The final PCR product was diluted to 2.5% in sterile water and analyzed on the ABI 3130 genetic analyzer (Life Technologies Europe BV, The Netherlands).

MS-AFLPs were screened in a total number of 320 plants, of which 317 plants yielded readable MS-AFLP fragments (see Table 2.S1 for number of samples per accession). Within each apomictic lineage, all selected samples were run through the MS-AFLP lab protocol in fully randomized order. We used for all samples of an apomictic lineage one digestion mix and after digestion proceeded directly with the ligation and pre-amplification steps. Technical duplicates of MS-AFLP analysis were performed for a randomly chosen subset of 15% samples in order to quantify the MS-AFLP error rates, and negative controls were included (10%) to check for peaks that indicate contamination signals and carry-over effects (Bonin et al. 2007).

Fragment Scoring

Fragments between 100 – 500 base pairs were scored using GeneMapper 5.0 (Life technologies Europe BV, the NL). Using overlaying peak profiles in GeneMapper, polymorphic loci were identified and included if at least one of the samples showed a peak height exceeding 25. After visually checking each locus a threshold peak height of 25 or 50 was chosen for each locus to score individual peaks as "present" if peak height exceeded the threshold. Loci were discarded if they were monomorphic or if they contained fragments that showed up in any of the negative controls. Loci were also discarded if they showed too many mismatches among technical duplicates: we allowed a maximum of three mismatches among the set of 24 pairs of technical duplicates. This resulted in MS-AFLP error rates of 1.65 % for *T. alatum* and 2.72 % for *T. hemicyclum*. The final data sets consisted of 49 polymorphic loci for *T. alatum* and 53 polymorphic loci for *T. hemicyclum*.

Statistical Analysis

Within apomictic lineage and per generation the status of each single marker was analysed using logistic regression models to test for significant stress and accession effects (R-function glm() with binomial error distribution and logit link function). P-values were corrected for multiple testing using false discovery rate control at FDR=0.05 (R-function p.adjust()). Multivariate analyses were performed based on pairwise distances calculated by counting the absolute number of inconsistent loci between individuals (R-function designdist()). Based on this distance matrix permutational multivariate analysis of variance (R-function adonis()) and analysis of multivariate homogeneity of group dispersions were performed (R-function betadisper()). The former analysis tests for different mean positions of experimental groups in multivariate MS-AFLP space while the latter analysis tests for differences between experimental groups in their amount of MS-AFLP variation irrespective of group mean positions. A principal coordinate analysis was plotted to visualize the multidimensional data (R-function pcoa() from package Ape).

To track individual methylation changes over generations we first inferred a consensus epigenotype, which represents the hypothesized MS-AFLP profile at the beginning of G1 of all plants from the same accession. We defined this consensus as the methylation state that was observed in plants from the control treatment in G1, for each accession separately, including only loci for which none or maximum one out of the 10 replicate plants showed a deviating marker status. This criterion excluded 1-3 loci per accession from the consensus analysis because they were too polymorphic across the G1 group to confidently call the consensus state. Any deviations of the detected MS-AFLP from the consensus that were observed in stress treatments and later generations were assumed to have arisen during the experiment. These methylation changes were counted and checked for their persistence in the next generations. For each accession separately, we fitted a generalized linear mixed model to test on the plant's proportion of MS-AFLP loci that deviated from consensus for effects of generation, G1 treatment, and the interaction generation x G1 treatment (PROC GENMOD in SAS 9.2, using type 3 analysis and likelihood ratio tests for significance).

Results

Drought and accession effects on methylation

The *Hpa*II profiles clustered by accession but not by stress treatment: no clear differentiation was found between the methylation profiles of drought-stressed and control plants (Table 2.1, visualized in Fig. 2.2). However, in both apomictic lineages the drought x accession interaction in the first generation was marginally significant (*T. alatum* P-value = 0.059, *T. hemicyclum* P-value = 0.074), suggesting that a weak drought effect may be present but not equally expressed in all accessions. Visual inspection of the PCoA clustering with group centroids in the first generation (supporting information, Fig. 2.S1) indicated that for *T. alatum* the lowland Czech accession (CZL) may be most responsive to drought while for *T. hemicyclum* the northern (FI) and medium-altitude (CZH) accession might be more responsive. But even in these accessions the response was weak, and any accession-dependency of the response to drought was not inherited, since the interaction effect had disappeared in the third generation.

Table 2.1 Permutational Multivariate Analysis of Variance based on MS-AFLP profiles of two apomictic dandelion lineages. Per generation and stress treatment, drought and salicylic acid, the proportion of variance explained is shown.

		T. alatum			*T.hemicyclum*		
	df	G1	G2	G3	G1	G2	G3
Drought experiment:							
Accession	2	0.913 ***		0.816 ***	0.914 ***		0.833 ***
Drought	1	0.001 ns		0.008 ns	<0.001 ns		0.002 ns
Accession x Drought	2	0.007 .		0.003 ns	0.006 .		0.005 ns
Salicylic Acid experiment:							
SA	1	0.073 ns	0.032 ns	<0.001 ns	0.049 ns	0.058 ns	0.045 ns

Based on function adonis () from R-package Vegan with 10,000 permutation steps.
Shown are R^2: proportion of variance explained and significance codes:
*** < 0.001; ** < 0.01; * < 0.05; . < 0.1; ns= not significant.

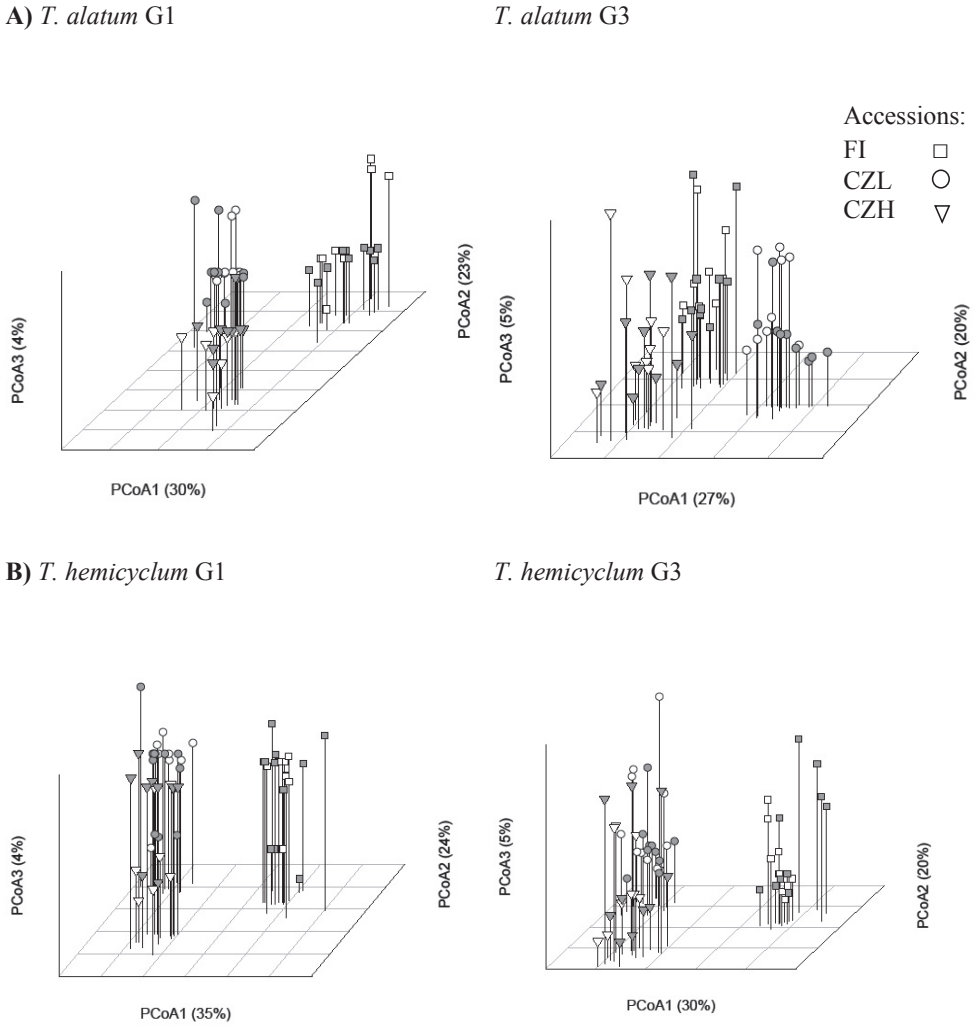


Figure 2.2 Principal Coordinate Analysis (PCoA) based on MS-AFLP profiles of drought stressed (grey symbols) and control plants (no fill) in the first, stressed generation and the unstressed progeny of the third generation. A) MS-AFLP profiles of *T. alatum*, B) MS-AFLP profiles of *T. hemicyclum*.

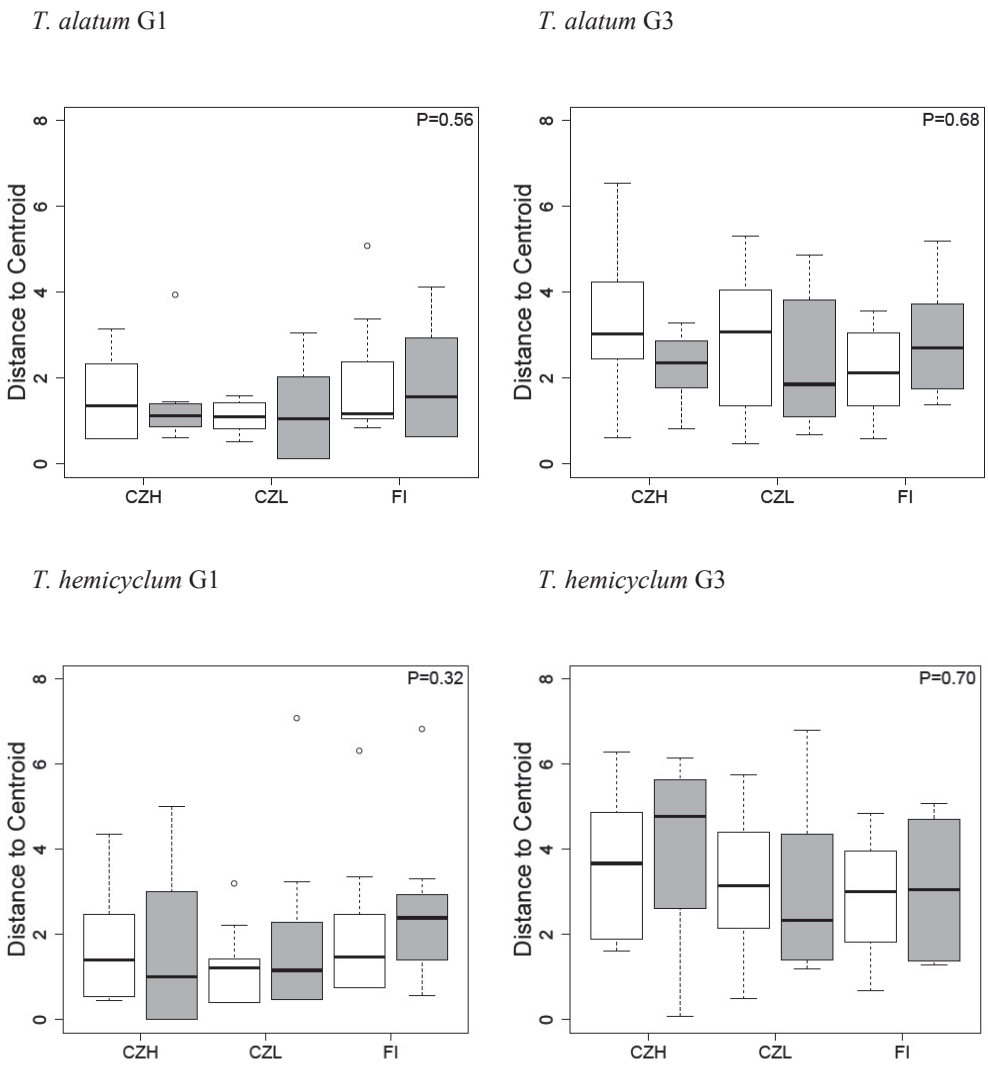


Figure 2.3 Drought stress- and accession-specific epigenetic variation. Values are distances to group centroids calculated by multivariate dispersion analysis on MS-AFLP profiles of two apomictic dandelion lineages from first and third generation. From left to right the boxplots show the dispersion of distances to centroid of the three accessions either in white boxplots = control or grey boxplots = drought stress conditions. P indicates the p-value of the treatment effect based on a permutation test with all accessions pooled together.

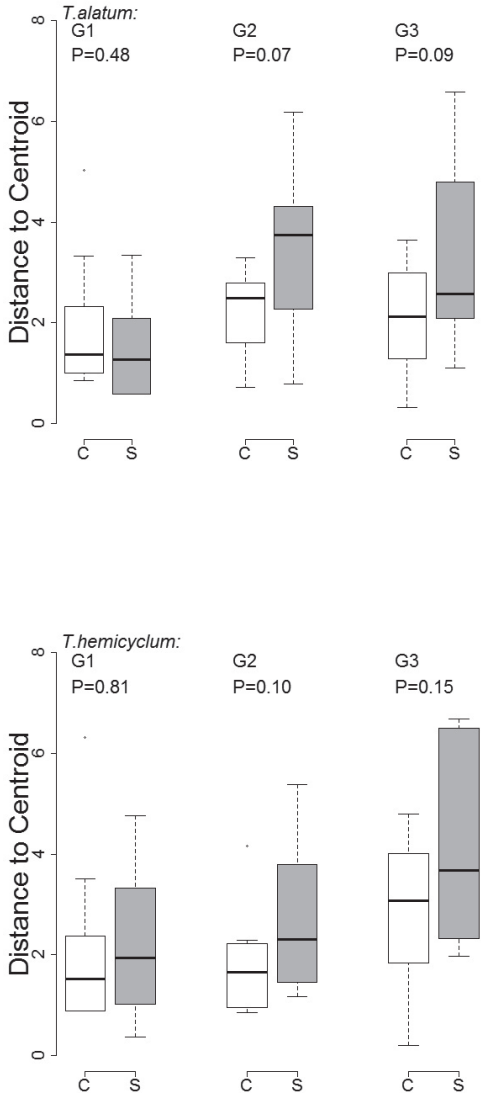


Figure 2.4 Salicylic acid stress-specific epigenetic variation. Values are distances to group centroids calculated by multivariate dispersion analysis on MS-AFLP profiles of two apomictic dandelions lineages and from three generations. From left to right the boxplots show the dispersion of distances to centroid of the accession FI separated in C (white boxplot) = control group and S (grey boxplot) = salicylic acid stressed group. P indicates the p-value of the treatment effect based on 1,000 permutations.

Besides causing a directed shift in methylation variation, treatments might also trigger an increased level of undirected (random) methylation changes. An increase in the number of random changes would promote differentiation in methylation profiles between replicate plants from the same experimental group. However, no such effect was observed in response to drought stress: multivariate dispersion did not differ significantly between control and drought groups (Fig. 2.3). Nevertheless, despite the lack of an inherited treatment effect, a clear buildup of methylation variation was observed between the first and third generation in both the control and the drought groups (Fig. 2.3). When pooled over treatments in order to test for generation differences, multivariate dispersion analysis revealed a significant increase of DNA methylation variation over generations for four of the six accessions: lowlands (CZL: *T. alatum* P-value: 0.003, *T. hemicyclum* P-value: 0.006) and medium altitude (CZH: *T. alatum* P-value: 0.014, *T. hemicyclum* P-value: 0.002); not significant for high latitude (FI: *T. alatum* P-value: 0.139, *T. hemicyclum* P-value: 0.198).

In addition to these multivariate analyses, we also performed a marker-by-marker analysis to test if MS-AFLP marker status associates with treatment or accession. After controlling for multiple testing at a false discovery threshold of 0.05 the single marker testing revealed that approximately a third of the analyzed loci show an accession effect (*T. alatum*: 16 loci in G1 and 17 loci G3, *T. hemicyclum*: 20 loci in G1 and 19 loci in G3), but none showed a drought effect.

Salicylic acid effect on methylation

The multivariate analysis of methylation variation following salicylic acid (SA) application (high latitude accessions, FI, only) showed no overall distinction between the control group and the SA-stressed plants, neither in the first generation that experienced the stress, nor in the subsequent generations (Table 2.1). Multivariate dispersion analysis (distance to centroid) showed no significant difference between SA-stressed and control plants, although a marginally significant trend was observed that offspring of SA-treated plants showed increased levels of dispersion compared to offspring of control plants (Fig. 2.4). We observed a buildup of DNA methylation variation over generations (pooled across control and stress groups, the generation effect for *T. alatum*: P-value = 0.017; for *T. hemicyclum*: P-value = 0.009).

The single marker tests revealed that only a few loci showed a response to salicylic acid treatment (*T. alatum*: 3 loci in G2 and 3 loci in G3, *T. hemicyclum*: 1 locus in G3), however they did not stand up to the multiple testing correction (false discovery rate > 0.05).

Tracking deviations from consensus

By comparing the status of MS-AFLP markers to an accession-specific consensus profile, which was based on G1 control plants, individual loci could be identified that showed a methylation change during the experiment. In both the drought and the SA experiments, methylation deviations were observed with a frequency of approximately 1-3% in G1 and up to 4-9% in G3 (Tables 2.2 and 2.3).

Table 2.2 Methylation changes observed in two apomictic dandelion lineages, that were exposed to drought stress in generation 1 and were propagated in a common environment to generation 3

	G1	G3	Transmitted to G3
T. alatum			
Total cases (markers x samples)	1,353	1,306	
Changes in Control cohort	16	51	*4*
Changes in Drought cohort	22	47	*8*
T. hemicyclum			
Total cases (markers x samples)	1,570	1,570	
Changes in Control cohort	25	124	*2*
Changes in Drought cohort	47	130	*10*

In Generation 1 the presence⁄absence profiles of methylation-sensitive amplified fragment length polymorphism (MS-AFLP) loci were evaluated against the consensus epigenotype. The counted changes represent the pooled sum over all accessions (~ 30 samples) and italic numbers show the changed methylations of G1 that were transmitted to G3.

Table 2.3 Methylation changes observed in two apomictic dandelion lineages, that were exposed to salicylic acid stress in generation 1 and propagated in a common environment to generation 3

	G1	G2	Transmitted to G2	G3	Transmitted to G3
T. alatum					
Total cases (markers x samples)	460	460		460	
Changes in Control cohort	7	12	*3*	10	*1*
Changes in SA cohort	6	24	*1*	21	*1*
T. hemicyclum					
Total cases (markers x samples)	530	530		530	
Changes in Control cohort	12	16	*2*	35	*0*
Changes in SA cohort	16	26	*4*	46	*2*

In Generation 1, the presence⁄absence profiles of methylation-sensitive amplified fragment length polymorphism (MS-AFLP) loci were evaluated against the consensus epigenotype. Italic numbers show the deviating methylations in G1 (= 10 samples) that were transmitted to G2 and G3 respectively.

For *T. hemicyclum* the total number of deviations from consensus per individual was significantly higher in the SA-treated plants and SA-descendants than in control plants and control-descendants ($P < 0.05$; Table 2.4). This SA effect was also marginally significant in *T. alatum* (P-value: 0.086; Table 2.4). No effect of drought stress was detected on the number of methylation changes per individual (Table 2.5). These analyses, that test deviations from the MS-AFLP consensus profile established for control plants in G1, were performed across all generations, meaning that the observed SA-effect is not necessarily restricted to the first generation. In fact the frequency of deviations from the consensus profiles showed more pronounced differences between control and SA group in G2 and G3 compared to G1 (Tables 2.2 and 2.3). For both drought and SA stress, the generation effect on deviations from the consensus was highly significant (Tables 2.4 and 2.5), showing increasing deviations from the consensus from G1 to G3 (see also Fig. 2.2). Of the methylation changes that occurred in G1 in response to drought or SA, 13%-36% were observed to remain in the changed state until the G3 generation (Tables 2.2 and 2.3).

Table 2.4 Generalized linear mixed model tests of treatment and generation effects on the number of deviating MS-AFLP (*Hpa*II) loci per individual.

		T. alatum		*T. hemicyclum*	
	Df	Chi-Square	sign	Chi-Square	Sign
Generation effect	2	10.60	**	30.28	***
SA effect	1	2.95	.	3.95	*
Generation x SA effect	2	2.11	ns	0.31	ns

Chi-square and significance for accession *N*.
Significance codes: *** < 0.001; ** < 0.01; * < 0.05; . < 0.1; ns=significant

Table 2.5 Generalized linear mixed model tests of treatment and generation effects on the number of deviating MS-AFLP (*Hpa*II) loci. Separate analyses were done per accession: *H, L* and *N*.

	T. alatum		*T. hemicyclum*	
	Chi-Square	sign	Chi-Square	sign
Generation effect in:				
CZH	14.31	***	55.62	***
CZL	16.33	***	44.25	***
FI	2.00	ns	21.70	***
Drought effect in:				
CZH	0.06	ns	2.64	ns
CZL	0.17	ns	1.81	ns
FI	0.52	ns	2.33	ns
Generation x Drought effect in:				
CZH	1.54	ns	1.81	ns
CZL	0.73	ns	3.08	.
FI	0.10	ns	0.39	ns

Chi-square and significance per accession (CZH: Czech Republic medium altitude, CZL: Czech Republic lowlands, FI: Finland). All degrees of freedom = 1, Significance codes: *** < 0.001; ** < 0.01; * < 0.05; . < 0.1; ns=significant

Discussion

The aim of this study was to evaluate the heritability of DNA methylation changes in response to environmental stimuli within apomictic dandelion lineages, which harbor limited genetic variation to adapt to changing environments. In addition we aimed at evaluating if the capacity for such inheritance is different in lineages that have successfully migrated into medium-altitude or high-latitude habitats. In two apomictic dandelion lineages drought stress showed marginally significant, accession specific direct stress effects in the methylation profile (accession x drought effect), but no transgenerational effect when screening responses of the third generation to the original stress treatments. Salicylic acid, which mimics effects of defense induction by biotrophic pathogens, promoted seemingly undirected DNA methylation changes in offspring plants leading to an increase in methylation variation in subsequent generations. This SA-induced methylation increase in subsequent generations was not detectable in the stressed plants themselves, suggesting a more complex underlying mechanism of the plants' response to SA than transgenerational stability of stress-induced modifications.

This study provides support for the induction of DNA methylation modifications by environmental stresses, both as a direct effect in stressed plants and via an (unidentified) inherited effect causing novel changes in their unstressed progeny. Depending on genotype and environmental exposure, up to one third of the DNA methylation changes observed in the first generation were stably inherited for at least two subsequent offspring generations, indicating the potential for epigenetic divergence within apomictic lineages. However, this estimate includes spontaneous DNA methylation changes that are unrelated to the environmental signal, and the effect of experimental treatments was generally weak, genotype-dependent, environment-specific and may involve different underlying mechanisms. An additional important finding of this study is that considerable levels of heritable DNA methylation variation build up irrespective of environments from generation to generation in this apomictic system.

Unambiguous demonstrations of environmentally-induced transmission of DNA methylations that result in a functional "stress memory" have remained elusive (Pecinka et al. 2010; Boyko & Kovalchuk 2011; Mirouze & Paszkowski 2011; Paszkowski & Grossniklaus 2011). Field studies have revealed associations between methylation variation and biotic, as well as abiotic characteristics of the habitat (Gao et al. 2010; Herrera & Bazaga 2010; Lira-Medeiros et al. 2010). However, in a widespread *Arabidopsis thaliana* haplotype, Hagmann et al. (2015) observed that heritable DNA methylation differences accumulated in a stochastic manner, like genetic divergence, while no inherited environmentally induced effects were detected. However, they used propagated plants that were several generations removed from the field-collected material. Thus the environmental epigenetic induction might have been only transient and disappeared again. In the case of the clonally reproducing Japanese knotweed plants were grown from field-collected roots directly, which revealed a substantial DNA methylation variation that was clustered by habitat while the plants showed limited genetic variation (Richards et al. 2012). In addition, (Schmitz et al. 2011) revealed the ability of DNA methylation variation to generate substantial transgenerational diversity in the extent

to which genes are transcribed, providing a mechanism for phenotypic diversity in the absence of genetic mutations. Although these studies show ambiguous results on the longevity and the stress sensitivity of DNA methylation inheritance, they nevertheless do not refute the hypothesis that epigenetics may enable asexual lineages to adapt to environmental changes, at least in the short run, and so overcome the assumed disadvantage of limited genetic variation.

One important factor in assessing the ecological and evolutionary relevance of epigenetic variation is to distinguish autonomous epigenetic variation from epigenetic variation that has a genetic basis. A recent study showed that even small genetic differences can be responsible for extensive genome-wide DNA methylation differences (Dubin et al. 2015). For instance heat-stressed *Arabidopsis* showed heritable phenotypic responses, presumably based on transmitted epigenetic effects, that differed depending on the genotype as well as the tissue tested and the stress response was shown to persist for two generations only (Lang-Mladek et al. 2010; Suter & Widmer 2013). Such relations between genetic and epigenetic variation make it difficult to attribute adaptive potential to epigenetic variation alone. Strategies to address this problem include the use of statistical methods to distinguish patterns of epigenetic variation that are independent from patterns of genetic variation (Richards et al. 2010) and the experimental use of completely inbred or asexually reproducing lineages (such as in this study). However, even with these strategies it is almost impossible to rule out underlying genetic variation as a factor without high-resolution genomic analysis. A genetic mechanism involved in epigenetic stress responses is for instance the regulation of transposable elements (TEs). TE transpositions, which are typically deleterious to the genome, are controlled by DNA methylations. The silenced state of TEs, which in turn can affect the expression of nearby genes, can persist through cell lines and across generations (Feng et al. 2010). Demethylations, and thereby the release of silenced TEs, have been shown in response to stress (Grandbastien 1998; Kalendar et al. 2000), which can result in altered transcription of genes close to the TE and can generate genetic variation by the transposed TEs. The ambiguous findings regarding the role and mechanism of epigenetic variation in plant populations call for more studies that link the causes and consequences of DNA methylation and try to disentangle sequence-independent effects from sequence-mediated effects.

In contrast to a previous study on effects of SA stress in apomictic (Verhoeven et al. 2010b) and *Arabidopsis thaliana* (Dowen et al. 2012), we could not detect clear direct stress-induced methylation changes in the SA-exposed plants themselves. The observed lack of a detectable response in the SA-exposed generation might derive from the low-resolution technique of MS-AFLPs, which detects only a small fraction of methylation changes. It is possible that there was a DNA methylation response to SA that we simply did not pick up with this technique – although we did pick up methylation effects in subsequent generations. Alternatively, our results might suggest that different underlying mechanisms are causing the varying SA stress responses. Our study shows that novel epimutations arose in the second and third generation after SA application. The mechanism for such a "delayed" effect of SA stress is unknown, but might be associated with heritably altered TE activity that causes continued transpositions and associated methylation changes in subsequent generations.

The differences between the SA stress responses observed by Verhoeven et al. (2010b) and by the current study might also be related to the age of the apomictic lineage used. Whereas the current study is based on natural apomictic genotypes, the genotype used in the previous study (AS34) was a synthetic apomict derived experimentally by crossing a sexually reproducing mother (diploid) with pollen from an apomictic father (triploid) and therefore underwent very recent hybridization and polyploidization. Such genomic events are associated with DNA methylation reprogramming and TE release which might affect responses to environmental stresses (Salmon et al. 2005; Verhoeven et al. 2010a).

Quite independent from stress-induced effects, we observed methylation variation that built up increasingly over the three tested generations indicating a considerable background rate of heritable epimutations. This provides evidence that DNA methylations can be stably transmitted and maintained for at least two generations. Using a methylome and genome screening in *A. thaliana*, Becker et al. (2011) found a high number of stochastic epimutations but also a frequent reversion of epimutations and a dependency on where and which type of DNA methylation (CG, CHG) was addressed. However, recent novel analyses in the same system have called the reported high reversal rates and lack of long-term stability into question (van der Graaf et al. 2015). Depending on multigenerational stability and on phenotypic consequences, the observed significant buildup of methylation variation over generations could play a relevant role for selection and adaptive responses within an apomictic lineage (Schmitz et al. 2011). Stochastic epimutations could potentially also result in epigenetic divergence between sub-lineages within apomictic lineages over microevolutionary time, which is consistent with the accession differences that we observed within single apomictic lineages.

Conclusion

This study reveals that stress environments can have effects on DNA methylation patterns in unexposed offspring plants, but also that the effects are highly context dependent. While drought tended to cause a genotype-specific response in the methylation profile of the stressed generation only, SA stressed plants showed effects (as increased methylation variation) that were expressed mostly in the successive generations. Apart from stress-related methylation variation, spontaneous epimutations added to a clear build-up across generations, confirming that methylation variation can be rapidly generated. Epimutations have been shown to occur at much higher rates than genetic mutations, generating variation that is potentially visible to natural selection. This could underlie the epigenetic deviance and ultimately in the within-lineage differentiation that we observed in the accessions tested. To what extent this epigenetic divergence is fully independent on genetic deviance has yet to be shown.

Acknowledgements
We thank Rutger Wilschut for providing microsatellite data and Bob Tráviníček and Juhani Räsänen for the seed collection. This study was supported by NWO-ALW grants: NWO-Ecogenomics 884.10.003 and 864.10.008 from the Netherlands Organisation for Scientific Research and by the Czech National Grant Agency, grant, No. GA13–13368S

Supporting information

Table 2.S1 Plant material sampling site

Sampling site	Accession	Original code	Apomictic lineage	Latitude	Longitude	m.a.s.l.	No. of samples
Central Bohemia, CZ	CZL	5/1	*T. alatum*	49° 50' 23" N	14° 31' 07" E	351	39
		1275	*T. hemicyclum*	50° 06' 31" N	15° 58' 14" E	302	40
White Carpathians, CZ	CZH	1284_S1	*T. alatum*	49° 16' 23" N	17° 58' 01" E	483	38
		1285	*T. hemicyclum*	49° 20' 13" N	18° 01' 17" E	373	40
South East Finland	FI	12/170	*T. alatum*	62° 11' 57" N	30° 35' 51" E	74	80
		12/168	*T. hemicyclum*	62° 10' 29" N	30° 35' 47" E	77	80

For accessions CZL and CZH: 2 generations x 2 treatments [drought, control] x 10 replicas; for accession FI: 2 generations drought x 10 replicas and 3 generations x 2 treatments [salicylic acid, control] x 10 replicates.

Table 2.S2 Microsatellites of all apomictic dandelion lineages and accessions used in chapters 2 and 3.

Lineage	Acc.	M1		M2		M3		M4	
T. alatum	FI	mst58	125	mst44B	185	mst31	238	mst78	164 172
T. alatum	CZH	mst58	125	mst44B	185	mst31	238	mst78	164 172
T. alatum	CZL	mst58	125	mst44B	185	mst31	238	mst78	164 172
T. hemicyclum	FI	mst58	104 123 125	mst44B	176 195	mst31	126 243	mst78	164 168
T. hemicyclum	CZH	mst58	104 123 125	mst44B	176 195	mst31	126 243	mst78	164 168
T. hemicyclum	CZL	mst58	104 123 125	mst44B	176 195	mst31	126 243	mst78	164 168

Lineage	Acc	M5		M6		M7		M8	
T. alatum	FI	mst61	136 138	mst67	203 221	mst72	**175** **209**	mst143	238 240 246
T. alatum	CZH	mst61	136 138	mst67	203 221	mst72	**175** **211**	mst143	238 240 246
T. alatum	CZL	mst61	136 138	mst67	203 221	mst72	**175** **201**	mst143	238 240 246
T. hemicyclum	FI	mst61	131 134 145	mst67	230 239 241	mst72	176 186 192	mst143	238 246
T. hemicyclum	CZH	mst61	131 134 145	mst67	230 239 241	mst72	176 186 192	mst143	238 246
T. hemicyclum	CZL	mst61	131 134 145	mst67	230 239 241	mst72	176 186 192	mst143	238 246

Bold numbers represent the only loci that deviated. Acc: Accessions; FI: Northern accession, Finland; CZH: Czech Republic high altitude; CZL: Czech Republic low altitude. For the sRNA screening in Chapter 3 only the accession FI, *T. hemicyclum* was used (grey highlighted).

Table 2.S3 Adapters and primers used for MS-AFLPs

Adapters[*]	**Sequence 5´- 3´**
*Eco*RI-adapter I	CTCGTAGACTGCGTACC
*Eco*RI-adapter II	AATTGGTACGCAGTC
*Hpa*II Adapter I	GATCATGAGTCCTGCT
*Hpa*II Adapter II	CGAGCAGGACTCATGA
Pre-selective primers	**Sequence 5´- 3´**
*Eco*RI-A	GACTGCGTACCAATTC**A**
*Eco*RI-T	GACTGCGTACCAATTC**T**
*Hpa*II -T	ATCATGAGTCCTGCTCGG**T**
Selective primers	**Sequence 5´- 3´**
*Eco*RI + AAC/ACA/AG/ACC	GACTGCGTACCAATTC**AAC/ACA/AG/ACC**
*Hpa*II + TCA/TAC/TAG	ATCATGAGTCCTGCTCGG**TCA/TAC/TAG**

[*] *Eco*RI adapters (Reyna Lopez *et al.* 1997), *Hpa*II adapters (Xiong *et al.* 1999). Following eight *Eco*RI/*Hpa*II primer combinations were used: ACA / TAC, ACA / TCA, AAC / TAG, AG / TCA, AG / TAC, ACC / TCA, ACC / TAG, ACC / TAC

Figure 2.S1 *HpaII* based distribution with group centroids of two apomictic dandelion genotypes and three accessions of the drought-stressed generation. Red dots represent the group centroids. Triangle show drought stressed plants and circles show controls.

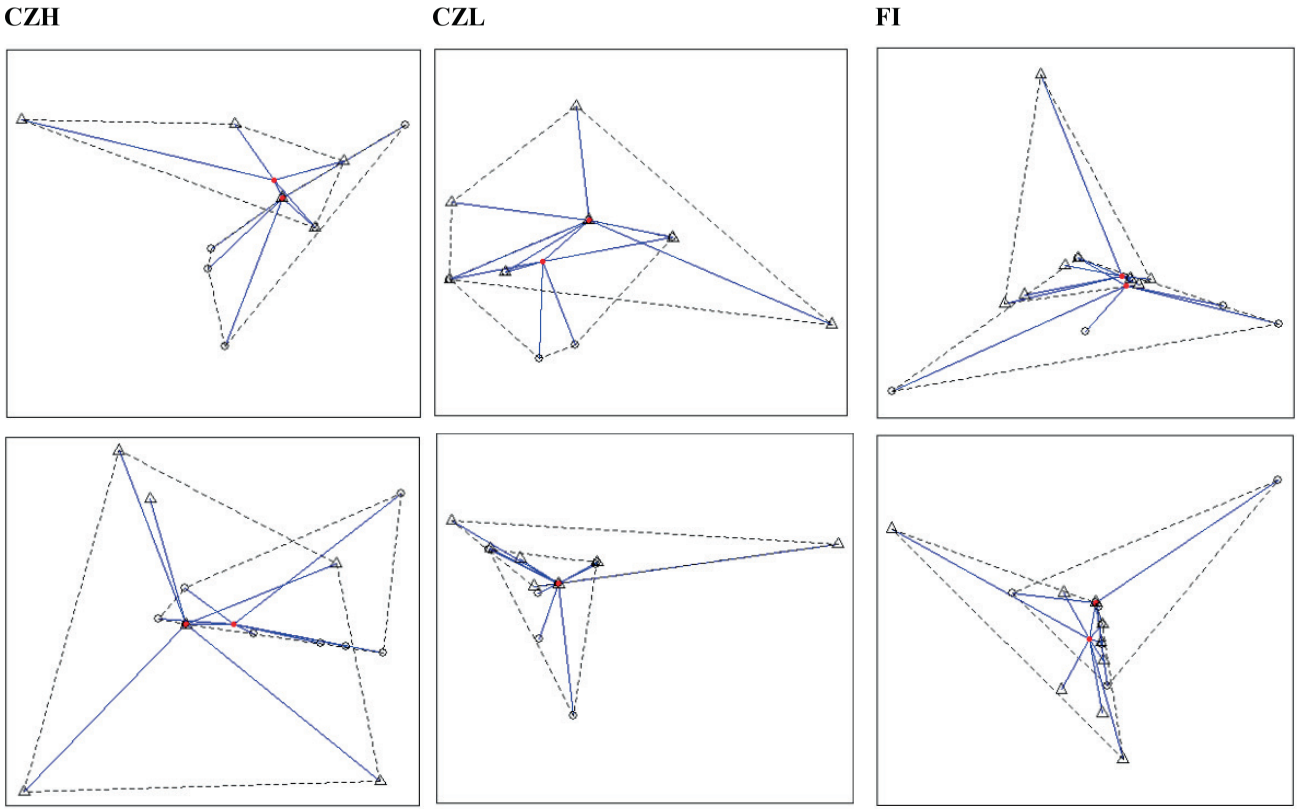
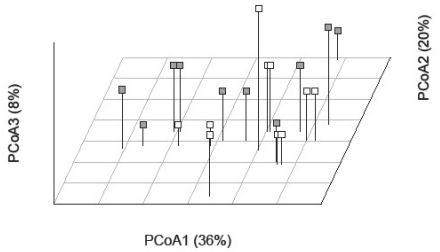
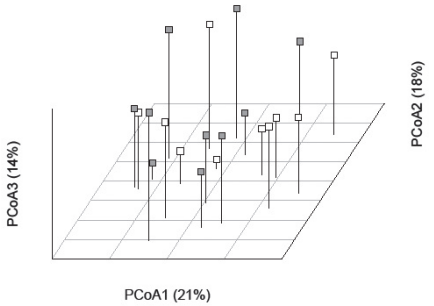


Figure 2.S2 Principal coordinate Analysis (PCoA) based on MS-AFLP profiles of salicylic acid stressed (grey filled rectangles) and control plants (no fill) in the first, stressed generation and the unstressed progeny of the two subsequent generations. All based on MS-AFLPs (*Hpa*II) profiles of accession FI of *T. alatum* and *T. hemicyclum*.

T. alatum G1



T. alatum G2



T. alatum G3

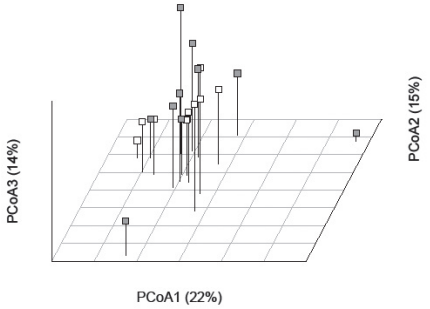
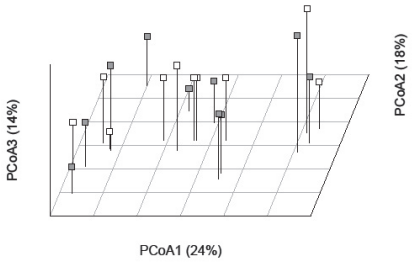
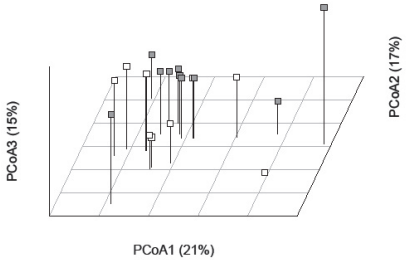


Figure 2.S2 continued

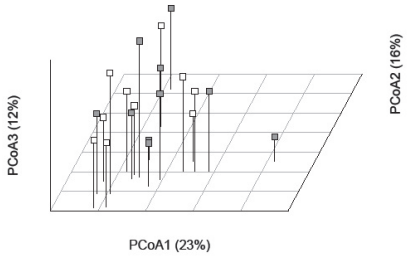
T. hemicyclum G1



T. hemicyclum G2



T. hemicyclum G3



3

Small RNAs reflect grandparental environment in apomictic dandelions

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Abstract

Carriers of epigenetic information can be profoundly altered by environmental stressors. Epigenetic mechanisms such as DNA methylation, histone modifications and small RNAs (sRNAs), can respond to stress signals with effects on activity of genes and transposable elements (TEs). It has furthermore been revealed that in some cases stress-induced DNA methylation modifications can be transmitted to subsequent generations even when the stress is no longer experienced. This transgenerational epigenetic inheritance might be especially important for plant species (due to their immobility) to quickly adjust to new environmental conditions. However, evidence for environment-transgenerational epigenetic inheritance is limited and the role of sRNAs in this "stress memory" remains little understood. An apomictic dandelion lineage was propagated and exposed to drought stress, salicylic acid (SA) treatment and a control environment and the progeny were grown unstressed for two generations. We screened sRNAs in the third generation and found consistent changes in the sRNA length composition in the offspring of treated groups compared to offspring of controls. Moreover, while individual genes did not show a clear change in sRNA abundance due to the grandparental treatments, the set of genes that showed the highest sRNA abundance changes was significantly enriched for GO (gene ontology) terms that were associated with stress-related functions. Our results demonstrate that ancestral environments can leave a sRNA footprint that lasts at least three generations. This observation adds to growing evidence that sRNAs may play an important role in multi-generational plant adjustment to stressful environments.

Introduction

Environmental stress can trigger responses that are mediated by changes in gene regulation. Some environmental responses are long-lived, for instance the "hardening off" phenomenon where mild stress stimuli induce resistance against stress treatment (Boyko & Kovalchuk 2011). Another example is the priming of plant defenses: upon mild pathogen infection some plants can enter a "primed" state, which is expressed as a quicker or more vigorous defense response upon a second infection later in life (Conrath et al. 2002). Epigenetic modifications with regulatory function, which include changes to DNA methylation, histone modifications and small RNAs, are candidate mechanisms to account for the maintenance of these stress responses. Such epigenetic marks can be stably transmitted through cell divisions and may enable long-term changes in gene regulation. Although many environmentally induced epigenetic changes are thought to be reset in plants during gametogenesis, some can persist and are stably transmitted to subsequent generations, even in the absence of the initial environmental stimuli (Luna et al. 2012; Verhoeven et al. 2010b; Holeski et al. 2012; Jablonka & Raz 2009; Bond & Baulcombe 2014).

Unraveling the epigenetic mechanisms of functionally relevant transgenerational stress responses is, because of its "Lamarckian" flavor, a topic of much recent interest, however, current evidence is limited (Heard & Martienssen 2014; Grossniklaus et al. 2013). For instance, inherited stress responses can be caused also by other mechanisms, such as maternal effects through *e.g.* seed size variation or through maternal hormonal inputs into the developing embryo (Galloway et al. 2009). Stress responses that cannot be explained by maternal effects, and that can rule out a direct exposure effect on, for instance, the germline or the developing embryo, are likely to involve transgenerational epigenetic inheritance (Holeski et al. 2012). Unlike DNA sequence variation, epigenetic patterns have a relatively high rate of mutation and reversion and therefore transgenerational epigenetic inheritance represent a more dynamic system (Becker et al. 2011). However, it is still unclear why certain DNA methylation changes persist across generations while others are reset and what the stable DNA methylation variation means for evolution on a population level (Baulcombe & Dean 2014).

Epigenetic inheritance to subsequent generations could involve DNA methylations that resist methylation resetting during gametogenesis and embryogenesis. Persistent DNA methylations have been found, in fact mostly in CG context (Feng et al. 2010). Heritable DNA methylations may play a significant role in the plants` "stress memory" since several studies have reported stress-induced DNA methylation modifications that were still detectable in offspring generations (Verhoeven et al. 2010b; Boyko et al. 2007; Bilichak et al. 2012; Kou et al. 2011). The actual function of these inherited epigenetic marks has, however, not been tested and some studies showed ambiguous results (see for instance chapter 2 of this thesis where stress-dependent epigenetic responses were found in apomictic dandelions). Overall, based on current plant studies there is little strong evidence that environment-induced epigenetic modifications are a common mechanism for the multi-generational propagation of adaptive stress responses. Moreover, Heard &

Martienssen (2014) state that epigenetic inheritance is mostly, if not always, a byproduct of germline defense strategies against TEs, viruses or transgenes.

As an alternative to meiotically stable DNA methylation, epigenetically generated signals such as small RNAs can migrate from parental tissues to the plant embryo where they could guide methylation in CG, CHG and CHH contexts at specific target loci (Bond & Baulcombe 2014; Ibarra et al. 2012; Calarco et al. 2012). Small RNAs are divided in length groups that are characterized by different functions and by different synthesis pathways, of which 21nt and 24nt RNAs are the best studied in plants. On the one hand, the 24nt RNAs often guide DNA methylations to genomic loci, referred to as the RNA dependent DNA methylation pathway (RdDM) (Zhai et al. 2008; Vu et al. 2013). On the other hand, 21nt RNAs typically target and degrade other types of RNA molecules such as messenger RNA or viral RNA, and therewith regulate the transcription of a gene or a virus, referred to as posttranscriptional regulation.

In the nematode *Caenorhabditis elegans* a heritable antiviral defense mechanism was found. It is based on pools of 21nt RNAs that are copied by RNA-dependent RNA polymerases and that are transmitted to subsequent generations (Rechavi et al. 2011). This mechanism also seems to control a heritable stress response in famished individuals of *C. elegans* where the starvation showed a lasting effect for at least three generations (Rechavi et al. 2014). Such a mechanism is unknown in plants, but there are some indications that also in plants sRNAs play a role in the expression of transgenerational environmental effects: mutants that are compromised in sRNA function failed to express transgenerational effects that were observed in wildtype plants (Luna et al. 2012; Rasmann et al. 2012).

Few studies revealed the potential of plant sRNAs to transfer from parental cells to germ cells or the embryo. Most of these studies associated the sRNA inheritance with RdDM and transposable elements (TEs) silencing (Vu et al. 2013). For instance, in pollen of *Arabidopsis* TE-silencing DNA methylation is lost in somatic companion cells, which results in activation of TEs. These active transposable elements then generate sRNAs that are transported to the germ cell, and it has been proposed that these sRNAs contribute to reestablishment of DNA methylation and efficient re-silencing of TEs in the zygote fertilized by this pollen (Slotkin 2009). Thus, in plants interaction between sRNAs and DNA methylation takes place in transmitting information between generations (Mirouze & Paszkowski 2011). Stress-induced sRNA inheritance has also been detected in some studies: Bilichak et al. (2015) observed sRNAs that migrated from soma cells in stressed tissues to germ cells and the same sRNAs were found back in leaf tissue of the developed offspring. Rasmann et al. (2012) showed epigenetic inheritance of induced defense and enhanced resistance against herbivory guided by transmitted sRNAs.

The aim of the present study is to gain more insight in the role of sRNAs in transgenerational stress effects by deep-sequencing sRNAs in second-generation offspring of apomictic dandelion plants that had been exposed to different environmental stresses. We used an apomictic dandelion lineage of the *Taraxacum officinale* group (*T. hemicyclum*) collected in Finland. Apomictic dandelion lineages reproduce asexually via clonal seeds and represent, just like other taxa with low levels of genetic variation, a

suitable model system to study epigenetic effects without the confounding effect of genetic variation (Richards et al. 2012; Lira-Medeiros et al. 2010; Johannes et al. 2009).

We applied two ecologically relevant stresses to plants of an apomictic dandelion lineage: drought stress and salicylic acid (SA) treatment. Stress related biotic interactions such as pathogen infection, which is mimicked via SA application (Delaney et al. 1994; Zhang et al. 2008), represents an important environmental factor in dandelion populations (Schemske et al. 2009; Verhoeven & Biere 2013). Spring droughts occur regularly, although in relatively mild form, in continental Finland (Potop et al. 2014). Under the current climate change these stresses are predicted to also become more severe and frequent (Stocker et al. 2013; Pautasso et al. 2012). The progeny of the stressed plants was grown for two generations in an unstressed environment. Chapter 2 of this thesis revealed, on the same material, stress and accession dependent DNA methylation modifications in stressed plants and in their offspring. We tested if the stresses applied on the grandparental generation are reflected in the sRNAs in the progeny after growing for two generations in an unstressed environment. While this study does not aim to expose the carrier of epigenetic information between generations, a grandparental effect of stress on sRNAs would demonstrate that environmental inputs have multigenerational consequences for the epigenetic regulation of plant genomes.

Material and methods

Plant material and growing conditions

Apomictic dandelions, *Taraxacum officinale*, produce seeds from unreduced egg cells *via* embryogenesis without fertilization by male gametes, also referred to as parthenogenesis. In this particular dandelion type of apomixis also the endosperm develops autonomously without fertilization (Koltunow 1993). The ploidy level is diploid for the sexuals and triploid for most apomicts (Richards 1973; Riddle & Richards 2002). New apomictic dandelion genotypes can arise through fertilization of sexually (diploid) reproducing dandelions with diploid pollen from (polyploid) apomicts in mixed populations. These crosses produce triploid offspring and some of these are functionally apomicts and can be founder individuals of new apomictic lineages (Tas & Van Dijk 1999). In Europe, hundreds of distinct apomictic lineages which are sometimes referred to as microspecies, have been described (Kirschner & Štěpánek 2011). Here we used the apomictic lineage *T. officinale hemicyclum*, for brevity hereafter referred to as *T. hemicyclum*, which was collected in Northeast Finland in spring 2013. Seeds were propagated for one generation under common greenhouse conditions and we confirmed the clonality within the lineage with eight microsatellite markers (see Table 2.S2 in supplementary information chapter2). Samples used in this study were included previously in a larger experiment that screened for DNA methylation variation using MS-AFLPs (see chapter 2).

Throughout all generations of the experiment we used the same protocol for seed collection and seed sterilization and the same temperature and light conditions for the germination, growth and vernalization stages. Seeds derived from the first produced seed head per plant and seeds were surface-sterilized for 5 minutes with 0.5% sodium

hypochlorite including 0.05% Tween20 (Sigma-Aldrich, Zwijndrecht, the Netherlands) and afterwards washed with demineralized water. Sterilized seeds were germinated on 0.8% agar plates for 10 days (14 h light / 10 h dark, 18 °C / 14 °C, 60% relative humidity on average, daylight maintained at a minimum of 30 µmol/m^2/s). Seedlings were individually transplanted to 9 x 9 x 10 cm pots containing a mixture of 80% potting soil and 20% pumice that was equalized to 210 ± 5 g. Nutrients were supplied with 1.5 g of fertilizing substrate Osmocote granules (Osmocote exact Mini, Everris international BV, the Netherlands, containing 15 N, 3.5 P, 9.1 % K and 1.2 Mg). Afterwards the seedlings were grown under the same condition as during germination but with a light level of approximately 315 µmol/m²/s and with several times per week of watering, depending on the rate of water loss. Prior to vernalization, rosette leaves were clipped back to 4-5 cm and the plants were put in a cold room at 4 °C (16 h daylight) for 5 weeks, with occasional watering depending on moisture loss.

Stress Experiment

For the protocol of the stress experiment, which includes seed collection, seed sterilization, germination, growth, vernalization and the stress (drought and salicylic acid) applications please refer to the methods part in chapter 2. Initially, we grew per treatment group 12 plants and propagated them by single-seed descent to the third generation. For the sRNA screening we randomly chose 4 individuals per treatment group from the available G3 plants.

Small RNA sequencing and analysis

Leaf tissue was sampled from five weeks old G3 plants. Sixteen leaf discs of 8 mm in diameter were punched from one young and fully-developed leaf. Leaf discs were snap frozen in liquid nitrogen and stored in -80°C until usage. From liquid nitrogen-ground leaf tissue total RNA was extracted using 1 ml of Trizol (Ambion, Life technologies, the Netherlands) according to the manufacturer protocol but with an additional precipitation step with isopropanol and 3M sodium acetate (pH 5.2) and subsequent washing steps with ethanol. The final pellet was dissolved in 50 µl DNase/RNase-free water. RNA quality was checked on agarose gel electrophoresis and concentration on a NanoDrop 1000 spectrophotometer. The library preparation kit from New England Biolabs (Ornat, Rechovot, Israel) was used with an initial 1 µg of RNA according to the manufacturer protocol. Barcodes-containing primers are used during the enrichment of adapter ligated DNA fragments in order to later recover reads from the pooled sequencing library. To select for sRNA with a length of 20-30nt, cuttings from a E-Gel EX 4% Agarose gel (Invitrogen, Life technologies, Israel) were taken between 140-150nt size fraction (accounting for two adapter sequences of 60nt each). The bands were cleaned up using MiniElute Gel Extraction kit (QIAGen, Eldan, Israel). After clean-up and a final quality check on the Bioanalyzer (Agilent Technologies, Eldan, Israel) using Agilent High Sensitivity DNA Kit, the 12 samples were pooled into a single sequencing library which was sequenced on two Illumina Hiseq2500 lanes. FastQC v0.11.3 software was used for preliminary quality check (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

The sequencing reads were processed using adapter trimming Cutadapt v 1.8 software (Martin 2011) and reads with a Phred quality score ≤ 33 and /or ambiguous base calls were eliminated. All reads with length <18nt and >30nt were filtered out. Reads were aligned with BWA (Li & Durbin 2009) to: i) a dandelion transcriptome (Ferreira de Carvalho et al. 2016) and ii) a small subset of dandelion genomic reference from BAC sequences, only considering perfect matches. Multiple mapping sRNA reads were assigned a random mapping location. The BAC information was based on 10 sequenced BAC clones from *Taraxacum officinale* (provided by Keygene BV Wageningen, the Netherlands), each of them containing several assembled contigs. This BAC information covers approximately 0.14% of the monoploid dandelion genome, which is estimated at 865 Mbases (Záveský et al. 2007). All consensus BAC sequences were pooled together and sequences were searched for repeats using Repeatmasker version 2.2.27 (with query species *Arabidopsis*), which resulted in 244 sequences consisting of 178 (6.7%) retroelements and 35 (0.4%) DNA transposons. The transcriptome was assembled *de novo* from RNAseq data (Ferreira de Carvalho et al. 2016), which resulted in a total of 123,232 transcripts of which 39,685 transcripts were annotated to TAIR genes (using BLASTn).

The relative number of different sRNA length classes was calculated and compared between groups of grandparental control and stress treatment (drought or SA). This analysis was performed with four sets of sRNAs: (1) all, unmapped sRNAs (2) sRNAs that mapped to genomic DNA sequences (contigs) within the BACs (3) sRNAs that mapped to annotated TEs within the genomic BAC sequences and (4) sRNAs that mapped to (TAIR) gene-annotated transcripts. The relative number of sRNAs was calculated using pools across all replicates within each treatment group and for each length class between 18 and 30nt separately. For 21nt for instance it is: number of 21nt reads / number of all length class reads between 18-30nt. The sRNA proportions from the control group were compared to the stress group: control versus drought and control versus SA. These differences in sRNA proportion were analyzed by testing whether the observed differences in sRNA proportion are larger than expected by chance. For this comparison random datasets without an effect between groups 'treatment' and 'control' are repeatedly drawn from a pool of all reads from all libraries and with sizes similar to the real observed 'treatment' and 'control' groups (bootstrapping repeated 10,000 times). P-values were obtained by evaluating the observed values against the distribution of bootstrapped sRNA proportions.

Differentiation (differential sRNA abundances) between control and stress group was tested at the level of four different test units resulting in following test sets: (1) unmapped sRNAs; differentiation test for each unique sRNA sequence, (2) BAC-mapped; contig-level differentiation test in sRNA reads that map to unique BAC contigs (3) BAC-mapped (TE annotated); TE-level differentiation test in sRNA reads that map to unique TE regions within BACs, (4) transcript-mapped (TAIR genes annotated); gene-level differentiation test in sRNA reads that map to unique TAIR annotated transcript within the dandelion transcriptome. These tests were done separately for 21nt and 24nt sRNAs and for all length classes combined (18-30nt). For the differential sRNA testing the

algorithm of DESeq2 (Love et al. 2014) was used with the false discovery rate of 0.1 as significance threshold.

A gene ontology (GO) enrichment analysis was performed to test whether genes with the largest differences in sRNA abundances between control and stress groups (the upper and lower 5% of genes) are enriched for specific gene categories. We used the annotations to the *Arabidopsis* genome, where the enrichment analysis was based on the R-package topGO (Alexa A and Rahnenfuhrer 2010) and the *Arabidopsis* annotation was based on the R-package GO.db (version 3.1.2. http://bioconductor.org/packages/GO.db/). To control for potential biases by using the full *Arabidopsis* gene set as a reference in the enrichment analysis, a baseline enrichment level was determined via random subsampling from the dandelion transcripts. This subsampling was repeated 10,000 times with sampling a number of random transcripts equal to the top 5% of the respective treatment set (control versus drought and control versus SA). We considered the enrichment to be significant if the FDR-adjusted p-values from the topGO enrichment analyses exceeded the 95% confidence interval of the bootstrapped subsamples.

Patterns of variation in sRNA composition and also in DNA methylation (data from chapter 2) were visualized with PCoAs that are based on Euclidean pairwise distances (design.dist () R-package Vegan and pcoa() R-package Ape). DNA methylation screening was done using the methylation sensitive AFLP marker *Hpa*II as described in chapter 2 on the same samples that are used here for sRNA sequencing. The sRNA pairwise distance matrix was based on log-transformed reads per million (RPM) values using only 21nt and 24nt sRNAs that have at least 5 reads per sample. Mantel tests were used to test the correlation between the pairwise distances of *Hpa*II and sRNAs (mantel() with 999 permutations from the R package Vegan).

Results

The RNA sequencing generated 208,194,361 reads with a size of 18 – 30 nt and quality scores above 33. Table 3.1 summarizes the read counts and unique sRNAs per library and Table 3.2 shows the number of mapped sRNAs, to either the BAC sequences, the TE regions within the BAC sequences or the transcriptome.

Table 3.1 Total number of sRNAs per library

Treatment	Replicate	Read count	Unique sRNAs	Unique RNAs with min. 5x coverage
Control:	R1	24,443,625	7,956,215	348,011
	R2	17,200,185	5,045,961	217,129
	R3	15,672,880	5,344,546	200,495
	R4	11,512,975	3,884,398	150,976
Drought:	R1	17,739,785	5,377,003	225,719
	R2	25,023,249	6,658,922	300,143
	R3	4,362,637	1,271,550	57,116
	R4	13,522,610	5,013,714	182,373
Salicylic acid:	R1	55,979,276	13,387,304	643,462
	R2	3,021,105	1,167,588	44,378
	R3	14,432,280	4,211,658	178,571
	R4	5,283,754	1,572,454	72,920

Table 3.2 Total number across 4 replicates per treatment of mapped sRNA reads for different test sets

Test Sets of sRNAs	**Treatment**	**18 – 30 nt**	**21nt**	**24nt**
BAC-mapped	Control	156,929	21,924	52,762
	Drought	130,534	18,450	44,612
	SA	146,359	20,308	50,353
BAC-mapped (TE annotated)	Control	25,853	4,288	7,986
	Drought	79,016	3,806	6,777
	SA	98,950	4,126	7,787
Transcript-mapped (TAIR genes annotated)	Control	1,094,017	204,438	187,055
	Drought	2,132,922	188,932	158,386
	SA	2,476,899	214,282	193,968

Treatment specific sRNAs abundance

Figure 3.1 summarizes the relative number of reads of different sRNA length classes for each of the treatment groups, showing overall relative high proportion of 21nt and 24nt sRNAs across the total set of analyzed sRNAs (Fig. 3.1A). TE regions are mostly associated with 24nt sRNAs (Fig. 3.1B), while genes are associated more with 21nt sRNAs (Fig. 3.1C). Bottom panels (Fig. 3.1D-F) indicate the significance of the grandparental stress induction on the fraction of 21nt and 24nt sRNAs (p-value = 0.0001 in all comparisons based on 10,000 times repeated bootstrapping). Specifically, the grandparental stress resulted in a reduction of 21nt and 24nt sRNAs in the TAIR-gene-annotated reads compared to the grandparental control group (3.1F). In the BAC/TEs-mapped reads the stress treatments in G1 induced a higher abundance of 21nt RNAs and lower abundance of 24nt RNAs; this pattern is more pronounced in the drought group (blue bars) than in the SA group (red bars).

The analysis of sRNAs that mapped to a genomic structure (BAC contigs, TE regions or genes), however, showed very few individual contigs, TEs or genes with significant sRNA enrichment or depletion due to grandparental stress treatment (Table 3.3).

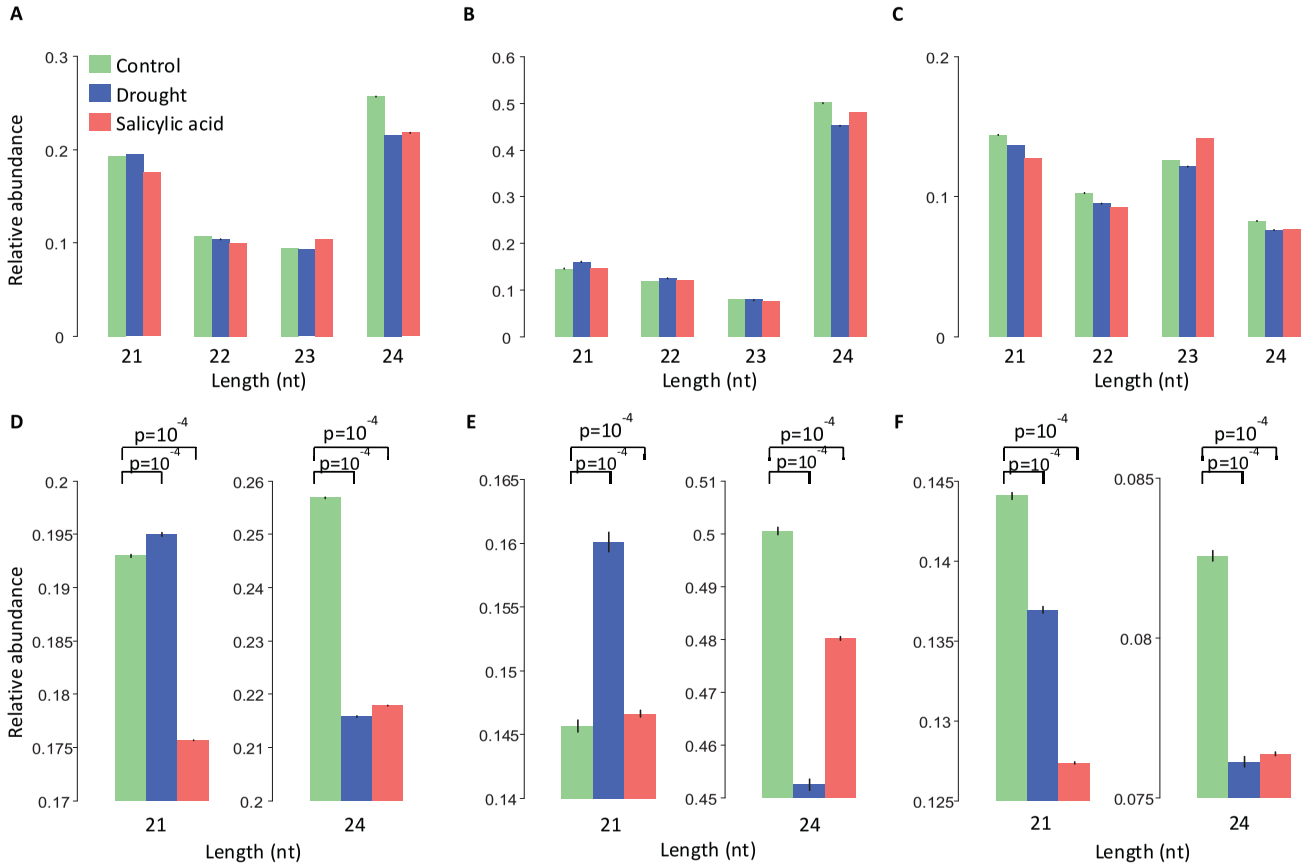

Figure 3.1 Relative abundance of sRNA length classes, shown for: all sRNAs (A and D), sRNAs mapped to annotated TEs (B and E) and sRNAs mapped to TAIR-annotated genes (C and F). The plots in the bottom (D, E and F) show the p-value for the bootstrap analysis performed for 21nt and 24 nt sRNA. Error bars represent 95%

Gene ontology (GO) enrichment analysis

Even when individual genes showed no significant enrichment or depletion of associated sRNAs due to grandparental treatment we performed a GO enrichment analysis to test whether the sRNA signal is associated with biological function. The GO enrichment analysis was based on comparing the 5% of most affected genes (5% of the lowest respectively highest log fold changes, that is the relative change in sRNAs mapping to a particular gene due to grandparental stress treatment). These 5% tails of the log fold change distributions contain 3,131 genes in the drought-control comparison (Fig. 3.3A) and 3,204 genes in the SA-control comparison (Fig. 3.3B). The gene ontology (GO) enrichment test is based on the comparison between these sets of most-affected genes with a random set of genes from the dandelion transcriptome (see methods for bootstrap procedure). Although almost no differentiated genomic loci were found when comparing the sRNA abundances between the treatments (Table 3.3), these gene sets with the most extreme changes in sRNA abundance do show a significant enrichment for specific biological functions.

Up to several hundreds of GO categories are enriched depending on the grandparental treatment and sRNA length class (Fig.3.2). Large overlap is observed in enriched GO categories in response to grandparental drought and SA stress, which suggests a generalized, non-stress specific response. Up- and downregulated genes for 21nt sRNAs were each enriched for ~400-500 GO categories in both the control-drought comparison and in the control-SA comparison. For 24nt sRNAs the downregulated genes were enriched for many more GO terms than the upregulated genes (Fig. 3.2).

We searched the list of enriched GO terms for specific keywords that are associated with the grandparental stresses: “water” and “drought” for drought treatment, “salicylic” and “hormone” for SA treatment and “response to stress”, “abiotic stimulus” and “wounding” for stress treatments in general (Fig. 3.3). Almost all these GO terms were significantly enriched after both the grandparental stress treatments (drought and SA), suggesting that these GO terms tend to show a more general rather than very stress-specific stress response. However, two GO categories indicate a more stress-specific pattern. The GO term 0009862 (“systemic acquired resistance, salicylic mediated signaling pathway”) shows a lower abundance of sRNAs only after grandparental SA treatment and not after grandparental drought treatment (suggesting an SA-induced depletion of sRNAs associated with these genes). The GO term 0009914 (“hormone transport”) shows a higher abundance of sRNAs only upon the grandparental SA stress. And although the observed p-value does not exceed 95% confidence interval of the random test sets, the GO term “wounding” indicates a higher abundance again only upon grandparental SA stress (Fig. 3.3D and C).

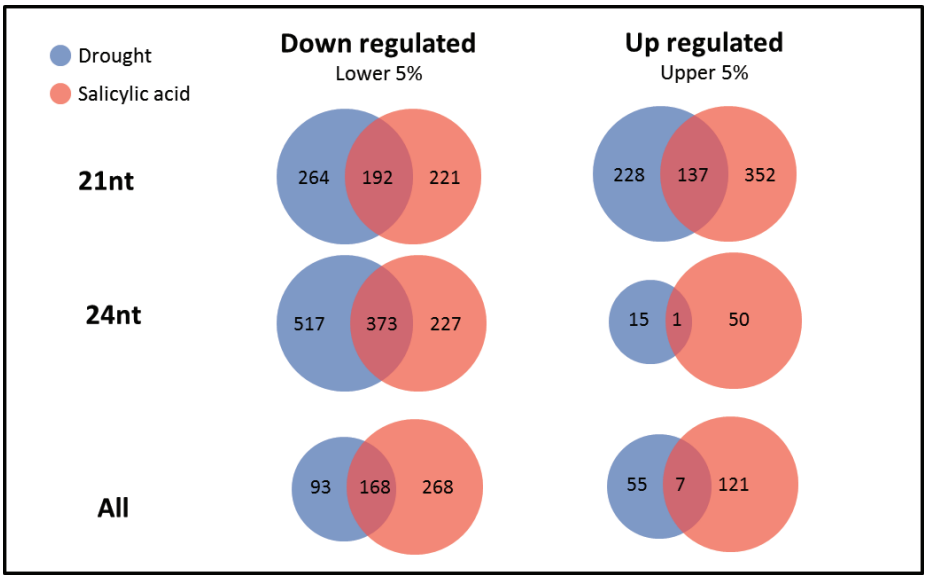

Figure 3.2 Overlap between all GO terms that are significantly enriched in the list of 5% most downregulated genes (reduction in mapped sRNAs due to grandparental stress treatment) and in the list of 5% most upregulated genes (increase in mapped sRNA due to grandparental stress treatment). Number of these significantly enriched GO terms are shown as Venn diagrams between drought and SA grandparental treatment for 21nt and 24nt sRNAs and for all length classes between 18 and 30nt. Considering just the 24nt sRNA, the downregulated transcripts share more terms (in number and proportion) between treatments than the upregulated 5%, indicating that the general stress response genes are mostly regulated under stress by a loss of this kind of sRNA.

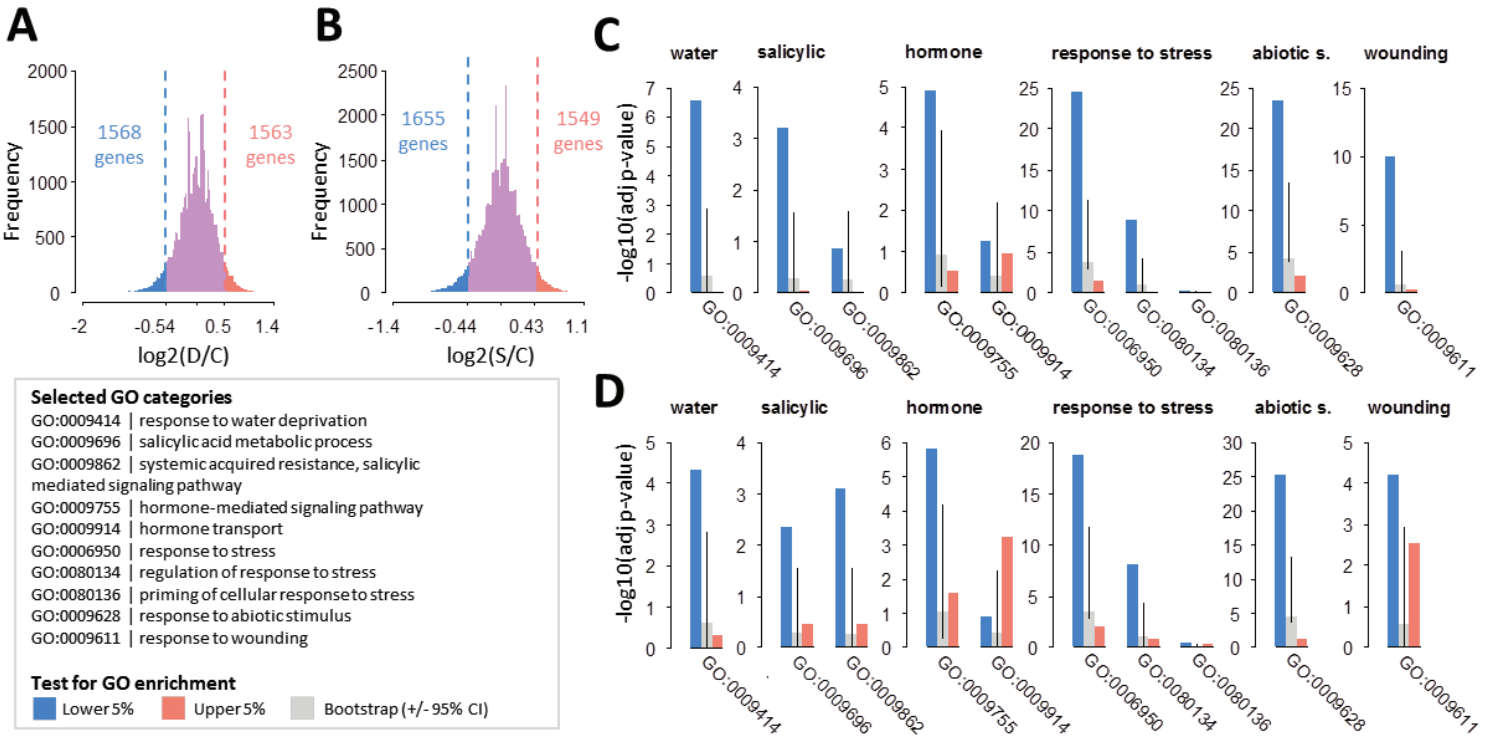

Figure 3.3 GO enrichment analysis. Frequency distributions of read fold change, based on all TAIR-annotated genes in de dandelion transcriptome, in C-vs-D (A) and in C-vs-S (B) comparisons. Panels C and D show the enrichment for meaningful GO categories for the genes in the 5% lower and 5% upper regions of the fold change spectrum against the results obtained via a bootstrap procedure. The results for drought and salicylic acid group are shown separately in figures C and D, respectively.

Correlation between Small RNA and DNA methylation

It is possible that transgenerational stress response may show correlated changes in both sRNA and DNA methylation patterns because these epigenetic mechanisms are linked via the RdDM pathway. Both sRNA variation (21nt and 24nt sRNAs) and DNA methylation variation (from the methylation sensitive AFLP marker *Hpa*II profile in chapter 2) are visualized using PCoAs in Figure 3.4. These PCoAs show no clear pattern that the grandparental stress groups differ from the grandparental control group. Considering the within-group variation the PCoAs of the 24nt and 21nt sRNAs reveal for the offspring of the SA-treated plants (black dots) a decreased variation compared to the other experimental groups. Mantel tests revealed correlations between the sRNA and the DNA methylation profiles that were marginally significant (based on 999 permutations the mantel tests revealed for 21nt R = 0.3674, p-value = 0.083 and for 24nt R = 0.3259, p-value = 0.098), which suggests that the transgenerational patterns in DNA methylation and in sRNAs might be linked to each other.

Table 3.2 Total number across 4 replicates per treatment of mapped sRNA reads for different test sets

Test Sets of sRNAs	Treatment	18 – 30 nt	21nt	24nt
BAC-mapped	Control	156,929	21,924	52,762
	Drought	130,534	18,450	44,612
	SA	146,359	20,308	50,353
BAC-mapped (TE annotated)	Control	25,853	4,288	7,986
	Drought	79,016	3,806	6,777
	SA	98,950	4,126	7,787
Transcript-mapped (TAIR genes annotated)	Control	1,094,017	204,438	187,055
	Drought	2,132,922	188,932	158,386
	SA	2,476,899	214,282	193,968

Table 3.3 Drought and SA induced differential expression of RNA-Seq data at the gene level, based on DESeq2 analysis(Love et al. 2014)

Test set of sRNAs	Test unit	Grandparental treatment	sRNA length class	No. of test units	Loci with significantly differentiated sRNAs abundances	
					Up-regulated Lfc > 0	Down-regulated Lfc < 0
Unmapped	Unique sRNA sequences	Drought	All	569,268	3	0
			21	102,759	2	0
			24	235,625	0	0
		SA	All	545,459	15	12
			21	99,072	5	8
			24	227,383	0	6
BAC-mapped	Unique BAC contigs	Drought	All	14,282	0	0
			21	1,447	0	0
			24	7,668	0	0
		SA	All	13,555	0	0
			21	1,560	0	0
			24	7,504	0	0

Lfc = log fold change, which means here the logarithm of the ratio: gene expression in treatment divided by gene expression in control; loci that show a lower p-value than false discovery rate 0.1 are summarized as loci with up and downregulated sRNAs abundances.

Table 3.3 Continued

Test set of sRNAs	Test unit	Grandparental treatment	sRNA length class	No. of test units	Loci with significantly differentiated sRNAs abundances	
					Up-regulated Lfc > 0	**Down-regulated** Lfc < 0
BAC-mapped (TE annotated)	Unique TE sequences (within BACs)	Drought	All	171	0	0
			21	139	0	1
			24	157	0	0
		SA	All	171	0	0
			21	130	0	0
			24	159	0	0
Transcript-mapped (TAIR genes annotated)	Unique genes	Drought	All	35,880	0	5
			21	27,153	0	0
			24	27,730	0	1
		SA	All	36,202	0	0
			21	27,975	0	0
			24	28,683	0	0

Lfc = log fold change, which means here the logarithm of the ratio: gene expression in treatment divided by gene expression in control; loci that show a lower p-value than false discovery rate 0.1 are summarized as loci with up and downregulated sRNAs abundances.

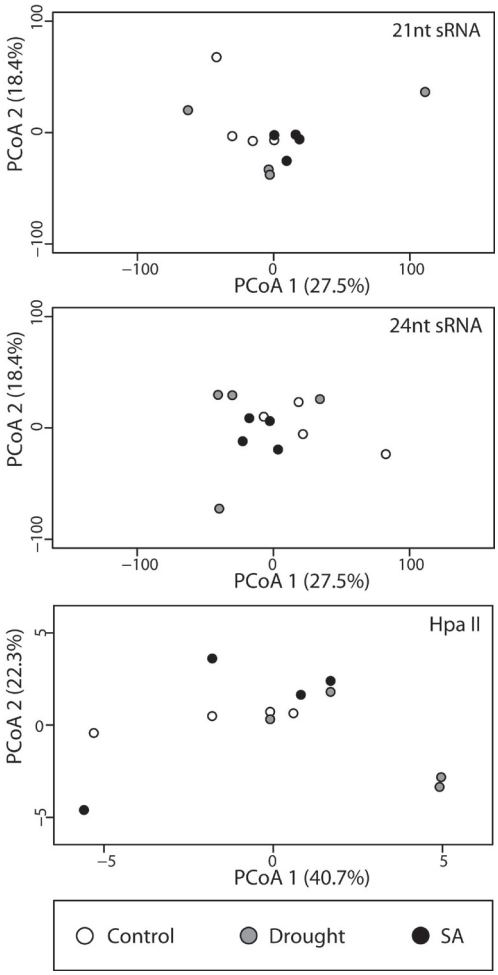

Figure 3.4 Principal Coordinate Analysis (PCoA) of sRNA and DNA methylation screenings in G3 plants. Symbol fillings indicate the different treatment under which the grandparental generation of these tested plants grew in: control or stress treatment (drought or SA); based on pairwise euclidean distances the profiles are shown for 21nt sRNA in the first panel, 24nt sRNAs in the second panel and *Hpa*II in the third panel; y-axis values of the *Hpa*II PCoA graph is jittered for visual aid.

Discussion

In addition to DNA methylations, also sRNAs can play a role in induced transgenerational epigenetic effects (Holeski et al. 2012). While distinct stress-related and transgenerational sRNA changes have been identified in two animal models, *C. elegans* (Rechavi et al. 2014) and *Drosophila* (Seong et al. 2011), in plants the involvement of sRNAs in stress memory and epigenetic inheritance remains less clear. The aim of the present study was to evaluate transgenerational effects in sRNA abundance upon grandparental stress treatment and comparing it to a previous DNA methylation screening (MS-AFLPs) of the same material. We exposed apomictic dandelion plants to drought stress or to salicylic acid for a single generation, after which offspring was grown for two generations under stress-free conditions. The sRNA profiles were screened in the grand-offspring stress-exposed plants. The results revealed that the sRNA length composition was affected by the stress treatments that the grandparental generation was exposed to. Furthermore, even though individual genes or genomic loci did not show strong changes in sRNA abundance due to grandparental treatments, we observed a functional signal present at the level of GO terms. The set of genes with the most extreme sRNA changes due to grandparental treatment was enriched for several hundred GO terms, including several GO terms with stress-specific functions. Thus, the stress experience left a footprint that was detectable in sRNA patterns, and therewith in the epigenetic regulation of the genome, two generations later.

The role of sRNAs in plants' transgenerational stress responses is so far poorly documented, although some studies have reported an involvement of sRNAs in transgenerational stress responses (Bilichak et al. 2015; Rasmann et al. 2012; Luna et al. 2012). Small RNAs with the length of 24 nucleotides are mostly involved in RNA-directed DNA methylation changes and their prominent target in *Arabidopsis thaliana* are TEs. These 24nt sRNAs play therefore an important role in silencing potentially damaging TEs. Furthermore, as an associated effect, activity of genes neighboring TE regions may be co-regulated by the methylation silencing (McCue & Slotkin 2012; Zilberman et al. 2007). Small RNAs with the length of 21 nucleotides are mostly involved in post-transcriptional silencing of plant genes or viral genomes (McCue & Slotkin 2012; Axtell 2013). Our results showed a decrease of gene-associated 21nt and 24nt sRNAs upon grandparental drought and SA stress, suggesting a release of gene and TE silencing (Fig 3.1F). That sRNAs in plants within a generation play an important role in gene and TE regulation upon stress is supported by several studies (Calarco & Martienssen 2011; McCue et al. 2012). To our knowledge, only one other sRNA-screening-study on progeny of salt-stressed *Arabidopsis* showed a transgenerational sRNA stress response (Bilichak et al. 2015). These first studies indicate a mediating role of sRNAs in stress responses within a plant generation and also between plant generations.

We screened and detected a stress-specific and transgenerational sRNA response. However, our data do not reveal the actual carrier of the information between generations. We only detect the carrier`s effect reflected in the sRNA production, which probably have epigenetic regulative effects on the genome. Evidence exist in literature that actual transmission of epigenetic information between generations might be migrating sRNAs (Bilichak et al. 2015; Calarco et al. 2012). Alternatively, DNA methylations may have simply

persisted through the methylation resetting during gametogenesis and embryogenesis and were thus transmitted to subsequent generations. Stress-induced and persistent DNA methylation modifications might then cause modified sRNA production in the offspring generations. This latter idea is supported by the observed (weak) correlation between transgenerational DNA methylation and sRNA patterns. A similar correlative effect has been shown in wild *Solanum* where 5`Azacytidine-treated plants (a demethylating agent) showed an effect on DNA methylations as well as on sRNAs (Marfil et al. 2012). Changes in sRNA production can in turn affect methylations via the RNA dependent DNA methylation (RdDM) machinery (Bond & Baulcombe 2014). The hint for correlation between sRNAs and DNA methylations (screened in chapter 2) may support these speculations, however due to the low sample size the statistical power to detect the correlation signal was quite low. Another possible caveat of the DNA methylation data is that it is based on MS-AFLP where fragments' location in the genome is unknown. Further studies are needed to reveal specific loci that show DNA methylation changes and changes in sRNA production.

The length composition of sRNAs showed significant stress effects (Fig. 3.1), while only very few genes showed a stress effect on sRNA abundances (Table 3.3). Thus, our data indicate that the transgenerational effect of stress exposure is very weak at individual genes. Indeed, coverage of the sRNAs may have been not high enough to detect important regions, and the limited number of replicates (n=4) can have contributed to a failure to detect subtle sRNA effects. However, the cumulative signal at many genes was strong enough to show significant effects of grandparental stress at the GO term level.

We suggest that sRNAs may play a relevant role in transgenerational responses to environmental changes, either as primary signal of information transfer or as downstream functional effects of stably transmitted DNA methylation at regulatory loci. Since epigenetic modifications might be more easily transmitted between generations under apomictic reproduction, transgenerational epigenetic stress responses may play a more important role under clonal, apomictic reproduction (*Taraxacum,* for instance) than under sexual reproduction (*Arabidopsis,* for instance). However, improved technical tools for non-model systems are needed to pinpoint the sRNA involvement in transgenerational effects. Such findings would further the understanding of sRNA signaling and transgenerational effects in the context of ecology and evolution.

Acknowledgements
We thank Rutger Wilschut for providing microsatellite data and Juhani Räsänen for the seed collection of the plant material. This study was supported by NWO-ALW grants: NWO-Ecogenomics 884.10.003 and 864.10.008 from the Netherlands Organisation for Scientific Research and by the Czech National Grant Agency, grant, No. GA13–13368S.

4

Testing heritable DNA methylation effects upon transplanting apomictic dandelions to native versus non-native field habitats

V. Preite, M. Glabischnig, C. Oplaat, W.H. van der Putten, K.J.F. Verhoeven

Abstract

Inherited epigenetic modifications, such as DNA methylations, have the potential to trigger stress responses across generations. However, there is limited evidence about epigenetic stress memory, and its relevance under natural field conditions is largely unexplored. Here, we evaluated whether heritable modifications of DNA methylation are triggered in apomictic dandelions by exposure to natural stress conditions. We reciprocally transplanted field-collected Czech and Dutch dandelion lineages in order to study plant responses to natural field conditions to which they had and had not adapted. The performance of plants confirmed adaptive differentiation between the populations, which supports the idea that growing conditions in non-native sites are perceived by the plants as more stressful than native growing conditions. Offspring of two-year old transplants were raised under common greenhouse conditions and screened for their DNA methylation profiles using MS-AFLPs. Different apomictic lineages showed clearly distinct MS-AFLP profiles, but no effects were detected of transplantation to the field or of transplantation into native versus non-native field conditions. We conclude that the majority of the MS-AFLP variation is associated with the underlying genetic variation and that the stresses perceived from the field environment may be too variable and/or mild to result in transgenerationally persistent DNA methylation changes. This result contrasts with previous findings from greenhouse experiments, where some genotypes showed stress-induced and heritable DNA methylation changes. Possibly only severe and specific stresses in an otherwise controlled environment trigger DNA methylation effects that are detectable across generations.

Introduction

Epigenetic modifications, such as DNA methylation variation, can contribute to altered gene expression without changing the primary DNA sequence. Such epigenetic mechanisms have been suspected to contribute to heritable phenotypic variation in natural populations, and thus may play a role in their evolutionary potential (Jablonka & Raz 2009; Rapp & Wendel 2005; Bossdorf et al. 2008). Environmental stresses can cause DNA methylation variation (Dowen et al. 2012), and a proportion of this epi-variation may be transmitted to the next generations (Cheng et al. 2004; Boyko et al. 2007; Kou et al. 2011; Verhoeven et al. 2010b). However, it is still unclear whether such stress-induced epigenetic inheritance is a common phenomenon and what relevance it has in natural population (Richards & Wendel 2011; Baulcombe & Dean 2014; Heard & Martienssen 2014).

Here, we conducted a reciprocal transplantation experiment to determine whether exposure to natural stress conditions causes heritable modifications of DNA methylation in apomictic dandelion lineages (*Taraxacum officinale*). Plants are particularly suitable for studying environment-induced epigenetic inheritance, because plant germ lines emerge from somatic tissue late in development. Animal germ lines, on the other hand, are set aside in an earlier stage of development. Therefore, genomic changes acquired during the life of a plant may be more likely to be transmitted to the next generation than in animals. In addition, the transmission rate of DNA methylation changes might be higher in plants because during plant gametogenesis and embryogenesis the methylation resetting is not as complete as in animal species (Feng et al. 2010).

Several studies in natural populations of plant and animal species revealed correlations between genome-wide patterns of DNA methylation with environmental variation (Paun et al. 2010; Herrera & Bazaga 2011; Schrey et al. 2012; Schulz et al. 2014, Massicotte et al. 2011; Raj et al. 2011; Hafer et al. 2011; Dombrovsky et al. 2009; Richards et al. 2012; Lira-Medeiros et al. 2010). Also chapter 5 of this thesis (Preite et al. 2015) has revealed some regional differentiation in the epigenetic profiles in natural populations of apomictic dandelions. Such correlative association between epigenetic profiles and environmental conditions could be explained in different ways. Since a considerable proportion of plant methylomes is determined by genetics, such as the position of transposable elements, genetic differentiation (due to neutral drift and selection) may cause genetically-determined epigenetic differentiation (Dubin et al. 2015). Alternatively, genetically-independent (autonomous) epigenetic effects can contribute to adaptation to local habitat differences, either when environments induce epigenetic modifications or when heritable epigenetic variation is shaped by environmental selection. The latter selection-based effects would require considerable multi-generational stability and phenotypic effects of the epigenetic modifications.

For testing the relevance of stress-induced epigenetic inheritance that could play a role in adaptation to local environmental conditions it is convenient to use asexually reproducing organisms. Confounding effects between genetic and epigenetic variation can be avoided using such asexually reproducing organisms, which are providing a genetically uniform background. In a previous greenhouse experiment salicylic acid (SA)

treated apomictic dandelions showed specific induced DNA methylation modifications that were heritable to the next generation (Verhoeven et al. 2010b). A multi-generation stress experiment with two field-collected apomictic dandelion lineages (chapter 2 of this thesis) revealed lineage- and stress-specificity of stress-induced heritable DNA methylation changes. For instance, the offspring generations of SA treated plants showed a significantly increased number of methylation modifications compared to offspring of control plants. However, it has not yet been established to which extent DNA methylations respond to stresses under more natural environmental conditions.

We tested in plants whether exposure to natural stress conditions causes heritable modifications of DNA methylation using a reciprocal transplantation experiment approach. Plants from multiple apomictic dandelion lineages (*Taraxacum officinale*) collected from two field sites in Czech Republic and the Netherlands were used. Apomictic descendants of mother plants were transplanted into both field environments and into a common greenhouse environment. This design enables testing of the effects of the field environment using greenhouse plants as a control. Furthermore, it enables testing of effects of transplantation into non-native field sites versus native field sites. Under the assumption that plants are locally adapted, they are expected to perceive a foreign environment as more stressful than the home environment to which they have adapted. Alternatively, it may be that the plants perform better at the foreign site when it is accompanied by a release from natural enemies.

Triploid apomictic dandelions propagate through unfertilized seeds and migrated northwards after the last ice age, from Central to North and North-Western Europe (Verduijn et al. 2004; Comes & Kadereit 1998). Numerous new apomictic lineages are generated (see methods section) leading to a large diversity of apomictic lineages in natural populations (Van der Hulst et al. 2001; Preite et al. 2015). Adaptation in such a system is presumably due to lineage sorting. However, within-lineage variation is restricted to de novo mutations which are gained since the incipience of the lineage. Additional sources of heritable variation, specifically epigenetic variation, might be relevant for adaptation at the within-lineage level.

The aim of the reciprocal transplantation experiment was to investigate whether heritable methylation changes can be induced through exposure to natural environmental stresses. We analysed the variation in plant performance and in epigenetic profiles within multiple apomictic lineages that were reciprocally transplanted between their native and non-native growing sites. We test the hypotheses that: 1) “home” plants outperform introduced plants in their native growing site, 2) transplanted individuals show DNA methylation patterns that differ from greenhouse-propagated control plants, 3) the stress experienced in a non-native growing site will result in more pronounced DNA methylation changes compared to the native growing site, 4) variation in methylation profiles correlates with differences in plant performance.

Material & Methods

Pre-experiment seed propagation

Figure 4.1 Map shows the locations (stars) of the two experimental gardens. Picture shows a transplanted apomictic dandelion, exemplary for each experimental garden. The yellow plastic stick was placed next to the plant for identification.

Dandelion (*Taraxacum officinale*) is a widespread perennial grassland plant species that exist in two forms that differ in their reproductive mechanisms: diploids that reproduce sexually and polyploids (mostly triploids) that reproduce asexually via unfertilized seeds, referred to as apomixes. These apomictic dandelions occur in Europe alongside sexuals in mixed populations in Central and Southern Europe. Mixed populations can generate new apomictic lineages through crosses between diploid mothers and polyploid (apomictic) fathers: when diploid pollen from polyploid fathers fertilizes haploid egg cells, new triploid genotypes arise and some of these triploid offspring plants are functional apomicts (Tas & Van Dijk 1999). Apomictic dandelions have a more widespread distribution area than their sexual conspecifics, resulting in solely apomictic populations in Northern Europe.

In spring 2011 seeds of apomictic dandelions were collected from fields at two locations where only apomictic dandelions occur: Benešov, Czech Republic (49.33302 N, 15.00314 E, at 655 meters above sea level), and Wageningen, the Netherlands (51.98938 N, 5.66966 E, at 12 m.a.s.l.). Both locations were previously used for agriculture and are now for several years kept as grasslands. We collected random seeds from both populations and these plants were propagated in a common greenhouse environment. All plants were confirmed to be triploid, and thus apomictic, by comparing the nuclear DNA content in their leaf tissue to a diploid reference plant using a flow cytometer (Tas & Van Dijk 1999). After twenty weeks of growth the plants were clipped back to 4-5 cm above the soil surface and transferred for 6 weeks to a cold room at 4 °C in order to promote vernalization. Afterwards, they were placed back into the greenhouse to set flowers and seeds were collected to be used for the transplantation experiment. Based on microsatellite-genotyping (see below) we selected 10 different genotypes per plant origin (10 CZ and 10 NL genotypes).

For microsatellite genotyping, per plant approximately 1 cm^2 of fresh leaf tissue was sampled and kept on ice in microtubes containing two 1/8" steel balls and after grinding, the samples were homogenized in CTAB buffer using a Tissuelyser II (Qiagen, the Netherlands) followed by washing and DNA precipitation steps (CTAB procedure by Rogstad (1992) with minor modifications by Vijverberg et al. (2004)). The DNA was stored at -20°C in 50 µl TE until DNA was collected for all generations. For the selection of distinct lineages eight previously, for dandelion genotyping, established microsatellites were used (msta31, msta44B, msta58, msta78, msta61, msta67, msta72, msta143; Falque et al. 1998; Vašut et al. 2004) and the first 4 microsatellites were used for a second genotyping at the end of the transplantation experiment to confirm correct sample identification of experimental transplants (see Table 4.1S, supporting information).

Transplantation experiment

In April 2012, in greenhouses near the transplantation sites (Czech Botanical Institute in Pruhonice CZ and NIOO facilities in Wageningen NL) 26 offspring individuals from each of the 20 lineages were propagated by placing seeds directly on a mixture of 80% potting soil and 20% pumice in seedling trays (individual cells 5cm diameter and 7.5cm deep). Three seeds were germinated per tray cell and after four weeks of growth the smallest plants were weeded out so that only one individual plant was left per tray cell. The plantlets were clipped back to 4-5 cm and placed outside for one week to acclimatize to ambient conditions. In May 2012, after mowing the field sites, at each transplantation site the 26 replicates of every apomictic lineage were planted, resulting in 520 plants per site (10 apomictic lineages x 2 origins x 26 replicates; planted in a complete randomized block design with 26 blocks and 1 replicate per lineage per block) and 1,040 plants for the whole experiment.

Figure 4.1 shows the location of the Czech and Dutch experimental sites (original map downloaded from http://d-maps.com/m/europa/europemax/eu-14) and an exemplary picture of a transplanted dandelion. The experimental sites were chosen in regions with relatively little human disturbance and were additionally protected by a sheep fence. The experiment lasted for two years and the plots were maintained by mowing twice a year. During March and April 2014, we hand-weeded non-experimental dandelions and grasses, that overgrew the transplants in the experimental plots and scored the survival of the transplants. During May and June 2014, towards the end of the flowering season, the number of flower stalks was scored at one time point and during two weeks seeds were collected daily.

To test for heritable DNA methylation modifications induced by the field environments, DNA methylation was screened in offspring of experimental field plants. The offspring was grown in a common greenhouse environment. A selection of apomictic lineages with sufficient number of seed-producing replicates in the field experiment was selected for this. The DNA was isolated and first genotyped by microsatellites to confirm that sampled individuals were in fact experimental transplants, and not wild plants growing in the experimental plots, and subsequently the material was screened by MS-AFLP. Since not many plants produced seeds at the end of the field experiment it was

necessary to restrict the methylation screening to 6 apomictic lineages. Furthermore, for the Dutch lineages, very few plants produced seeds at the Czech site, therefore the Czech site was dropped for the analysis of Dutch genotypes. For 3 Dutch apomictic lineages (nl-13, nl-16, nl-3) enough reproducing individuals at their home site were found. And 3 Czech apomictic lineages were selected that showed a reasonable number of replicates (minimum of 4 individuals) which produced seeds at their home site as well as at the Dutch field site (cz-26, cz-28, cz-42). Microsatellite profiles of these 6 apomictic lineages are shown in Table 4.S1, Supporting Information.

Table 4.1 Number of plants subjected to MS-AFLP analysis per apomictic lineage, plant origin and growing sites of the transplants

Apomictic lineage	Plant origin	Growing Site		
		Benešov, CZ	Wageningen, NL	Greenhouse (control)
Cz_26	Cz	4	8	7
Cz_28	Cz	5	9	9
Cz_42	Cz	4	10	10
Nl_3	Nl	-	9	10
Nl_13	Nl	-	9	10
Nl_16	Nl	-	10	10

The table shows the number of plants that yielded MS-AFLP fragments; these are individuals that survived in the fields and were correctly resampled (from initially 26 replicates per lineage). The last column represents number of plants that yielded MS-AFLP fragments from the parallel batch that was grown in the greenhouse as controls.

MS-AFLP

From the six tested lineages 10 replicates per lineage were grown under common greenhouse conditions as a control group. After eight weeks of growth, for each individual plant DNA isolation was prepared (see chapter 2 for CTAB DNA isolation procedure) using leaf punches from the third fully developed leaf. We excluded DNA samples that indicated contamination (Nanodrop 260/230 < 1). Based on these criteria 4-10 replicates per apomictic lineage per growing-site were used for the MS-AFLP screening (see Table 4.1; see chapter 2 for MS-AFLP procedure). In total, MS-AFLP data was obtained for 124 samples: 6 apomictic lineages x 3 growing sites (Benešov CZ, Wageningen NL and Greenhouse) x 4-10 replicates. For each DNA sample two *Hpa*II digestions were run and the duplicate pairs were randomly assigned to three separate PCR plates. To exclude sample-unrelated peaks 10 negative controls were added.

We used the methylation-sensitive restriction enzyme *Hpa*II to capture methylation variation (as in chapter 2). Usually MS-AFLPs are run in parallel batches

with *Hpa*II and a second methylation-sensitive restriction enzyme *Msp*I, which enables the distinction between methylation polymorphisms and DNA sequence polymorphisms.We assumed that within the apomictic lineages used in this experiment genetic variation that might have arisen within the experiment is negligible and that we can therefore interpret *Hpa*II variation as methylation polymorphism (following Verhoeven et al. 2010b). Seven *Eco*RI / *Hpa*II primer combinations were used (ACA / TAC, ACA / TCA, AAC / TAG, AG / TCA, AG / TAC, ACC / TCA, ACC / TAG). Table 2.S3 in chapter 2 (Supporting information) summarizes all adapters and primers.

Fragment scoring

Fragments were scored between 50-500 base pairs using GeneMapper 5.0 (Life technologies Europe BV, the NL). Since the primer combinations used in this experiment generally showed different qualities with different origin of samples we identified polymorphic loci separately for the Czech and the Dutch lineages, using overlaying peak profiles in GeneMapper. This resulted in one Czech and one Dutch bin set against which the Czech and the Dutch samples were evaluated, respectively, for polymorphisms. Loci were excluded when the maximum peak height (relative fluorescence unit) was below 100 across all samples and when the loci clearly showed up in the negative controls. Individual peaks were scored as "present" if peak height (relative fluorescence unit) exceeded 100. Since we ran all sample in duplicates we included singletons when both duplicates were consistent.

On the assumption that with AFLP approaches the possibility is higher for false negatives than for false positives, inconsistencies between the duplicates were scored as 1, "present". Few samples (3 samples in Czech lineages and 2 samples in Dutch lineages) showed a strikingly low number of fragments and were discarded since they were considered as technical failures. In preliminary multivariate data analysis we observed a PCR-plate-specific effect. We subsequently tested each locus for association with PCR-plate using logistic regression and we excluded all loci from further analysis that showed a significant PCR-plate effect ($P < 0.05$ for 41 loci within Czech lineages and for 38 loci within Dutch lineages). Error rate was recorded in the final set of polymorphic loci as percentage of discrepancies in fragment scores (0/1 status) across the duplicates. In total for the Czech lineages we analysed 66 samples with 74 polymorphic loci (including 18 singletons) and an error rate of 8.3%, while for the Dutch lineages we analysed 57 samples with 77 loci (including 22 singletons) and an error rate of 3.4%.

Statistical analysis of HpaII profiles

The following analyses were performed separately per origin, that is all plants originating from Czech or Dutch lineages: The 0/1 status of each locus was tested with logistic regression models for a pattern associated with growing site (greenhouse, Czech Republic and the Netherlands), the apomictic lineage (3 Czech respectively Dutch lineages) and the growing site x lineage interaction. This was done with the R-function glm() using a binomial error distribution and a logit link function. The p-values where corrected for multiple testing at a false discovery rate of 0.05 with the R-function p.adjust().

Multivariate analyses were performed based on pairwise distances in MS-AFLP profiles. The pairwise distances were calculated by counting the absolute number of inconsistent loci between individuals (R-function designdist(), package Vegan).

Stress-induced DNA methylation changes could occur as targeted stress-specific changes (causing a shift in methylation profiles that is similar among replicated plants) or as an increased rate of random epimutations (Richards 2006). To reveal these different patterns the DNA methylation profiles of the different experimental groups need to be tested for group-mean differences and for differences in the within-group dispersion. Differences between experimental groups were tested using a Permutational Multivariate Anova using the R-function adonis() with 10,000 permutations. To test whether groups have different levels of variation, irrespective of differences in group means (multivariate analogue to Levene's homogeneity of variances test) a permutation test for homogeneity of multivariate dispersion was done with 999 permutations (R-functions betadisper() and permutest(), package Vegan). To visualize the multidimensional data a principal coordinate analysis was performed (R-function pcoa(), package Ape) based on the same pairwise distances as the multivariate analysis mentioned above.

Statistical analysis of plant performance

The number of plants that flowered, noted in all 10 Czech and Dutch lineages, was divided by the total of transplants (26) to estimate the "proportion of flowering plants", which thus includes survival to the reproductive state two years after the transplantation. Generalized linear mixed models (GLIMMIX procedure in SAS 9.3, SAS Institute, Cary NC) were used to test the growing site effect (Wageningen or Benešov growing sites), the origin effect (Wageningen or Benešov populations) and the growing site x origin interaction effect on the proportion of flowering plants. In the model the lineages are included (10 lineages nested within origin) as a random factor, and as fixed factors we included growing site, origin and the growing site x origin effect. For "proportion of flowering plants" the model was run with a binomial error distribution and for "number of flowers produced per plant" the model was run with a negative binomial error distribution. The latter error distribution is suitable for over-dispersed count data. On the subset of plants whose offspring was screened for MS-AFLPs a Mantel test was applied to test the correlation between offspring DNA methylation profiles and number of flowers produced (R-function mantel(), package Vegan). The pairwise distances for number of flowers was calculated by the default R-function dist() and for the MS-AFLP (*Hpa*II) pairwise distances were calculated as described for the multivariate analysis above.

Results

Transplantation effects on fitness traits

For both fitness proxies "proportion of flowered plants" and "flowers per plant" the interaction growing site x origin was significant, indicating that the performance at each growing site was dependent on the plant´s origin (Table 4.2). Figure 4.2 shows the

reaction norms of the analysed fitness traits for all 20 transplanted lineages at the Czech and the Dutch growing-site. At the Czech site the local lineages tended to show more plants that reproduce at the end of the two-year experiment, whereas at the Dutch site no such home-site advantage was found. The number of flowers per plant (from the subset of plants that reached the reproductive state) shows a significant effect associated with the growing site (Table 4.2). At both growing-sites the local lineages produced in average (dashed lines, Figure 4.2) more flowers than the foreign lineages. This suggests that the local lineages have higher reproductive fitness, indicating a local adaptation pattern. Overall, these analyses on plant performances reveal a pattern that is consistent with local adaption, especially at the Czech growing-site where the local plants outperformed the introduced plants.

Table 4.2 Generalized linear model results for the fitness proxies, testing for growing site and plant origin effects. Proportion of plants that flowered and the number of flowers were measured at the end of the transplantation experiment.

	Growing site effect			**Origin effect**			**Growing site x origin interaction effect**		
	Df	**F value**	**Sign.**	**Df**	**F value**	**Sign.**	**Df**	**F value**	**Sign.**
Proportion flowered	1	147.69	***	1	7.91	*	1	6.89	*
Number of flowers	1	368.7	***	1	21.31	ns	1	368.7	*

Results are shown for the fixed effects in the generalized linear mixed model with genotype (nested within origin) as a random factor. Sign.:Significance codes: *** P < 0.001; ** P < 0.01; * P < 0.05; . P < 0.1; ns= not significant

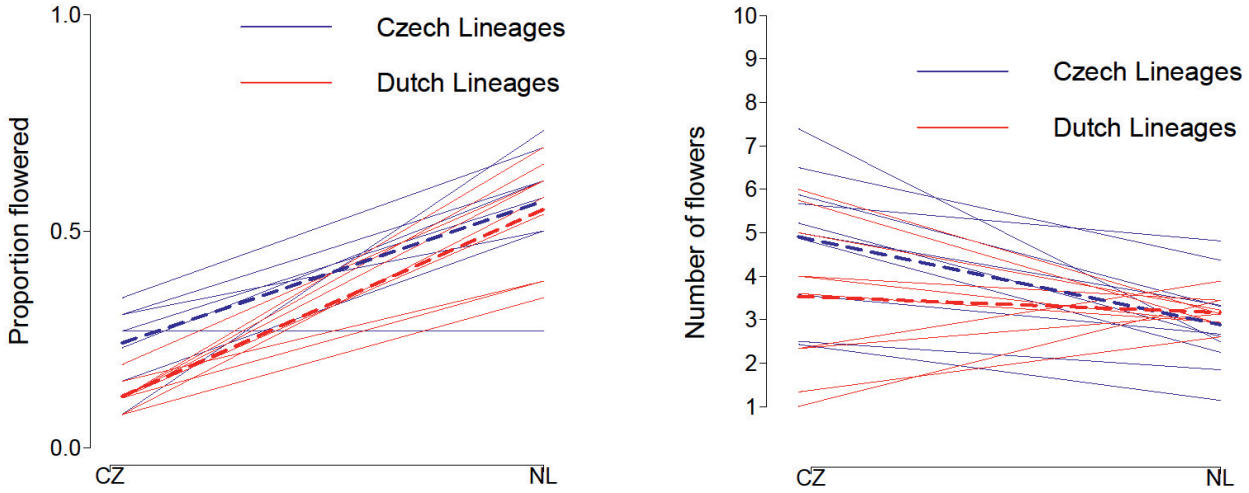

Figure 4.2 Reaction norms for proportion of flowered plants (left panel) and mean number of flowers per flowering plant (right panel). Both traits are shown for each apomictic dandelion lineage (10 lineages from Czech origin and 10 lineages from Dutch origin) and for the mean value per origin (bold dashed lines).

Table 4.3 Single-marker tests of habitat and lineage effects on *Hpa*II marker status using generalized linear models. Shown are the number of significant loci (P-value adjusted for multiple testing at FDR 0.05) in Czech and Dutch apomictic dandelion lineages that associate with growing site and lineage.

	Total no. of loci	**Growing site effect**	**Lineage effect**	**Growing site x lineage interaction effect**
Cz-lineages	74	0	33 (45%)	0
Cz_26	74	0		
Cz _28	74	0		
Cz_42	74	0		
Nl-lineages	77	0	35 (46%)	0
Nl_3	75*	0		
Nl_13	74*	0		
Nl_16	77	0		

The proportion of differentiated *Hpa*II loci is indicated in brackets. * The deviance from the total number of loci (77) is due to some loci where the statistical model failed to converge.

Transplantation effects on DNA methylations

The *Hpa*II profiles of the apomictic lineages with Czech and Dutch origin reveal clustering by lineage (Figure 4.3 and Table 4.4). The offspring methylation profiles of the Dutch lineages further show a combined *Hpa*II cluster of the two lineages nl-3 and nl-13, while nl-16 is clustered separately (Figure 4.3). However, no differences were detected between greenhouse and any of the two field environments. At the within-lineage level the analysis reveals that offspring DNA methylation profiles do not differ between growing sites for any of the tested lineages (Table 4.4, no significant growing site effects and no interaction growing site x lineage effect). Within-group variation in offspring *Hpa*II profiles was of similar magnitude between lineages and, at the within-lineage level, between environments (Table 4.5). After correction for multiple testing, almost half the scored heritable *Hpa*II fragments are associated with apomictic lineage (Table 4.3) while within lineages no fragment was associated with parental growing site. At the lineage level these analyses indicated no differences in offspring DNA methylations between plants growing under greenhouse or field conditions. Furthermore, the heritable DNA methylation patterns did not differ between plants growing under native or non-native growing site conditions (analysis done only for the Czech lineages).

Within each of the six apomictic dandelion lineages the *Hpa*II profile was compared to the fitness proxy "number of flowers produced". Within each of the six apomictic lineages, and for the subset of plants that produced flowers in the field, mantel tests showed no significant correlation between the number of flowers produced and the *Hpa*II profiles of their offspring (cz26: r -0.2321, p 0.96; cz28: r 0.002445, p 0.351; cz42: r -0.215, p 0.934; w3: r -0.2413, p 0.974; w13: r -0.1042, p 0.418; w16: r 0.2057, p 0.22).

Table 4.4 Permutational multivariate analysis of variance results based on apomictic dandelion *Hpa*II profiles in offspring of plants that were grown in different growing sites. The table shows degrees of freedom, proportion of variance explained (R^2) and significance of the growing site and lineage effects on *Hpa*II profiles in Czech and Dutch apomictic dandelion lineages.

	Growing site effect			**Lineage effect**			**Growing site x lineage interaction effect**		
	Df	**R^2**	**Sign.**	**Df**	**R^2**	**Sign.**	**Df**	**R^2**	**Sign.**
Cz-lineages	2	0.011	ns	2	0.747	***	4	0.016	ns
Cz_26	2	0.079	ns						
Cz_28	2	0.125	ns						
Cz_42	2	0.098	ns						
Nl-lineages	1	0.001	ns	2	0.912	***	2	<0.001	ns
Nl_3	1	0.008	ns						
Nl_13	1	0.017	ns						
Nl_16	1	0.023	ns						

Based on function adonis () from R-package Vegan with 10,000 permutation steps. Sign.: Significance codes: *** P < 0.001; ** < 0.01; * < 0.05; . < 0.1; ns= not significant

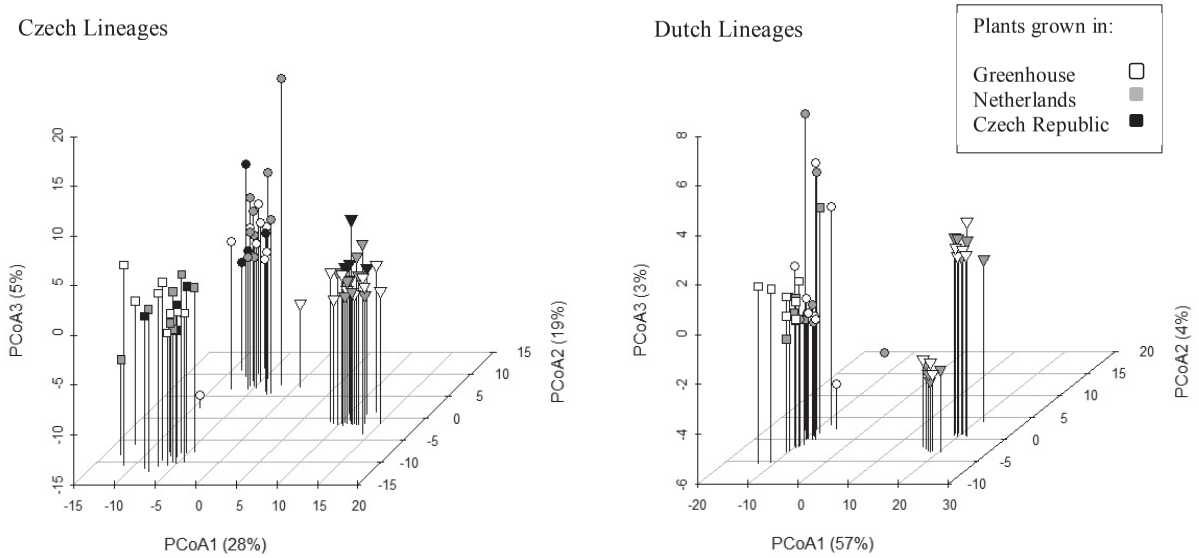

Figure 4.3 Principle coordinate analysis (PCoA) based on apomictic dandelion *Hpa*II profiles in offspring of plants that were grown in different growing sites. The graphs show three Czech (left panel) and three Dutch lineages (right panel) distinguished by different symbols (rectangle: cz_26 and nl_13; circle: cz_28 and nl_3; triangle: cz_42 and nl_16). Grey fill identifies lineages that have been propagated in the greenhouse, black fill are lineages that have been transplanted in their native site and no fill identifies lineages transplanted to a non-native field in the Netherlands.

Discussion

Because previous experiments on apomictic dandelions indicated the existence of stress-inducible and heritable DNA methylations under greenhouse conditions (Verhoeven et al. 2010b and chapter 2) we investigated if exposing apomictic dandelions to novel natural environments triggers heritable changes in DNA methylations as well. We performed a reciprocal transplantation experiment by growing apomictic lineages from Czech Republic and The Netherlands for two years at different growing sites: in their own and in each other's natural growing site, and in a greenhouse as a control. Based on ten apomictic dandelion lineages from each origin we found indications for adaptive differentiation of the populations. Especially in the Czech growing site the local lineages outperformed the foreign plants by showing more plants that reached the reproductive state and produced more flowers per plant. For three lineages of each origin a DNA methylation screening was performed, using MS-AFLPs (*Hpa*II). In contrast to our hypothesis, no heritable DNA methylation effects were detected of translocating apomictic dandelions: neither transplanting into natural growing sites per se (compared to greenhouse plants) nor transplanting into non-native versus native growing sites caused detectable changes in DNA methylation that persisted to offspring. However, the DNA methylation profiles showed distinct clusters associated with the tested apomictic lineages, indicating a strong genotype effect.

Table 4.5 Permutational multivariate dispersion analysis based on apomictic dandelion *Hpa*II profiles in offspring of plants that were grown in different growing sites. Three Czech and Dutch apomictic dandelion lineages were tested.

	Growing site effect	**Lineage effect**
Cz lineages	0.742	0.509
Cz_26	0.865	
Cz_28	0.632	
Cz_42	0.738	
Nl-lineages	0.703	0.295
Nl_3	0.290	
Nl_13	0.533	
Nl_16	0.603	

Numbers are the p-value of the treatment effect based on function betadisper () from R-package Vegan with 1,000 permutations

The aim of the reciprocal transplantation experiment was to test whether the apomictic dandelions show patterns of local adaptation and whether exposure to natural growing site conditions triggers heritable DNA methylation changes. If plants are locally adapted then growing at a non-native growing site would be perceived as stressful. We can therefore also test whether transplantation into non-native sites triggers more changes than transplantation into native sites. Analyses of fitness proxies (survival to reproduction and flower production per plant) indicated adaptive divergence. With the MS-AFLP screening the tested apomictic lineages showed distinct lineage-specific DNA-methylation clusters, indicating a strong genotype effect. However, within lineages no DNA methylation differentiation was found by growing site. Although the detected adaptive phenotype divergence suggested that non-native growing site are perceived as a more stressful environments, no transgenerational DNA methylation changes were found in the offspring of translocated plants.

Studies on DNA methylation in natural plant populations have revealed clear environmentally induced DNA methylation variation (Dowen et al. 2012; Richard et al. 2012; Foust et al. 2016). However, until now limited information is available about the heritability of stress-induced modifications. In previous greenhouse experiments heritable DNA methylation effects were found in apomictic dandelions (Verhoeven et al. 2010b; chapter 2), but these heritable effects seem to be stress and genotype specific (chapter 2). Thus, although the MS-AFLPs resolution is limited we can expect to detect epigenetic effects when the signal is strong. Therefore, the fact that no environmental signal was found with the MS-AFLP screening in this chapter is at odds with previous studies on apomictic dandelions. However, results should not be discussed without considering that overall DNA methylation patterns should not vary too much, because DNA methylation silences deleterious transposable elements (TEs). These defensive DNA methylations are important for the genome integrity and, therefore, need to be stably transmitted. There may be a trade-off between the benefit of DNA methylation variation, possibly causing adaptive phenotypic variation, and the risk of re-activation of deleterious TEs.

The lack of DNA methylation differentiation in offspring of transplanted dandelions might be due to absence of growing site-related stress-induced DNA methylation changes. On the other hand, it is possible that if it caused an epigenetic stress response it was not inherited to their offspring. In the MS-AFLP screening of chapter 2 we discovered a genotype dependency. It is possible that the apomictic dandelion lineages tested here were genotypes that are not very susceptible to the transplantation stress. The absence of DNA methylation differentiation might also indicate that the transplantation was not perceived as very stressful by the plants, meaning that it did not cause stress-induced (or stress-induced but not heritable) DNA methylation changes. The environmental signals that the transplants experienced during growth under field conditions might not have been severe or consistent enough to trigger detectable DNA methylation modifications. A single severe stress in an otherwise controlled and stable environment, such as in the greenhouse experiments in the chapters 2 and 3, might more easily trigger detectable DNA methylation changes.

Although no transplantation-specific differentiated DNA methylations within apomictic dandelion lineages were observed, a clear lineage-specific DNA methylation pattern appeared. In Figure 4.3 the Dutch lineages nl_3 and nl_13 revealed a cluster while nl_16 is clustered away as a distinct group. The same pattern is visible when inspecting the microsatellite fragments in Table 4.S1, further suggesting strong genotypic determination of DNA methylation patterns. DNA methylation differences can follow genomic differences because of several reasons: (1) silencing of TE inserts that have variable positions between genotypes (2) divergence in loci that control DNA methylation elsewhere in the genome (Dubin 2015), or (3) accumulation of stochastic methylation mutations (van der Graaf 2015), which could lead to asexual lineage-specific methylation patterns.

Although MS-AFLP screenings have been used successfully for several plant studies (e.g. Cervera et al. 2002; Salmon et al. 2008; Herrera et al. 2011; Massicotte et al. 2011) the limitation of this method is a rather low resolution and the lack of information on the genomic context of the methylation differences. It might be possible that in this experiment DNA methylation effects were present that went undetected by the MS-AFLP method. A better method, which would also yield single-nucleotide information about the methylation status of

a DNA region in addition, would be next-generation sequencing combined with a bisulfite treatment. Bisulfite treatment transforms unmethylated cytosines, but not methylated cytosines, into uracil. Such an in-depth screening using bisulfite sequencing has allowed for the identification of epigenetic quantitative trait loci in experimental *Arabidopsis* epiRILs, (Cortijo et al. 2014) but is now also starting to be applied to non-model plant systems (Platt et al. 2015; Trucchi et al. 2016; van Gurp et al. 2016).

Conclusion

The DNA methylation profiles tested in the offspring of reciprocally transplanted apomictic dandelion lineages clustered by lineage, but not by environment. Thus the differences in DNA methylation patterns are reflecting their genetic differences, but not their parental environments. Although some home-site advantages and thus adaptive divergence was observed between populations, no transplantation-induced heritable DNA methylations were detected. We conclude that plant growth in the native and non-native growing sites did not cause a stressful enough environment to trigger detectable levels of stress-induced and heritable DNA methylation changes. Thus, although stress-induced DNA methylation changes have been reported previously in dandelion greenhouse experiments, we conclude that heritability of stress-induced DNA methylation modifications is likely not a general or common phenomenon under natural conditions. Alternatively, it might be that undetected DNA methylation modification occurred that were not picked up by the MS-AFLP method, possibly even affecting the observed phenotypic differences. Studies using high-resolution methods, such as next-generation bisulfite sequencing are necessary to investigate the role of stress-induced epigenetic inheritance in more detail. Overall, this reciprocal transplantation experiment on apomictic dandelion lineages suggest that spontaneous and/or genetically determined, instead of stress-induced, DNA methylation changes are the prevailing system behind heritable epigenetic variation in nature.

Acknowledgment:

We acknowledge Miroslav Šrůtek, Jan Štěpánek and Prof. Jan Kirschner for their help in managing the field site in Czech Republic. Many thanks to Julie Ferreira de Carvalho, Dennis Kälble and Sylvia Drok for their contribution to the field work. This study was supported by NWO-ALW grants: NWO-Ecogenomics 884.10.003 and 864.10.008 from the Netherlands Organisation for Scientific Research.

Supporting information

Table 4.S1 Microsatellite profiles of the 6 apomictic dandelion lineages used for the MS-AFLP study. Ordered by apomictic lineage, first the 3 Czech lineages followed by the 3 Dutch lineages. CZ: Czech site, Benešov; NL: Dutch site, Wageningen; G: greenhouse, control group grown under greenhouse conditions. Question marks represent uncertainties, which we neglect as missing data.

Site	Sample	msat31					msat44B					msat58					msat78				
CZ	c26-6	238	0	0	0	0	?	182	0	0	0	104	133	0	0	0	154	164	0	0	0
CZ	c26-10	238	0	0	0	0	0	0	0	0	0	104	133	0	0	0	154	164	0	0	0
CZ	c26-19	238	0	0	0	0	182	?	?	0	0	104	133	0	0	0	154	164	0	0	0
CZ	c26-20	238	0	0	0	0	182	0	0	0	0	104	133	0	0	0	154	164	0	0	0
G	c26-2	238	0	0	0	0	182	0	0	0	0	104	133	0	0	0	154	164	0	0	0
G	c26-4	238	0	0	0	0	?	0	0	0	0	104	133	0	0	0	154	164	0	0	0
G	c26-6	238	0	0	0	0	?	182	0	0	0	104	133	0	0	0	154	164	0	0	0
G	c26-7	238	0	0	0	0	?	0	0	0	0	104	133	0	0	0	154	164	0	0	0
G	c26-8	238	0	0	0	0	?	182	0	0	0	104	133	0	0	0	154	164	0	0	0
G	c26-9	238	0	0	0	0	182	?	0	0	0	104	133	0	0	0	154	164	0	0	0
G	c26-10	238	0	0	0	0	?	182	0	0	0	104	133	0	0	0	154	164	0	0	0
NL	c26-2	?	0	0	0	0	?	0	0	0	0	104	133	0	0	0	154	164	0	0	0
NL	c26-3	238	0	0	0	0	?	0	0	0	0	104	133	0	0	0	154	164	0	0	0
NL	c26-11	238	0	0	0	0	182	?	0	0	0	104	133	0	0	0	154	164	0	0	0
NL	c26-13	238	0	0	0	0	?	0	0	0	0	104	133	0	0	0	154	164	0	0	0
NL	c26-14	238	0	0	0	0	182	?	0	0	0	104	133	0	0	0	154	164	0	0	0
NL	c26-21	238	0	0	0	0	0	0	0	0	0	104	133	0	0	0	154	164	0	0	0
NL	c26-22	238	0	0	0	0	?	182	0	0	0	104	133	0	0	0	154	164	0	0	0
NL	c26-26	238	0	0	0	0	182	0	0	0	0	104	133	0	0	0	154	164	0	0	0
CZ	c28-1	261	0	0	0	0	187	0	0	0	0	104	132	0	0	0	164	168	0	0	0
CZ	c28-19	?	261	0	0	0	187	0	0	0	0	104	132	0	0	0	164	?	0	0	0
CZ	c28-21	261	0	0	0	0	187	0	0	0	0	104	132	0	0	0	164	168	0	0	0

Table 4.S1 continues.

Site	Sample	msat31					msat44B					msat58					msat78				
CZ	c28-24	261	0	0	0	0	187	0	0	0	0	104	132	0	0	0	164	0	0	0	0
CZ	c28-27	261	0	0	0	0	170	187	0	0	0	104	132	0	0	0	164	0	0	0	0
G	c28-1	261	0	0	0	0	187	0	0	0	0	104	132	0	0	0	164	168	0	0	0
G	c28-2	261	0	0	0	0	187	0	0	0	0	104	132	0	0	0	164	0	0	0	0
G	c28-3	261	0	0	0	0	187	0	0	0	0	104	132	0	0	0	?	164	168	0	0
G	c28-4	?	261	0	0	0	187	0	0	0	0	104	132	0	0	0	164	168	0	0	0
G	c28-6	261	0	0	0	0	187	0	0	0	0	104	132	0	0	0	164	168	0	0	0
G	c28-7	261	0	0	0	0	187	0	0	0	0	104	132	0	0	0	164	168	0	0	0
G	c28-8	261	0	0	0	0	187	0	0	0	0	104	0	0	0	0	164	0	0	0	0
G	c28-9	261	0	0	0	0	187	0	0	0	0	104	132	0	0	0	164	0	0	0	0
G	c28-10	261	0	0	0	0	?	170	187	0	0	104	132	0	0	0	?	164	168	0	0
NL	c28-2	261	0	0	0	0	187	0	0	0	0	104	132	0	0	0	?	164	168	0	0
NL	c28-4	261	0	0	0	0	187	0	0	0	0	104	132	0	0	0	164	168	0	0	0
NL	c28-5	261	0	0	0	0	187	0	0	0	0	104	132	0	0	0	164	168	0	0	0
NL	c28-6	?	261	0	0	0	170	?	187	0	0	104	132	0	0	0	?	164	168	0	0
NL	c28-11	261	0	0	0	0	187	0	0	0	0	104	132	0	0	0	164	0	0	0	0
NL	c28-15	261	0	0	0	0	187	0	0	0	0	104	0	0	0	0	164	0	0	0	0
NL	c28-16	218	251	261	0	0	162	170	187	0	0	104	123	132	0	0	147	151	164	0	0
NL	c28-22	261	0	0	0	0	187	0	0	0	0	104	132	0	0	0	164	0	0	0	0
NL	c28-30	261	0	0	0	0	187	0	0	0	0	104	132	0	0	0	164	0	0	0	0
CZ	c42-7	247	297	0	0	0	170	193	0	0	0	104	124	128	0	0	160	169	172	0	0
CZ	c42-11	247	297	0	0	0	193	0	0	0	0	104	124	128	0	0	160	169	172	0	0
CZ	c42-14	247	297	0	0	0	193	0	0	0	0	104	124	128	0	0	160	169	172	0	0
CZ	c42-24	247	297	0	0	0	193	0	0	0	0	104	124	128	0	0	160	169	172	0	0
G	c42-1	247	297	0	0	0	193	0	0	0	0	104	124	128	0	0	160	169	172	0	0

Table 4.S1 continues.

Site	Sample	msat31					msat44B					msat58					msat78				
G	c42-2	247	297	0	0	0	170	193	0	0	0	104	124	128	0	0	160	169	172	0	0
G	c42-3	247	297	0	0	0	170	193	0	0	0	104	124	128	0	0	160	169	172	0	0
G	c42-4	247	297	0	0	0	?	170	193	0	0	104	124	128	0	0	160	169	172	0	0
G	c42-5	247	297	0	0	0	170	193	0	0	0	104	124	128	0	0	160	169	172	0	0
G	c42-6	247	255	297	0	0	193	0	0	0	0	104	117	124	128	0	160	164	169	172	0
G	c42-7	247	297	0	0	0	193	0	0	0	0	104	124	128	0	0	160	169	172	0	0
G	c42-8	247	297	0	0	0	170	193	0	0	0	104	124	128	0	0	160	169	172	0	0
G	c42-9	247	?	0	0	0	170	193	0	0	0	104	124	128	0	0	160	169	172	0	0
G	c42-10	247	297	0	0	0	193	0	0	0	0	104	124	128	0	0	160	169	172	0	0
NL	c42-5	247	0	0	0	0	193	0	0	0	0	104	124	128	0	0	160	169	172	0	0
NL	c42-11	247	297	0	0	0	170	193	0	0	0	104	124	128	0	0	160	169	172	0	0
NL	c42-13	247	297	0	0	0	193	0	0	0	0	104	124	128	0	0	160	169	172	0	0
NL	c42-14	247	297	0	0	0	170	193	0	0	0	104	124	128	0	0	160	169	172	0	0
NL	c42-16	247	?	0	0	0	170	193	0	0	0	104	124	128	0	0	160	169	172	0	0
NL	c42-18	247	297	0	0	0	170	193	0	0	0	104	124	128	0	0	160	169	172	0	0
NL	c42-19	247	297	0	0	0	193	0	0	0	0	104	124	128	0	0	160	169	172	0	0
NL	c42-21	247	297	0	0	0	170	193	0	0	0	104	124	128	0	0	160	169	172	0	0
G	w13-1	218	251	0	0	0	?	162	189	0	0	123	?	0	0	0	147	151	164	0	0
G	w13-2	218	251	0	0	0	?	162	0	0	0	123	0	0	0	0	147	151	164	0	0
G	w13-3	218	251	0	0	0	?	162	0	0	0	123	0	0	0	0	147	151	164	0	0
G	w13-4	218	251	0	0	0	?	162	0	0	0	123	0	0	0	0	147	151	164	0	0
G	w13-5	218	251	0	0	0	?	162	0	0	0	123	0	0	0	0	147	151	164	0	0
G	w13-6	218	251	0	0	0	?	162	0	0	0	104	123	0	0	0	147	151	164	0	0
G	w13-7	251	0	0	0	0	?	0	0	0	0	123	0	0	0	0	147	151	164	0	0
G	w13-9	251	0	0	0	0	?	?	0	0	0	123	0	0	0	0	147	151	164	0	0

Table 4.S1 continues.

Site	Sample	msat31					msat44B					msat58					msat78				
G	w13-10	218	251	255	0	0	?	?	162	189	191	104	117	123	0	0	147	151	164	174	0
NL	w13-5	218	251	0	0	0	?	162	0	0	0	123	0	0	0	0	147	151	164	0	0
NL	w13-7	251	0	0	0	0	0	0	0	0	0	123	0	0	0	0	147	151	164	0	0
NL	w13-8	251	0	0	0	0	162	0	0	0	0	123	0	0	0	0	147	151	164	0	0
NL	w13-9	218	251	0	0	0	?	162	0	0	0	123	?	0	0	0	147	151	164	0	0
NL	w13-11	251	0	0	0	0	?	0	0	0	0	123	?	0	0	0	147	151	164	0	0
NL	w13-18	218	253	0	0	0	?	162	0	0	0	123	?	0	0	0	147	151	164	0	0
NL	w13-24	251	0	0	0	0	?	162	0	0	0	123	0	0	0	0	147	151	164	0	0
NL	w13-25	218	251	0	0	0	?	162	0	0	0	123	0	0	0	0	147	151	164	0	0
NL	w13-28	251	0	0	0	0	?	?	0	0	0	123	0	0	0	0	147	151	164	0	0
G	w16-1	239	253	0	0	0	?	0	0	0	0	124	128	0	0	0	147	168	176	0	0
G	w16-2	239	253	0	0	0	?	0	0	0	0	124	128	0	0	0	147	168	176	0	0
G	w16-3	239	253	0	0	0	?	0	0	0	0	124	128	0	0	0	147	?	168	176	0
G	w16-4	239	253	255	0	0	191	?	0	0	0	104	117	124	128	0	147	164	168	174	176
G	w16-5	239	253	0	0	0	?	0	0	0	0	124	128	0	0	0	147	168	176	0	0
G	w16-6	239	253	0	0	0	?	191	0	0	0	104	117	124	128	0	147	168	176	0	0
G	w16-7	239	253	0	0	0	?	0	0	0	0	124	128	0	0	0	147	168	176	0	0
G	w16-8	239	253	0	0	0	?	191	0	0	0	104	117	124	128	0	147	168	176	0	0
G	w16-9	239	253	0	0	0	191	?	0	0	0	104	117	124	128	0	147	164	168	176	0
G	w16-10	239	253	0	0	0	?	0	0	0	0	124	128	0	0	0	147	168	176	0	0
NL	w16-5	239	253	0	0	0	?	?	0	0	0	124	128	0	0	0	147	168	176	0	0
NL	w16-6	239	253	0	0	0	?	?	0	0	0	104	124	128	0	0	147	168	176	0	0
NL	w16-7	239	253	0	0	0	0	0	0	0	0	124	128	0	0	0	147	168	176	0	0
NL	w16-8	239	253	0	0	0	?	189	191	0	0	104	117	124	128	0	147	168	176	0	0
NL	w16-20	239	253	0	0	0	?	?	?	0	0	124	128	?	0	0	147	168	176	0	0

Table 4.S1 continues.

Site	Sample	msat31					msat44B					msat58					msat78				
NL	w16-24	239	253	0	0	0	0	0	0	0	0	124	128	0	0	0	147	168	176	0	0
NL	w16-25	239	253	0	0	0	?	0	0	0	0	124	128	0	0	0	147	168	176	0	0
NL	w16-27	239	253	0	0	0	?	?	0	0	0	124	128	0	0	0	147	168	176	0	0
NL	w16-28	239	253	0	0	0	?	?	0	0	0	124	128	0	0	0	147	168	176	0	0
G	w3-1	251	0	0	0	0	?	162	0	0	0	123	?	0	0	0	147	151	164	0	0
G	w3-2	218	251	0	0	0	?	?	162	0	0	123	0	0	0	0	147	151	164	0	0
G	w3-3	0	0	0	0	0	0	0	0	0	0	123	0	0	0	0	147	151	0	0	0
G	w3-4	218	251	0	0	0	?	0	0	0	0	123	0	0	0	0	147	151	164	0	0
G	w3-5	218	?	251	0	0	?	162	0	0	0	123	0	0	0	0	147	151	164	0	0
G	w3-6	218	251	0	0	0	?	162	0	0	0	123	?	0	0	0	147	151	164	0	0
G	w3-7	218	251	255	0	0	?	162	182	191	?	104	117	123	0	0	147	151	164	174	0
G	w3-8	218	251	0	0	0	?	162	0	0	0	123	?	0	0	0	147	151	164	0	0
G	w3-9	218	247	251	0	0	?	162	0	0	0	123	0	0	0	0	147	151	164	0	0
G	w3-10	218	251	0	0	0	?	162	0	0	0	123	0	0	0	0	147	151	164	0	0
NL	w3-5	218	251	0	0	0	?	162	0	0	0	123	?	0	0	0	147	151	164	0	0
NL	w3-7	251	0	0	0	0	?	?	162	0	0	123	0	0	0	0	147	151	164	0	0
NL	w3-18	251	0	0	0	0	?	162	0	0	0	123	?	0	0	0	147	151	164	0	0
NL	w3-20	251	0	0	0	0	?	162	0	0	0	123	?	0	0	0	147	151	164	0	0
NL	w3-21	?	?	0	0	0	?	162	189	0	0	123	?	0	0	0	147	151	164	0	0
NL	w3-22	218	251	0	0	0	?	0	0	0	0	123	?	0	0	0	147	151	164	0	0
NL	w3-26	218	251	0	0	0	?	?	162	0	0	123	0	0	0	0	147	151	164	0	0
NL	w3-27	218	251	0	0	0	?	162	0	0	0	123	0	0	0	0	147	151	164	0	0
NL	w3-28	218	251	0	0	0	?	0	0	0	0	123	0	0	0	0	147	151	164	0	0
NL	w3-29	218	251	0	0	0	?	162	0	0	0	123	?	0	0	0	147	151	164	0	0

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The epigenetic footprint of poleward range-expanding plants in apomictic dandelions

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Published in Molecular Ecology (2015) 24, 4406–4418

Abstract

Epigenetic modifications, such as DNA methylation variation, can generate heritable phenotypic variation independent of the underlying genetic code. However, epigenetic variation in natural plant populations is poorly documented and little understood. Here, we test if northward range expansion of obligate apomicts of the common dandelion (*Taraxacum officinale*) is associated with DNA methylation variation. We characterized and compared patterns of genetic and DNA-methylation variation in greenhouse-reared offspring of *T. officinale* that were collected along a latitudinal transect of northward range expansion in Europe. Genetic AFLP and epigenetic MS-AFLP markers revealed high levels of local diversity and modest but significant heritable differentiation between sampling locations and between the Southern, Central and Northern regions of the transect. Patterns of genetic and epigenetic variation were significantly correlated, reflecting the genetic control over epigenetic variation and/or the accumulation of lineage-specific spontaneous epimutations, which may be selectively neutral. In addition, we identified a small component of DNA methylation differentiation along the transect that is independent of genetic variation. This epigenetic differentiation might reflect environment-specific induction or, in case the DNA methylation variation affects relevant traits and fitness, selection of heritable DNA methylation variants. Such generated epigenetic variants might contribute to the adaptive capacity of individual asexual lineages under changing environments. Our results highlight the potential of heritable DNA methylation variation to contribute to population differentiation along ecological gradients. Further studies are needed using higher-resolution methods to understand the functional significance of such natural occurring epigenetic differentiation.

Introduction

Plant species have the ability to respond to a changing climate by phenotypic plasticity, adaptation, and migration towards more suitable habitats (Nicotra et al. 2010). In practice these processes are intermingled, for example adaptive changes may arise during migration. At the leading edge of the migration front during range expansion plants have to adapt to novel biotic and abiotic conditions (Davis & Shaw 2001). Numerous asexual plant species consist of individual clonal genotypes of which many have successfully colonized a wide range of new habitats (Hollingsworth & Bailey 2000; Ahmad et al. 2008). Because of their limited within-lineage genetic variation, asexuals largely rely on the capacity of phenotypic plasticity to cope with new environmental conditions (Castonguay & Angers 2012). An important question is to determine what factors enable asexuals to successfully persist in changing climatic conditions.

Recent findings suggest that epigenetic mechanisms, such as histone modification and DNA methylation, may represent an additional source of phenotypic variation that is relevant for both within-generation phenotypic plasticity and if stably transmitted also for heritable adaptation (Angers et al. 2010). Epigenetic mechanisms play an important role in regulating gene expression and stabilizing the genome by repressing harmful genetic elements, like transposable elements (Henderson & Jacobsen 2007). In plants, DNA methylation variation also shows substantial heritability (Cervera et al. 2002, Anway et al. 2005; Jablonka & Raz 2009, Johannes et al. 2009, Cortijo et al. 2014). Without changing the genetic code epigenetic mechanisms can generate stable phenotypic variation contributing to phenotypic plasticity both in sexual (Bossdorf et al. 2010; Zhang et al. 2013) and asexual species (Angers et al. 2010; Latzel & Klimešová 2010). Especially in plants, stable DNA methylation variation can account for heritable trait differences that persist for multiple generations (Cubas et al. 1999; Cortijo et al. 2014). Variation in epigenetics may not only arise spontaneously (Becker et al. 2011; Schmitz & Ecker 2012) but also be environmentally induced (Verhoeven et al. 2010b; Dowen et al. 2012; Sahu et al. 2013).

The current knowledge of epigenetic variation derives mainly from studies using model species under controlled conditions. It has been only recently that studies have started to focus on patterns of DNA methylation variation in natural systems, in order to understand the evolutionary and ecological role of epigenetics (Bossdorf et al. 2008; Richards 2008; Bossdorf & Zhang 2011). Studies in natural plant populations have shown that DNA methylation variation can be correlated with ecological stresses (Herrera & Bazaga 2011) and habitats (Gao et al. 2010; Lira-Medeiros et al. 2010, Paun et al. 2010). Further, common garden studies on the clonally reproducing and invasive Japanese knotweed revealed habitat differentiation by DNA methylation and only limited genetic variation (Richards et al. 2012). These findings suggest that epigenetic variation may enable individual asexual lineages to adapt under changing environments.

The focus of this paper is on epigenetic variation during range expansion in asexual plant species. We compare patterns of genetic and heritable DNA methylation variation in natural populations of apomictic dandelions (*Taraxacum officinale*) along a

geographic transect of their range expansion. *T. officinale* reproduces sexually or asexually via apomixis, i.e. production of non-fertilized seeds (Asker & Jerling 1992), and in Europe it shows a pattern of geographic parthenogenesis reflecting postglacial range expansion (Menken et al. 1995; Verduijn et al. 2004). After the last retreat of land ice, approx. 10,000 years ago obligate apomicts migrated from glacial refugia towards Northern Europe (Comes & Kadereit 1998). Triploid apomictic lineages co-occur with diploid sexually reproducing dandelions in Central-Southern Europe, the area of the glacial refugia, and new apomictic (triploid) lineages arise through hybridization between sexual mothers and apomictic pollen donors (Richards 1973, Mogie & Ford 1988, Tas & Van Dijk 1999). It is believed that apomictic lineages are continuously formed in these mixed populations and together with the northwards migration this process may account for the high levels of clonal diversity typically observed in Northern European populations of apomictic dandelions (Van der Hulst et al. 2001). Previous work on apomictic dandelions showed that heritable DNA methylation changes can be triggered by exposure to ecological stresses (Verhoeven et al. 2010b) and by the hybridization of sexual and asexual dandelions, which gives rise to novel apomictic plants (Verhoeven et al. 2010a).

Heritable epigenetic modifications can be functionally targeted (for instance when a specific environmental cue triggers a specific epigenetic modification) or essentially random (Shea et al. 2011) and range from transient to very stable across generations (Becker et al. 2011; Cortijo et al. 2014). Targeted epigenetic effects could function as an underlying mechanism for specific stress responses, inherited stress "memory" and transgenerational phenotypic plasticity. By contrast, random epigenetic variation, if stably inherited, could function as a basis for natural selection on epimutations (Hirsch et al. 2012). Environmental stress, such as exposure to novel habitats during range expansion, can change DNA methylations, histones modifications, transposon silencing and gene expression, which subsequently generates random and novel genetic and epigenetic variation (Rapp & Wendel 2005, Bilichak et al. 2012). In that case we would expect that DNA methylation variation is increased in apomictic dandelions of northern regions due to their history of encountering novel biotic and abioitic environments during their northward range expansion. We would also expect DNA methylation variation to differentiate along the geographic transect. As environmental conditions change, DNA methylations can specifically be modified and can result in different epigenetic patterns associated with the habitats along the range expansion gradient. In addition, selection acting on random but stable epimutations may also contribute to differentiation between habitats in the DNA methylation profile.

Here, we studied epigenetic variation along a geographic transect of northward migration of apomictic dandelion in north-western Europe. We used offspring from field derived plants to analyse the heritable component of DNA methylation variation. With this experimental design, the heritable component of DNA methylation variation that we capture potentially includes both DNA methylation polymorphisms that are stably transmitted for many generations (e.g. Cortijo et al. 2014) and also possible environmentally-induced methylation modifications associated with the maternal growing

environment (Verhoeven et al. 2010b). Specifically we tested the following hypotheses: (1) Northern populations show higher levels of DNA methylation variability than southern populations. Such a pattern could arise because of higher levels of stress-induced DNA methylation modifications in the lineages' novel northern environments. (2) Regions along the transect are epigenetically differentiated. This could arise from environment-specific DNA methylation patterns. (3) DNA methylation variation patterns are partly autonomous, i.e. independent of underlying genetic variation. DNA methylation variation can be controlled by, or act independent of underlying genetic polymorphisms (Richards 2006). A relevant issue is therefore the degree of independence from the underlying genetic code in epigenetic variation. Many of the known epialleles are associated with silencing of transposable elements that can affect the expression of nearby genes (Paszkowski & Grossniklaus 2011). However, some features of the DNA methylome show autonomous variation independent of genetic variation (Cubas et al. 1999; Kalisz & Purugganan 2004; Marfil et al. 2012; Schmitz et al. 2013). Sequence-independent epialleles can potentially allow for adaptive dynamics that cannot be explained by the genetic code alone (Bossdorf et al. 2008), which may be particularly relevant in asexuals that have limited within-lineage genetic variation (Castonguay & Angers 2012; Verhoeven & Preite 2014).

Material and methods

Study species and sampling design

The common dandelion, *T. officinale,* is a widespread perennial plant species that is dispersed through seeds. For the description of the taxon *T. officinale*, formerly grouped in the sections *Vulgaria* and *Ruderalia*, see Kirschner & Štěpánek (2011). In spring 2011, we collected seeds from apomictic dandelions in ten areas (which we refer to as populations) along a south-north transect from Luxembourg to Central Sweden (Fig. 5.1). This transect covers a large portion of the apomicts' distribution in north-western Europe. The southernmost part of the transect is situated close to the area of mixed sexual-asexual populations in south-central Europe where new apomicts can arise from sexual ancestors (Menken et al. 1995; Verduijn et al. 2004). Within each population we collected one seed head from each of 16 different fields within an approximate 5-10 km radius to obtain an unbiased sample of the genetic diversity of the local population (Table 5.1). Sampling localities were usually pastures and some fallows,

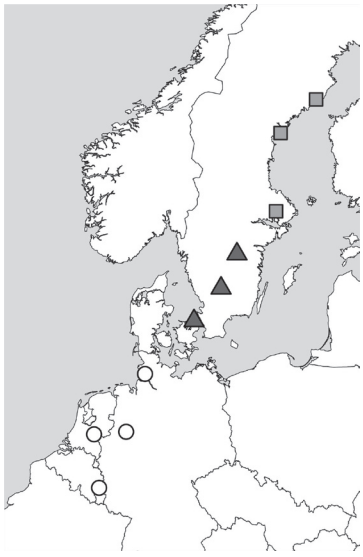

Figure 5.1 Sampling localities grouped in three regions indicated by: white circle South, black triangle Centre and grey rectangle North. For further description of the localities see Table 5.1.

road sides and forest glades. From each field-collected seed head we grew one offspring. Seeds were germinated on moist filter paper in Petri dishes for 11 - 16 days (10 h dark : 14 h light; 15°C : 20°C). Individual seedlings were transplanted into 1L pots containing a mixture of 80% potting soil and 20% pumice. The plants were grown for three months in a fully randomized design in the greenhouse (8 h dark : 16 h light; 16°C : 21°C) and watered several times per week, depending on the rate of water loss. In addition, plants received 50 ml of half-strength Hoagland nutrient solution once a week. All plants were confirmed to be triploid, and thus apomictic, using a flow cytometer (Partec Ploidy Analyser) by checking their nuclear DNA content against a diploid reference plant (Tas & Van Dijk 1999)

Table 5.1 Overview of the sampled apomictic dandelions

ID	Region	Population	Latitude	Longitude
N_3	North	Umeå, SE	63°49'33.06"N	20°15'46.94"E
N_2	North	Söderrå, SE	62°37´56.17"N	17°56´27.13"E
N_1	North	Uppsala, SE	59°51´30.82"N	17°38´20.15"E
C_3	Centre	Skänninge, SE	58°23´42.97"N	15°05´11.80"E
C_2	Centre	Värnamo, SE	57°10´59.38"N	14°02´52.15"E
C_1	Centre	Mårum, DK	56°01'35.80"N	12°16'51.78"E
S_4	South	Meldorf, GE	54°05'23.93"N	9°04'31.76"E
S_3	South	Ostbevern, GE	52°02'11.44"N	7°50'32.57"E
S_2	South	Heteren, NL	51°56'56.18"N	5°45'03.24"E
S_1	South	Hosingen, LU	50°03'11.34"N	6°04'40.66"E

For each of the 10 areas 16 plants were propagated in the greenhouse. DNA extracted from fresh leave tissue was used to analyze the genetic variation with amplified fragment length polymorphism (AFLP) and the epigenetic variation with methylation sensitive AFLP (MS-AFLP). Two samples failed to give reliable AFLP fragments (in C1 and S3) resulting in 158 samples and 160 samples for MS-AFLPs.

DNA isolation, AFLP and MS-AFLP

DNA was isolated from approximately 1 cm^2 of leaf tissue following the CTAB procedure by Rogstad (1992) with minor modifications (Vijverberg et al. 2004). The leaf tissue was collected in microtubes, kept on ice, which contained two 1/8" steel balls. Afterwards the leaf tissue was homogenized in the CTAB buffer using a Tissuelyser II (Qiagen, the Netherlands). The DNA pellets were dissolved in 50 µl TE and stored at -20°C until usage.

While the AFLP protocol uses the enzyme *Mse*I as the frequent cutter (Vos et al. 1995) the MS-AFLP protocol uses the DNA methylation sensitive enzymes *Msp*I and *Hpa*II in parallel batches (Xiong et al. 1999; Keyte et al. 2006), each in combination with the same rare cutter *Eco*RI (Reyna-Lopez et al. 1997). *Msp*I and *Hpa*II are isoschizomers

that recognize the same tetranucleotide sequence, 5´-CCGG, whereas the cytosines can be methylated on one or both DNA strands, referred as hemi and fully methylated. *Msp*I and *Hpa*II cut depending on the exact methlyation status of the restriction site (e.g. see Schulz et al. 2013): both enzymes cut if the restriction site is free from cytosine methylations (type I), only *Msp*I cuts if the internal cytosine is hemi- or fully methylated (type II), only *Hpa*II cuts if the external cytosine is hemi-methylated (tpye III), and additionally, sites that are fully methylated at the external cytosine or hemi- or fully methylated at both internal and external cytosines are not accessible for *Hpa*II and *Msp*I (type IV). The advantage of screening with both isoschizomers is the possibility to distinguish DNA methylation polymorphism from genetic polymorphism, where the fragment is absent due to mutation at the restriction site (Schulz et al. 2013).

The protocol for AFLP and MS-AFLP was adapted from Keyte et al. (2006) with some modifications. Based on previous pilot tests we selected four *Eco*RI / *Mse*I primer combinations for AFLP analysis (AAC / CTA, AAC / CAA, AAC / CTT, ACC / CTA) and seven *Eco*RI / *Msp*I-*Hpa*II primer combinations for the MS-AFLP analysis (ACA / TAC, ACA / TCA, AAC / TAG, AG / TCA, AG / TAC, ACC / TCA, ACC / TAG). In Table 5.S1 (supporting information) all adapters and primers used for the AFLP and MS-AFLP protocol are summarized. 50 ng of DNA was digested for three hours at 37°C in a total volume of 20 µl with ten units of *Eco*RI (100´000 U/ml), *Mse*I (50´000 U/ml), *Msp*I (100´000 U/ml) or *Hpa*II (50´000 U/ml). The corresponding buffer was added to the digestion mix and on top of that for the digestion with *Mse*I we added 2 µg of BSA (restriction enzymes, restriction buffer and BSA, New England BioLabs, Bioke, the Netherlands). Adapters were then ligated in a total reaction volume of 30 µl containing: 1 Unit of T4 DNA ligase and corresponding ligase buffer (ThermoFisher scientific, the Netherlands), 3.75 pmol of *Eco*RI adapter and respectively 37.5 pmol of *Mse*I or *Msp*I/*Hpa*II adapter for 18 hours at 22 °C followed by 10 minutes at 65°C. The ligation product was diluted to 15% in sterile water. Pre-amplification was performed in a total volume of 50 µl using: 1 x buffer, 125 nmol MgCl2, 2.5 U Taq DNA polymerase (all from GC biotech BV, the Netherlands), 10 nmol dNTPs (ThemoFisher scientific), 15 pmol of each pre-selective primer (Table 5.S1, supporting information) and 10 µl of diluted ligation product. The reaction started with 2 minutes hold at 72°C followed by 20 cycles of 30 sec at 94°C, 30 sec at 56°C, 2 min at 72°C and finished with 10 min incubation at 60°C and hold at 10°C. These pre-amplified products were diluted to 5% in sterile water. The selective amplification was performed in a total volume of 25 µl containing: 1 x buffer, 37.5 nmol MgCl2, 1.25 U Taq DNA polymerase (all from GC biotech B.V., the Netherlands), 7.5 nmol dNTPs (ThermoFisher scientific, the Netherlands), 10µg BSA, 5 pmol labelled selective *Eco*RI primer, 20 pmol selective *Mse*I, *Hpa*II/*Msp*I primer and 5 µl diluted PCR product. The selective amplification was started with 2 min hold at 94°C, followed by 10 cycles of 30 sec at 94°C, 30 sec at 65°C, 2 min at 72°C and 25 cycles with 30 sec at 94°C, 30 sec at 56°C, 2 min at 72°C and ended with 10 min at 60°C before hold at 10°C. The final PCR product was diluted to 2.5% in sterile water and analysed on the ABI 3130 genetic analyser (Life Technologies Europe BV, the Netherlands).

To avoid systematic biases we used a randomized block design to run all samples through the MS-AFLP and AFLP protocols. The samples were divided into four blocks and each block was divided into four sub-blocks, each sub-block containing one individual per population. Additionally 10% of the total number of samples was run as technical duplicates in order to quantify error rates and 10% as negative controls to check for peaks that indicate contamination signals and carry over effects (Bonin et al. 2007). Samples were fully randomized within sub-blocks, and blocks went through the lab protocols sequentially. This procedure ensured that any block-specific technical biases are randomly distributed over the ten populations and do not cause a specific bias that is correlated with the transect.

Fragment Scoring

The fragments were analysed and scored using GeneMapper 3.7 (Life technologies Europe BV). Fragments between 100 – 500 base pairs were scored and fragments that showed up in any of the negative controls were discarded. We used a semi-automated bin setting to identify marker loci as bins that had at least one sample showing a peak height above 50. Markers were scored as "present" when peak height exceeded a relative peak-specific threshold (mean peak height minus two times the standard deviation), and if peak height exceeded a minimum absolute threshold of 10. Monomorphic loci, singletons and doubletons (i.e. when only 1-2 samples had a deviating status) were discarded. In a preliminary data analysis we detected a significant block-specific bias: a subset of fragments was present in nearly all samples from one block but never in samples from any of the other blocks. We subsequently tested each marker for association with blocks using logistic regression and we excluded all markers from further analysis that showed a significant block effect ($P < 0.05$). Additionally we discarded all loci from analysis that showed more than two mismatches across the 16 pairs of duplicates. This resulted in a final data set of 85 polymorphic AFLP loci in 158 samples and 96 polymorphic MS-AFLP loci in 160 samples (Table 5.1).

The profiles for the selected *Msp*I and *Hpa*II markers were combined into a matrix of the four possible methylation conditions: type I) fragment is present in both *Msp*I and *Hpa*II profiles, type II) fragment is present only in *Msp*I profile, type III) fragment is present only in *Hpa*II profile and type IV) absence of fragment from both profiles. Type II is often interpreted as evidence for CG methylation and type III is often interpreted as CHG methylation (Schulz et al. 2013) but this interpretation is questioned (Fulneček & Kovařík 2014). Type IV can have multiple causes: both inner and outer cytosines are methylated on one DNA strand, the outer cytosine is fully methylated, i.e. on both strands, and a true fragment absence due to a sequence polymorphism in the restriction site condition (Salmon et al. 2008). Due to its uninformative status we excluded fragments of type IV from logistic regression analysis (see below). For multivariate analyses of cytosine methylations, we followed the analysis approach of Schulz et al. (2013) and we recoded the MS-AFLP combined matrix into two datasets: dataset M containing methylated loci where the methylated state (type II and III) equals 1 and the unmethylated state (type I) equals 0, and dataset U representing unmethylated loci where

type I is scored as 1 and type II and III are scored as 0. Both matrices M and U contain the same information, but downstream analysis based on pairwise distance metrics that emphasize shared 1´s can differ between the M and U coding. As pointed out by Schulz et al. (2013), functionally different patterns may emerge when emphasizing shared methylated sites or shared unmethylated sites in the genome. In both M and U matrices, ambiguous type IV loci were coded as zeros following Schulz et al. (2013). Scoring error rates based on the 16 replicate samples were 4.3%, for the AFLP profile, 6.5% for the *Msp*I profile and 5.0% for *Hpa*II. Because we used a randomized design for the greenhouse experiment and for the lab protocols the scoring errors are randomly distributed over the experimental design and therefore may cause undesired noise but no systematic bias in the results. Additionally, we evaluated how the patterns detected in our study are affected by using different criteria of repeatability and error rates. Using lower error rates reduced the number of loci retained in the analyses considerably, leading to undesirably small data sets. However, we found that results remained qualitatively well comparable (Tables 5.S2 - 5.S7, Supporting information).

Statistical Analysis

To check for broad geographic patterns in Europe we partitioned the transect into three regions: South, Centre and North (Fig. 5.1, Fig. 5.3) and we performed several analyses to detect differentiation and diversity patterns at the regional level. In addition, more fine-grained patterns were analysed at the levels of population or/and latitude. Firstly, clonal lineages were identified using GENOTYPE based on the AFLP profiles. Assuming some level of scoring error and within-lineage mutation, this program uses the empirical distribution of all pairwise genetic distances between samples to set an appropriate threshold for lineage assignment; this distribution is typically bimodal as a result of within-lineage variation and genetic variation between lineages and the appropriate threshold lies in between (Meirmans & Van Tienderen 2004). Pairwise distances between individuals were based on dice similarities: 1-(2a/(2a+b)), where a is the number of shared 1's and b the number of the number of loci with discordant information. In our data ten mismatches out of a total of 85 polymorphic loci were allowed as a maximum distance between lineage members (Fig. 5.S1, Supporting information). Clonal diversity within populations and within regions was captured as the number of clonal lineages divided by total number of plants per group. Shannon-Weaver indices were calculated for small sample sizes as an additional measure of clonal diversity (Chao & Shen 2003) using GENODIVE (Meirmans & Van Tienderen 2004). The regional differences within these Shannon-Weaver indices were then tested using a bootstrapping approach, i.e. resampling the individuals from the regions and comparing the indices (Manly 1991).

Secondly, multivariate analyses were performed that detect genomic diversity by quantitatively analysing the calculated pairwise dice similarity scores. PCoAs were plotted for AFLP and MS-AFLP (M&U) profiles based on the first two dimension calculated with the R function pcoa() from the package Ape with an additive constant to modify the non-diagonal distances to Euclidean (Cailliez 1983) and hence can be represented in n - 1 dimensions. A hierarchical AMOVA (R-function amova() from the

package Pegas with 10000 permutations) was performed to evaluate genetic (AFLP loci) and epigenetic variation (MS-AFLP loci from M and U profiles) among regions, among populations-within-regions and within populations. Fst was calculated an averaged across all loci for the AFLP and M profiles (fst() R-function from package Vegan). Permutation tests for homogeneity of multivariate dispersion were calculated with 999 permutations (betadisper() and permutest() R-functions from package Vegan). This is a multivariate analogue to Levene's homogeneity of variances test; it evaluates whether different groups have different levels of variation, irrespective of differences in group means.

Correlations between AFLP, MS-AFLP and geographic distances were tested using mantel tests (mantel() with 999 permutations from the R package Vegan). Geographic distances were either coded as km distances between the 10 populations, or as proxies for regional distances: same region = 0, adjacent regions =1, and non-adjacent regions = 2. Of special interest is the partial mantel correlation test (partialmantel() with 999 permutations also from Vegan) of MS-AFLP loci and geographic or regional distances, after controlling for the effect of genetic distances. This captures epigenetic differentiation that is uncorrelated with genetic differentiation.

In addition to the pairwise distances based multivariate analyses that describe genome-wide patterns of variation, we analysed single markers individually. Logistic regression models evaluate if the marker status of the M profile of MS-AFLP and the AFLP profile associates with region, population and the latitude of the sampling site. As mentioned above we handled here the ambiguous Type IV status as missing data for the M profile. This analysis was performed with the R-function *glm*() using binomial error distribution and a logit link function. The pvalues where corrected for multiple testing at a false discovery rate of 0.05 with the function *p.adjust*().

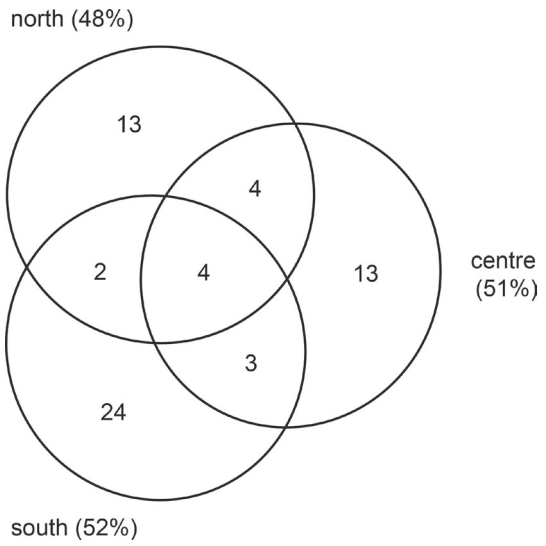

Figure 5.2 Venn Diagram with number of clonal lineages per region. In total 63 clonal lineages were found based on the amplified fragment length polymorphism (AFLP) dataset. In brackets the percentage of clonal diversity within region is shown: number of clonal lineages divided by total number of plants per region.

Results

Clonal Diversity

The AFLP profile consisting of 158 samples revealed 63 clonal lineages (with a maximum of 10 marker differences allowed within lineage, see Fig. 5S.1 supporting information). The 15-16 sampled plants per population represented on average 9-13 different clonal lineages. The regional clonal diversity showed a weak decrease from South (52%) to Centre (51%) to North (48%). This decrease in diversity was supported

Table 5.2 Variance Partitioning (AMOVA) for amplified fragment length polymorphisms (AFLP) and methylated/unmethylated profiles based on methylation sensitive AFLPs (MS-AFLP)

		Among regions	Among population – within regions	Within population
AFLP	Df	2	7	148
	SSD	0.174	0.223	2.988
	Molecular Variation in %	4.8	3.4	91.8
	Significance	**	ns	
MS-AFLP Methylated	Df	2	7	150
	SSD	0.771	1.286	19.936
	Molecular Variation in %	2.1	2.0	95.9
	Significance	**	ns	
MS-AFLP Unmethylated	Df	2	7	150
	SSD	0.164	0.540	7.693
	Molecular Variation in %	0.2	3.1	96.7
	Significance	ns	**	

Table shows the output of R-function amova() from package Pegas. Df: degrees of freedom. SSD: Sum of square deviation. Molecular variation percentages derive from variance components sigma2. Significance shown as p-values deriving from 10,000 permutations: <0.001 ***, <0.01 **, <0.05 *

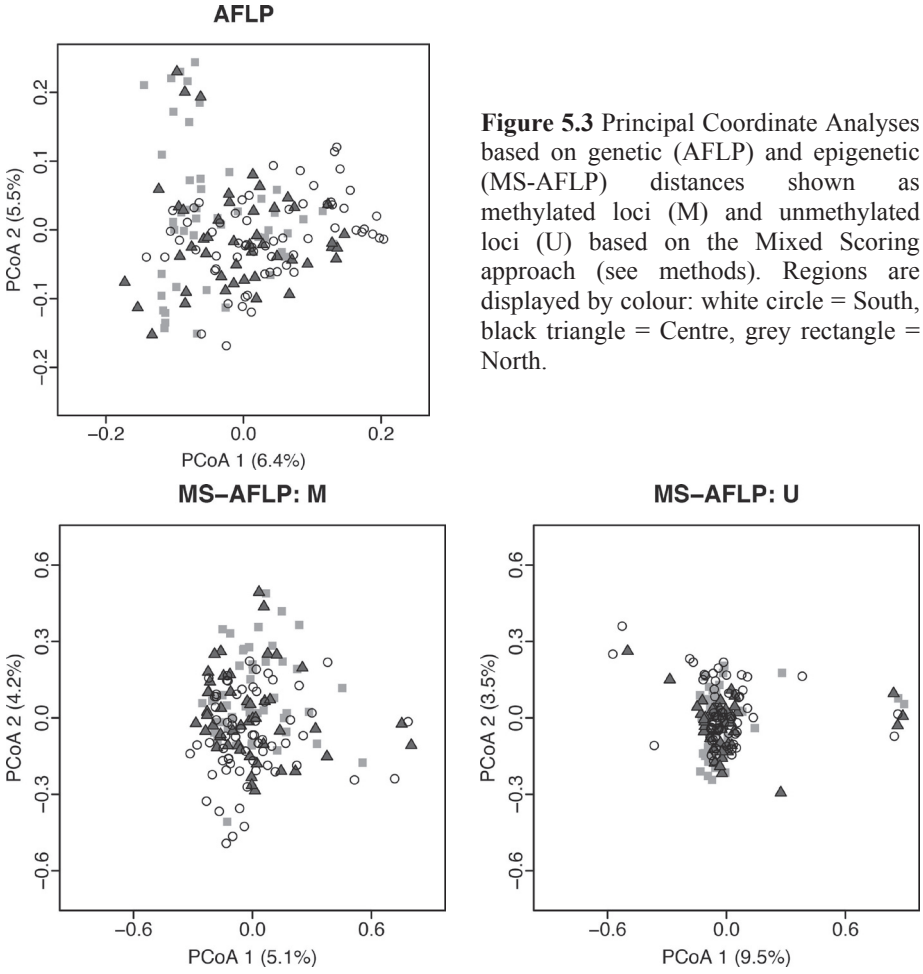

Figure 5.3 Principal Coordinate Analyses based on genetic (AFLP) and epigenetic (MS-AFLP) distances shown as methylated loci (M) and unmethylated loci (U) based on the Mixed Scoring approach (see methods). Regions are displayed by colour: white circle = South, black triangle = Centre, grey rectangle = North.

by a decrease in the corrected Shannon-Weaver index: South (1.6), Centre (1.5) and North (1.4) with a significant clinal pattern along the transect: South > Centre ($P = 0.05$) and South > North ($P < 0.01$; p values based on bootstrapped indices with 9,999 permutations). Most clonal lineages occurred exclusively in a single region. Thirteen clonal lineages occurred in multiple regions: four widespread lineages were found in allthree regions, Centre and North shared four lineages, Center and South shared three lineages and North and South shared two lineages (Fig. 5.2, Table 5.S8, Supporting information).

Genetic and Epigenetic Variation

AMOVA revealed that the great majority of the molecular variation (92% of the genetic variance, and 96% of the epigenetic M and 97% of the epigenetic U profiles) was partitioned within populations while the small remaining portion was partitioned among populations-within-regions and among regions (Table 5.2). Lack of strong regional

differentiation is also visible in the principal coordinate analysis (PcoA) plots that are based on pairwise AFLP and MS-AFLP distances (Fig. 5.3). Despite the small percentage of variation partitioned among regions, these variance components were significant for the genetic and methylated variation profiles (Table 5.2). The regional differentiation was somewhat more pronounced in the AFLP than in the methylated data; genetic regional differentiation of 4.8% compared to methylated regional differentiation of 2.1%. Analysis of single markers also showed stronger genetic than epigenetic differentiation, with only few MS-AFLP markers significantly associated with regions (Table 5.3). Consistent with the limited regional differentiation, measures of genetic subdivision among populations showed low values: $Fst_{AFLP} = 0.04$ and $Fst_{MS\text{-}AFLP} = 0.027$, indicating high migration across the populations.

Within regions, levels of MS-AFLP variation were higher than levels of AFLP variation, especially in the M-profiles (Fig. 5.4). The analysis of within-region genomic diversity, i.e. average distance to the regions´ centroid, did not show a clinal pattern along the south-to-north transect (permutation test: $P > 0.05$; Fig. 5.4).

Table 5.3 Results for single-marker tests using generalized linear models. Number of significant loci (P-value adjusted for multiple testing at FDR 0.05) and proportion of differentiated genetic and epigenetic loci that associate with population, region or latitude.

	Significantly differentiated:	
	AFLPs (n=85)	**MS-AFLPs (n=96)**
Differentiation by:		
Population	15 loci - 18%	Ns
Region	12 loci - 14%	2 loci – 2.1%
Latitude	5 loci - 6%	Ns

n = total number of polymorphic loci

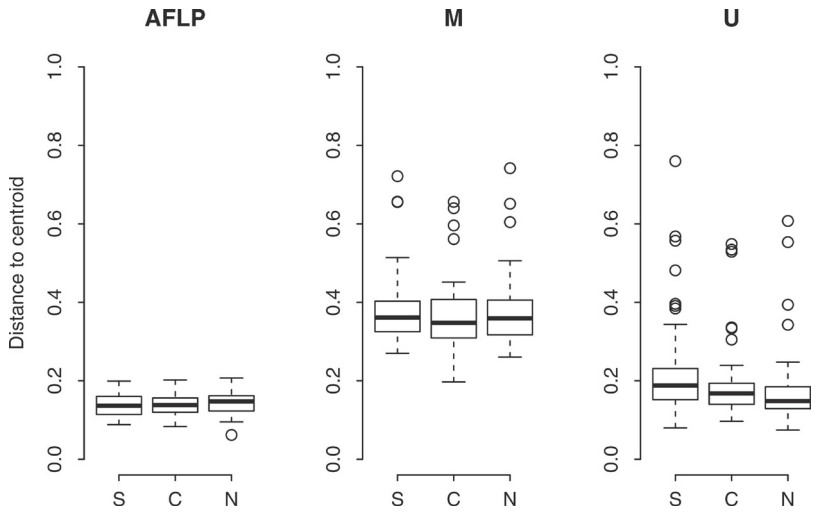

Figure 5.4 Within-region genomic diversity. Boxplots represent the average distance to centroid calculated at the regional level. Data for S (South), C (Centre) and N (North) regions, and for AFLP, M (methylated) and U (unmethylated) profiles.

Genetic and Epigenetic Correlation

Because the regions North, Centre and South are distributed along a linear transect, differentiation between these regions may derive from adaptation to the regional conditions or from neutral isolation by distance. Correlations between genetic and geographic distances (km distances between populations) were weak yet significant(R = 0.106, P = 0.001) and epigenetic variation showed an even weaker correlation with geographic distance (using the epigenetic M profile: R = 0.048, $P < 0.05$ but not significant for U profiles). Additionally, we used partial mantel tests to detect geographic patterns of epigenetic variation after controlling for genetic effects, i.e. we looked for geographic patterns in the MS-AFLP data that did not simply mirror geographic patterns in the genetic data. When tested at the level of populations, no evidence was found for such an autonomous epigenetic pattern when testing the correlation between autonomous MS-AFLP profiles and geographic km distance. When tested at the regional level, after correcting for AFLP variation a significant correlation was observed between MS-AFLP profiles and regional distances (M profile: partial mantel correlation R = 0.049, $P < 0.05$; not significant for the U profile MS-AFLp data).

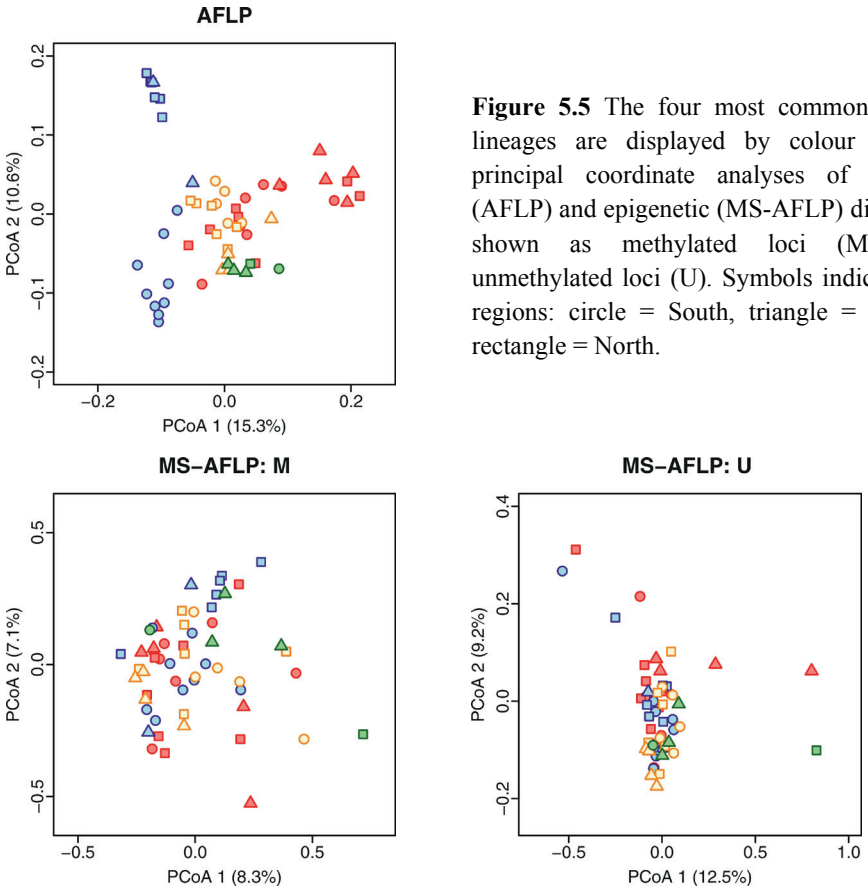

Figure 5.5 The four most common clonal lineages are displayed by colour in the principal coordinate analyses of genetic (AFLP) and epigenetic (MS-AFLP) distances shown as methylated loci (M) and unmethylated loci (U). Symbols indicate the regions: circle = South, triangle = Centre, rectangle = North.

Visualization of the four most common clonal lineages (occurring in all three regions, see Table 5.S8, supporting information) shows genetic clustering but limited clustering based on their epigenetic profiles (Fig. 5.5). While there is a clear overall correlation between AFLP and MS-AFLP profiles across all clonal lineages (Mantel test correlation between AFLP and M profiles: $R = 0.163$, $P = 0.001$; AFLP and U profiles: $R = 0.068$, $P = 0.07$), this absence of obvious epigenetic clustering supports the idea that there is also some fraction of the DNA methylation variation that is independent from genetic background.

Discussion

In this study we explored patterns of epigenetic variation in apomictic dandelion populations along a northward range expansion gradient. We hypothesized that range expansion could result in certain patterns of epigenetic variation: (1) increased levels of epigenetic variation towards the north and (2) in regional epigenetic differentiation. We found limited evidence for regional sequence-independent epigenetic differentiation and no gradient in levels of epigenetic variation. While much of the heritable epigenetic variation was intertwined with genetic variation, a fraction of the DNA methylation differentiation between regions along the transect was not associated with genetic variation. This autonomous fraction of epigenetic variation is interesting because it shows

a potential contribution to phenotypic variation and plasticity beyond what can be explained by genetic variation; however, this fraction is quite small.

Genetic and epigenetic patterns in apomictic dandelions

The analysis of the offspring of sampled apomictic dandelions along the latitudinal transects revealed very high clonal diversity. The continuing formation of novel apomictic lineages from mixed sexual-apomictic populations that subsequently migrate northwards probably accounts for the high clonal diversity observed in all sampling locations, which is consistent with previous reports (Van der Hulst et al. 2001). We observed a moderate decrease in clonal diversity towards the north, possibly reflecting clonal selection in response to environmental variability when migrating away from the location of origin.

The hypothesis that epigenetic variation increases towards the north was not supported. This may be because exposure to novel climatic conditions does not trigger enhanced levels of epigenetic variation. An alternative explanation is that such an epigenetic signal is very transient and is not reflected anymore in present-day standing variation. Also, increased biotic stress exposure towards the north might be partly counteracted by reduced abiotic stress levels along the same gradient (Verhoeven & Biere 2013), resulting in similar overall levels of stress along the transect. Modest levels of differentiation in epigenetic variation was observed at a regional, local and clinal level along the transect as hypothesized, which suggests environment-related epigenetic patterns. Such environment-associated epigenetic differentiation could arise from either induction of heritable epigenetic modifications by the environment or divergent selection on stable epimutations.

Autonomous epigenetic variation

The correlation between genetic and epigenetic variation observed in our study shows that a large part of the epigenetic variation is not sequence-independent which may imply that much of the epigenetic variation is not autonomous but rather under genetic control (Richards 2006). To date, epigenetic polymorphism have generally shown association with DNA sequence, e.g. with transposable elements (Paszkowski & Grossniklaus 2011; Schmitz et al. 2013). It is important to point out that the observed correlation can either derive from genetic control over DNA methylation patterns, or from the build-up of lineage-specific (and potentially autonomous) epimutations within clonal lineages that may also create statistical associations between genetic and epigenetic patterns. It has been shown for *Arabidopsis thaliana* that differences in DNA methylation status can accumulate over generations similar to, but less stable than genetic mutations (Becker et al. 2011). In our study, in addition to sequence-associated DNA methylation, small but significant regional epigenetic differentiation persisted after controlling for the correlation with genetic variation. This portion of epigenetic variation likely reflects epigenetic differentiation that is not under genetic control, and such autonomous, heritable epigenetic differentiation may contribute to phenotypic variation that cannot be explained by genetic variation alone. It has been proposed that such additional epigenetically

mediated phenotypic variation could play a role in plant adaptation to rapidly changing conditions (Bossdorf et al. 2008, Massicotte & Angers 2012). We detected this autonomous fraction of epigenetic variation only in the M-profiles (which emphasizes shared cytosine methylation between plants) and not in the U-profiles (which emphasizes cytosine that are not methylated). In the interpretation of Schulz et al. (2013) this could indicate a larger contribution to differentiation of epigenetically silenced loci compared to transcriptionally active loci.

Natural epigenetic variation

Several recent studies have revealed an association of natural epigenetic variation with environment-specific traits within genetically uniform groups (Gao et al. 2010; Lira-Medeiros et al. 2010; Richards et al. 2012). Studies in natural populations of sexually reproducing plants demonstrated a correlation between genetic and epigenetic markers while a proportion of epigenetic variation showed sequence-independent differentiation (Li et al. 2008; Herrera & Bazaga 2010; Schulz et al. 2014). Also in natural populations of animal species and in nectar inhabiting yeast some evidence for distinct and ecologically relevant epigenetic patterns was found (Herrera et al. 2011; Massicotte et al. 2011; Schrey et al. 2012). Our study differs from these and related studies because our study was not based directly on field-collected material (or vegetatively derived offspring), but on natural DNA methylation variation that persists through apomictic seed production. Hence our findings contribute to the field of ecological epigenetics in natural populations by pointing out epigenetic differentiation in the component of natural DNA methylome variation that is heritable, which arguably is the most relevant fraction of epigenetic variation for adaptation (Bossdorf et al. 2008).

The detection of linear patterns of genetic variation and the presence of widespread clonal lineages along the transect are in line with the post-glacial latitudinal range expansion of *T. officinale*. However, alternative historical migration routes may exist as well; apomictic dandelions also persisted in glacial refugia in south-eastern Europe and may have colonized Sweden entering from the north and migrating to the south. In support of this possibility, we observed a distinct group of a few samples in the AFLP dataset (upper left corner in Fig. 5.3 A) that might reflect plants originating from a different glacial refugium. However, these plants did not have deviating MS-AFLP profiles (Fig. 5.3 B & C). If these individuals would indeed represent a group of plants with a different historical background, the similarity of their epigenetic profiles would further support our main conclusion that regional epigenetic differentiation exists partly independent of the genetic background.

Detecting cytosine methylations

To compare genetic and epigenetic variation we ascertained the detection of purely epigenetic variation by scoring a combination of the two methylation sensitive enzyme

profiles (*Msp*I and *Hpa*II). The ability to detect purely epigenetic variation (autonomous from genetic variation) is an important strength of MS-AFLPs and this method has been used successfully to describe patterns of epigenetic variation in a wide range of different species (e.g. Cervera et al. 2002; Salmon et al. 2008; Herrera et al. 2011; Massicotte et al. 2011). However, there are also a number of technical limitations of MS-AFLPs, including the relatively low numbers of loci and the lack of information about sequence context (Becker et al. 2011; Schrey et al. 2013). Better methylome screening is possible, e.g. whole genome bisulfite sequencing (Becker et al. 2011) or reduced representation bisulfite sequencing (Meissner et al. 2005). However, these and other sequencing-based methods are not yet cost effective when using sample sizes typical for ecological population studies and are challenging to use in species without a reference genome.

Conclusion

Natural populations of apomictic dandelions along a northward range expansion gradient revealed high levels of heritable genetic and epigenetic variation, but limited regionally structured variation and no enhanced epigenetic variation with increasing latitude. Therefore, we did not find evidence of increased levels of inherited DNA methylations in northern, potentially more stressful, environments. The observed regional differentiation is partly correlated with genetics and partly non-correlated. In addition to within-lineage genetic variation it is this sequence-independent epigenetic variation that may contribute to phenotypic variation and adaptation in asexual plant lineages. Studies like ours can demonstrate the potential of epigenetic variation in natural populations, but to understand its functional consequences studies that link DNA methylations to their function and detect epigenetic variation at higher resolution, are necessary.

Acknowledgements

We thank Kitty Vijverberg and Niels Wagemaker for advice on the DNA extraction method and for support on the MS-AFLP protocol. Thanks to Thomas van Gurp, Maria Johansson and Farag Mahmoud El Sabbagh for their help during the seed collection trip and to Tanja Bakx-Schotman and Slavica Milanovic-Ivanovic for their help in collecting leaf material for DNA extraction. Thanks to Patrick Meirmans for support on using the program Genotype and to Christina Richards for useful comments on the manuscript. This work was supported by NWO-ALW grants: NWO-Ecogenomics 884.10.003 and 864.10.008 from the Netherlands Organisation for Scientific Research.

Supporting information

Table 5.S1 Adapters and primers used for AFLPs and MS-AFLPs

Adapters*	**Sequence 5´- 3´**
*Eco*RI-adapter I	CTCGTAGACTGCGTACC
*Eco*RI-adapter II	AATTGGTACGCAGTC
*Mse*I-adapter I	GACGATGAGTCCTGAG
*Mse*I-adapter II	TACTCAGGACTCAT
*Hpa*II and *Msp*I-Adapter I	GATCATGAGTCCTGCT
*Hpa*II and *Msp*I-Adapter II	CGAGCAGGACTCATGA
Pre-selective primers	**Sequence 5´- 3´**
*Eco*RI-A	GACTGCGTACCAATTC**A**
*Eco*RI-T	GACTGCGTACCAATTC**T**
*Mse*I-C	GACGATGAGTCCTGAGTAA**C**
*Hpa*II and *Msp*I –T	ATCATGAGTCCTGCTCGG**T**
Selective primers	**Sequence 5´- 3´**
*Eco*RI + AAC/ACA/AG/ACC	GACTGCGTACCAATTC**AAC/ACA/AG/ACC**
*Mse*I + CTA/CAA/CTT	GATGAGTCCTGAGTAA**CTA/CAA/CTT**
*Hpa*II / *Msp*I + TCA/TAC/TAG	ATCATGAGTCCTGCTCGG**TCA/TAC/TAG**

*_Eco_RI adapters (Reyna Lopez et al. 1997), *Mse*I adapter (Vos et al. 1995), *Hpa*II and *Msp*I adapters (Xiong et al. 1999).

Table 5.S2 Scoring error per error criteria

	AFLP	**MS-AFLP**	
Error criteria	***Mse*I**	***Hpa*II**	***Msp*I**
E_1	2.36	2.89	3.36
E_2	4.27	5.23	6.35
E_4	7.05	8.13	9.18

Scoring errors are shown in %. Error criteria denote the number of mismatches across the 16 duplicated samples. Datasets were divided by following error criteria: one (E_1), two (E_2) or four (E_4) mismatches allowed across the duplicated samples.

Table 5.S3 Significantly differentiated loci associated with population, region and latitude

Differentiated by	Error criteria	AFLP		MS-AFLP	
		Total number of loci	Number of significant loci	Total number of loci	Number of significant loci
Population	E_1	70	19 / 12	52	3 / 0
	E_2	85	23 / 15	96	8 / 0
	E_4	101	25 / 17	142	14 / 0
Region	E_1	70	25 / 14	52	7 / 1
	E_2	85	29 / 12	96	18 / 2
	E_4	101	33 / 13	142	28 / 2
Latitude	E_1	70	19 / 7	52	15 / 0
	E_2	85	22 / 5	96	22 / 0
	E_4	101	27 / 8	142	30 / 0

Individual markers were tested using generalized linear models. Number of significant loci are shown as unadjusted / fdr (false discovery rate) adjusted loci with p-values below 0.05. Datasets were divided by following error criteria: one (E_1), two (E_2) or four (E_4) mismatches allowed across the duplicated samples.

Table 5.S4 Variance Partitioning (AMOVA) for amplified fragment length polymorphisms (AFLP) and methylated/unmethylated profiles based on methylations sensitive AFLPs (MS-AFLP)

		AFLP		MS-AFLP			
				Methylated		Unmethylated	
	Error Criteria						
Among region	E_1	6	***	0	ns	1	ns
	E_2	5	**	2	**	0	ns
	E_4	4	***	2	*	0.5	ns
Among population within regions	E_1	3	ns	3	**	1	ns
	E_2	3	ns	2	ns	3	*
	E_4	3	ns	2	ns	3	*
Within population	E_1	91		97		98	
	E_2	92		96		97	
	E_4	93		96		96.5	

P-values deriving from 10´000 permutations: <0.001 ***, <0.01 **, <0.05 *, not significant ns. Datasets were divided by following error criteria: one (E_1), two (E_2) or four (E_4) mismatches allowed across the duplicated samples.

Table 5.S5 Analysis of homogeneity of multivariate dispersion: P-values from permutation tests on the distances to group centroids

Error criteria	AFLP	MS-AFLP	
		Methylated	Unmethylated
E_1	0.651	0.645	0.588
E_2	0.425	0.751	0.164
E_4	0.039	0.610	0.046

P-values deriving from 999 permutations. Datasets were divided by following error criteria: one (E_1), two (E_2) or four (E_4) mismatches allowed across the duplicated samples.

Table 5.S6 Mantel tests between genetic and epigenetic profile

Error criteria	Number of Samples	Correlation between:	R^2 and significance
E_1	158	AFLP + Methylated	0.15 **
		AFLP + Unmethylated	0.07 ns
E_2	158	AFLP + Methylated	0.16 **
		AFLP + Unmethylated	0.07 ns
E_4	154	AFLP + Methylated	0.15 **
		AFLP + Unmethylated	0.03 ns

P-values deriving from 999 permutations: <0.001 ***, <0.01 **, <0.05 *, not significant ns. Datasets were divided by following error criteria: one (E_1), two (E_2) or four (E_4) mismatches allowed across the duplicated sample.s.

Table 5.S7 Mantel tests and Partial Mantel test between genetic/epigenetic profile and geographic distances

	Error criteria	AFLP	MS-AFLP	
			Methylated	Unmethylated
Mantel tests:				
Geographic distances (km)	E_1	0.105 **	0.015 ns	-0.012 ns
	E_2	0.106 **	0.048 *	-0.012 ns
	E_4	0.099 **	0.043 ns	-0.018 ns
Regional distances	E_1	0.102 **	0.030 ns	-0.001 ns
	E_2	0.102 **	0.060 *	< 0.001 ns
	E_4	0.104 **	0.065 *	-0.007 ns
Partial Mantel test:				
Geographic distances (km) corrected for AFLP correlation	E_1		0.003 ns	-0.018 ns
	E_2		0.036 ns	-0.018 ns
	E_4		0.027 ns	-0.025 ns
Regional distances corrected for AFLP correlation	E_1		0.018 ns	-0.008 ns
	E_2		0.049 *	-0.005 ns
	E_4		0.049 **	-0.015 ns

Pearson´s correlation coefficient followed by P-values which derive from 999 permutations: <0.001 ***, <0.01 **, <0.05 *, not significant ns. Datasets were divided by following error criteria: one (E_1), two (E_2) or four (E_4) mismatches allowed across the duplicated samples.

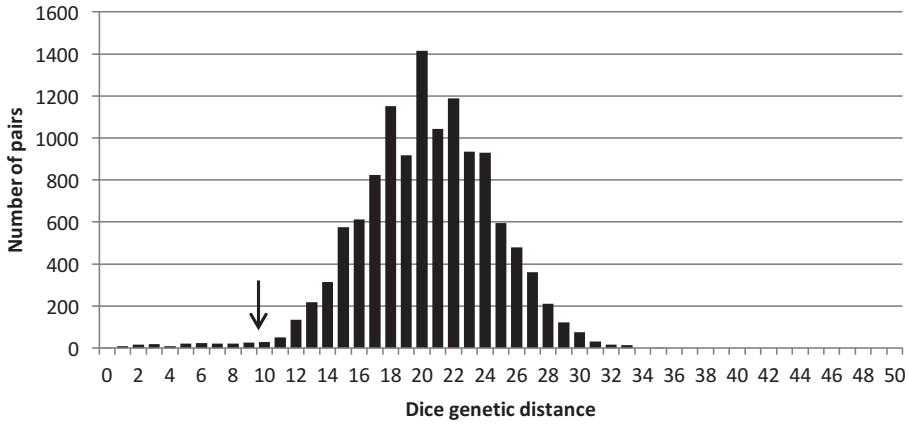

Figure 5.S1 Selecting maximum genetic distance between lineage members. Shown is a histogram of Dice distances between all possible pairs of samples. Arrow indicates the genetic distance that we selected as the apropriate yet conservative threshold for clonal assignment: up to 10 genotyping differences were allowed between clone members (due to within-clone mutation and genotyping errors).

Table 5.S8: Clonal lineage assignment. Genotyping and its alignment across the sampling locations based on AFLP profile from apomictic dandelions. Highlighted cells indicate the clonal lineages that occurred across two regions (grey) or three regions (dark grey).

ID	Plant	Clonal lineage	Alignment	ID	Plant	Clonal lineage	Alignment
S_1	1	G_1	SCN	S_3	4	G_20	S
S_1	2	G_2	S	S_3	8	G_15	S
S_1	3	G_3	SCN	S_3	9	G_21	S
S_1	5	G_4	SCN	S_3	10	G_15	S
S_1	6	G_5	S	S_3	11	G_22	S
S_1	7	G_6	S	S_3	12	G_1	SCN
S_1	9	G_7	SN	S_3	13	G_3	SCN
S_1	10	G_8	S	S_3	14	G_23	S
S_1	12	G_4	SCN	S_3	15	G_24	S
S_1	13	G_3	SCN	S_3	17	G_25	S
S_1	14	G_9	S	S_3	18	**NA**	**NA**
S_1	15	G_4	SCN	S_3	19	G_4	SCN
S_1	16	G_10	S	S_3	20	G_26	S
S_1	17	G_11	S	S_4	1	G_1	SCN
S_1	19	G_4	SCN	S_4	3	G_27	CS
S_1	20	G_1	SCN	S_4	4	G_28	CS
S_2	2	G_12	SN	S_4	5	G_1	SCN
S_2	3	G_12	SN	S_4	9	G_4	SCN
S_2	5	G_13	S	S_4	10	G_4	SCN
S_2	6	G_1	SCN	S_4	11	G_29	S
S_2	7	G_14	S	S_4	12	G_30	S
S_2	9	G_12	SN	S_4	13	G_31	CS
S_2	10	G_15	S	S_4	14	G_28	CS
S_2	12	G_4	SCN	S_4	15	G_18	S
S_2	13	G_55	SCN	S_4	16	G_32	S
S_2	14	G_12	SN	S_4	17	G_33	S
S_2	15	G_12	SN	S_4	18	G_32	S
S_2	16	G_3	SCN	S_4	19	G_1	SCN
S_2	17	G_17	S	S_4	20	G_30	S
S_2	18	G_18	S				
S_2	19	G_12	SN				
S_2	20	G_4	SCN				
S_3	1	G_19	S				
S_3	2	G_3	SCN				
S_3	3	G_6	S				

ID	Plant	Clonal lineage	Alignment	ID	Plant	Clonal lineage	Alignment
C_1	1	G_4	SCN	C_3	10	G_4	SCN
C_1	2	G_1	SCN	C_3	12	G_49	C
C_1	3	G_27	CS	C_3	13	G_4	SCN
C_1	4	G_1	SCN	C_3	14	G_4	SCN
C_1	5	G_1	SCN	C_3	15	G_3	SCN
C_1	6	G_3	SCN	C_3	17	G_49	C
C_1	7	G_34	C	C_3	18	G_50	C
C_1	8	G_35	C	C_3	19	G_36	CN
C_1	11	G_36	CN	C_3	20	G_36	CN
C_1	12	G_3	SCN				
C_1	13	**NA**	**NA**				
C_1	16	G_31	CS				
C_1	17	G_37	C				
C_1	18	G_38	C				
C_1	19	G_55	SCN				
C_1	20	G_4	SCN				
C_2	1	G_39	C				
C_2	3	G_4	SCN				
C_2	4	G_40	CN				
C_2	5	G_3	SCN				
C_2	7	G_41	C				
C_2	8	G_1	SCN				
C_2	9	G_3	SCN				
C_2	10	G_42	CN				
C_2	13	G_43	C				
C_2	14	G_1	SCN				
C_2	15	G_44	C				
C_2	16	G_3	SCN				
C_2	17	G_45	C				
C_2	18	G_1	SCN				
C_2	19	G_31	CS				
C_2	20	G_46	C				
C_3	1	G_47	CN				
C_3	2	G_42	CN				
C_3	4	G_40	CN				
C_3	5	G_48	C				
C_3	6	G_28	CS				
C_3	7	G_42	CN				
C_3	9	G_1	SCN				

ID	Plant	Clonal lineage	Alignment	ID	Plant	Clonal lineage	Alignment
N_1	1	G_1	SCN	N_3	9	G_55	SCN
N_1	2	G_51	N	N_3	11	G_3	SCN
N_1	3	G_52	N	N_3	12	G_60	N
N_1	5	G_53	N	N_3	13	G_63	N
N_1	6	G_12	SN	N_3	14	G_64	N
N_1	7	G_1	SCN	N_3	16	G_3	SCN
N_1	8	G_54	N	N_3	18	G_47	CN
N_1	10	G_53	N	N_3	19	G_42	CN
N_1	11	G_53	N	N_3	20	G_56	N
N_1	12	G_42	CN				
N_1	14	G_3	SCN				
N_1	15	G_53	N				
N_1	16	G_42	CN				
N_1	17	G_42	CN				
N_1	18	G_36	CN				
N_1	20	G_42	CN				
N_2	2	G_55	SCN				
N_2	3	G_3	SCN				
N_2	4	G_4	SCN				
N_2	5	G_56	N				
N_2	7	G_42	CN				
N_2	8	G_1	SCN				
N_2	9	G_57	N				
N_2	10	G_1	SCN				
N_2	11	G_1	SCN				
N_2	12	G_58	N				
N_2	15	G_59	N				
N_2	16	G_42	CN				
N_2	17	G_59	N				
N_2	18	G_55	SCN				
N_2	19	G_60	N				
N_2	20	G_4	SCN				
N_3	1	G_7	SN				
N_3	2	G_36	CN				
N_3	3	G_42	CN				
N_3	5	G_61	N				
N_3	6	G_40	CN				
N_3	7	G_56	N				
N_3	8	G_62	N				

General Discussion

6

Research status of epigenetic inheritance and current open questions

The theory of Neo-Darwinism is the commonly accepted explanation of how species can adapt to changing environments. It is understood that the evolutionary process is based on natural selection acting on heritable genetic variation that arises through spontaneous mutations in the genetic code and the best genetically adapted individuals will reproduce successfully. However, recent studies suggest that not only genetic effects are causing heritable variation. Epigenetic mechanisms, such as DNA methylation, also showed evidence for heritable epigenetic variation. In my thesis I focus on heritable epigenetic variation across generations, to which I refer as transgenerational epigenetic effects.

Epigenetic modifications can be induced by environmental changes (Dowen et al. 2012), to which I refer as induction-based epigenetic inheritance (or detection-based effects, following Shea et al. 2011, in the sense that the organism detects a modified environment and responds accordingly via epigenetic changes). Induction-based epigenetic inheritance is one possible mechanism underlying transgenerational phenotypic plasticity, which could sustain an adaptive response to environmental change into offspring generations even when the environmental cue is not present anymore. Even if only transient, such environmentally sensitive epigenetic adjustments might play a facilitating role in the performance of organisms under fast changing conditions (Boyko et al. 2011; Boyko et al. 2007). Especially when genetic adaptation alone is not able to keep up with rapid environmental changes, epigenetic information from the ancestral environment could enhance survival of populations (Furrow and Feldman 2013). Induction-based epigenetic inheritance expressed as adaptive phenotypic variation suggests a type of Lamarckian-flavoured component to evolution, also called ‘soft inheritance’ (Richards 2006).

Transgenerational epigenetic effects can also arise through spontaneous, stress-unrelated, changes (Becker et al. 2011; van der Graaf et al. 2015). Such spontaneous epi-alleles can, if transmitted stably enough, accumulate across generations and could be in principle subjected to selection and drift. Random-based heritable epi-variation that is shaped by selection I refer to as selection-based epigenetic inheritance (see selection-based effects, Shea et al. 2011). One way in which spontaneous as well as induced epigenetic changes may affect the process of adaptation is by "holding" an adaptive phenotype for several generations, which can subsequently affect selection on genetic mutations to stabilize the phenotype (Pal and Miklos 1999; Klironomos et al. 2013).

Evidence for transgenerational epigenetic effects causing changes in gene expression was found in plants as well as in animal studies (Johannes et al. 2009; Akimoto et al. 2007; Rechavi et al. 2011). It is known that during ontogenesis DNA methylation resetting and reprogramming takes place, but this resetting is more pronounced in animal than in plant species (Feng et al. 2010). In plants DNA methylation-based epigenetic modifications might thus be easier transmitted to the subsequent generation. Significant progress in the understanding of epigenetic inheritance in plants is based mostly on molecular genetic studies in laboratory strains and these findings should be taken as indications of the potential importance of epigenetic mechanisms in nature. Studies on non-model systems have started to emerge, involving

experiments under natural conditions with field-collected material to further the understanding on the role of epigenetic inheritance under natural conditions (Bossdorf et al. 2010; Richards et al. 2012; Herrera & Bazaga 2011; Angers et al. 2012). Additionally, in recent studies the potential of epigenetic inheritance in natural accessions of the model plant *Arabidopsis* has been investigated (Dubin et al. 2015; Hagmann et al. 2015). However, many basic questions have remained unclear: For how many generations are stress-induced epigenetic changes inherited? Is stress-induced epigenetic inheritance a common phenomenon? Is it relevant in natural environments? And to what extent does heritable epigenetic variation in natural populations play a role in selection and adaptation, adding adaptive dynamics that cannot be explained by genetic variation alone?

Epigenetic inheritance in apomictic dandelions – insights from this thesis

This thesis builds on previous findings in apomictic dandelions (*Taraxacum officinale* Wig.) that showed stress-induced DNA methylation changes persisting into offspring of biotically and abiotically stressed apomictic dandelions (Verhoeven et al. 2010b). This perennial plant species consists of apomictic lineages, which reproduce asexually through clonal seeds. Apomictic offspring are, due to the asexual reproductive mode, genetically uniform and apomictic lineages thus present suitable systems to investigate epigenetic effects that are not confounded with genetic variation. Furthermore, it is shown in plants that environmental stimuli can induce methylation changes within a plant's lifecycle. Because a plant's reproductive cell lineage is derived from somatic tissue late in development, induced epigenetic changes that occur during a plant's lifecycle can be transmitted to its progeny without having the embryo or the germline directly exposed to the initial stress signal.

Apomictic dandelion lineages colonized successfully vast areas in Northern Europe following the last retreat of the land ice. Many different apomictic dandelion lineages exist, and natural dandelion populations typically show a high level of clonal diversity (van der Hulst et al. 2001; chaper 5 this thesis). This suggests that adaptation by selection at the population-level is presumably based mostly on apomictic lineage sorting. Within an individual lineage, the classical way of adaptation by selection is assumed to be severely constrained due to their limited genetic variation. However, within-lineage epigenetic variation that arose through stress-induced or spontaneous epigenetic effects could at least partly compensate this limited adaptive potential. For instance, within-lineage epigenetic variation might facilitate the adaptation of apomictic lineages to changing environments, either through short-term effects or through long-term effects (by selection on stable epigenetic variants, which can modify the dynamics of genetic adaptation).

In this PhD thesis I specifically investigated DNA methylation and small RNAs in multi-generation experiments using field-collected material from dandelion populations. For plant species without available reference genome, such as dandelions, methylation sensitive AFLPs (MS-AFLPs) are used to screen DNA methylation variation. When studying induction-based transgenerational epigenetic effects, it has been suggested to

evaluate transgenerational effects in the third unexposed offspring generation for mammal species and in the second offspring generation for plant species, to rule out direct environment induction. This is because in mammals, if a pregnant female is exposed, its embryo and also the germ line within the embryo are exposed as well. A direct environmental induction could thus be visible for two offspring generations in mammals. But in plants, since they set apart their germline only late in development, the second offspring generation would not be directly exposed to the stress (Paszkowski and Grossniklaus 2011).

I carried out four experiments that addressed transgenerational epigenetic inheritance in dandelion. In a follow-up multi-generation experiment of Verhoeven et al. (2010b) I screened DNA methylations and sRNAs in several apomictic dandelion lineages which were exposed in their grandparental generation to drought stress and salicylic acid (SA) treatment, which mimics plant responses to pathogen attack. To test whether induction-based epigenetic inheritance would be detectable also under natural conditions I used a reciprocal field transplantation approach. I tested whether exposing dandelions to field versus greenhouse conditions triggers heritable epigenetic variation. And more specifically, I investigated whether the translocation of apomictic dandelions into a non-native, presumably more stressful, but natural, habitat induces heritable epigenetic variation. Finally, across a north-south transect in Europe the genetic and epigenetic structure in natural dandelion populations was screened. Since epigenetic variation could be, to some extent, unlinked from genetic variation I specifically tested if DNA methylation variation along the geographic transect simply mirrors genetic variation, or if it shows a deviating pattern. A deviating pattern indicates an uncoupling of heritable epigenetic variation from underlying genetic variation, suggesting potential for a unique contribution of epigenetics to population differentiation.

Overall, the four experiments in this thesis revealed evidence for induction-based transgenerational epigenetic inheritance in apomictic dandelions – but the results also showed that such effects are not ubiquitous. Chapters 2 and 3 revealed evidence for stress-induced epigenetic effects (DNA methylation and sRNA changes) that were inherited across generations within apomictic dandelion lineages. However, the heritable DNA methylation effects were not present in all experimental tests, they differed between drought stress and salicylic acid stress (which mimics pathogen attack), and between different apomictic lineages. Also, transgenerational effects were sometimes observed in absence of a detectable effect in the exposed generation itself. Nevertheless, some stress-related epigenetic effects were detected in the second offspring generation that grew under stress-free conditions and in these cases I can rule out direct environmental induction of the developing embryos (or their germ lines). Chapter 4 revealed no evidence for transgenerational epigenetic effects induced by natural field environments, suggesting that heritable modifications may be generally limited under natural conditions. Finally Chapter 5 showed a regional pattern in epigenetic differentiation in the standing variation of heritable DNA methylations in natural populations of apomictic dandelions across Europe. Furthermore, although a large part of the heritable DNA methylation differentiation was correlated with underlying genetic differentiation, a fraction of

heritable DNA methylation variation was detected that is independent, thus possibly autonomous from genetic differentiation. This suggests the potential for a unique contribution of epigenetics to population differentiation.

Context-dependency in transgenerational DNA methylation effects upon severe drought stress and salicylic acid treatment

In the multi-generation stress experiment the drought stress and salicylic acid (SA) treatment, which mimics pathogen attack, was applied during the plants' vegetative state in the first generation. Both stress treatments indicated a strong lineage and stress dependency of the epigenetic response. A weak drought response may be present, but not equally expressed in the tested lineages and the stress signal was lost again in the second offspring generation. SA treatment revealed a stress-related increased rate of DNA methylation changes in the two offspring generations, but no stress signal was found in the stressed generation itself. Furthermore, I discovered for both treatment and control groups also a stress-unrelated increase in DNA methylation variation in the offspring. Despite the context-dependency, the discovery of SA-specific DNA methylation changes in two unstressed generations provides evidence for the existence of induction-based epigenetic inheritance, not confounded by direct stress induction to the embryo or the germline.

In contrast to previous studies on effects of SA on apomictic dandelion (Verhoeven et al. 2010b) and *Arabidopsis thaliana* (Dowen et al. 2012), and although an increased rate of DNA methylation changes was observed in offspring of SA-exposed plants, my multi-generation experiment did not detect clear stress-induced methylation changes in the SA-exposed plants themselves. It is not clear what explains this difference in results. The genotypes used in my research might differ in the sensitivity to environmental stress or in the way how induced epigenetic modifications are inherited. The dandelion genotype used in the previous study (Verhoeven et al 2010b) was a synthetic apomict derived experimentally by crossing a sexual mother (diploid) with diploid pollen from an apomictic father and therefore underwent very recent polyploidization. Such genomic events can be associated with DNA methylation reprogramming and TE release which might affect responses to environmental stresses (Salmon et al. 2005; Verhoeven et al. 2010a). Additionally, the low-resolution technique (MS-AFLPs) might have failed to detect many of the stress-induced DNA methylation changes in the lineages I used. The observed lack of a detectable response in the SA-exposed generation might also derive from a different underlying mechanism causing a "delayed" effect of SA stress. Although the underlying mechanism is not established yet, one possible explanation for a delayed stress response could be that there are stress-induced changes in transposable element (TE) silencing. Such changes in TE silencing can in turn increase further transpositions in subsequent generations where TE-associated methylation changes would then increase in order to re-silence the TEs (Johannes et al 2009).

Transgenerational effects in sRNA induced by grandparental drought and salicylic acid treatment

Besides DNA methylations also sRNAs have the potential to be transmitted between generations, when maternal or paternal sRNAs migrate to germ cells or to the embryo (Slotkin et al. 2009). However, the role of sRNAs in transgenerational stress responses is unclear. Indications for stress-induced and heritable sRNA changes were found in direct progeny of heat-stressed *Brassica rapa* (Bilichak et al. 2015). Additionally, sRNA biogenesis mutants in *Arabidopsis* revealed an interference of the otherwise successful transgenerational herbivory resistance (Rasmann 2012). In chapter 3, sRNAs were screened in the second unexposed offspring generation after stress exposure which revealed sRNAs associated with the grandparental stress treatment. The grandparental stress effect was reflected as shifts in the relative abundances of 21nt and 24nt sRNAs. In addition, shifts in sRNA populations were not random but showed a functional signal and were associated with gene functions related to the grandparental stress. The 21nt and 24nt sRNAs, which are the sRNAs length-classes with known functions in gene and TE regulation, tended to show a reduction in the offspring of stressed plants compared to offspring of control plants. The sRNA screening in the study of Bilichak and coworkers (2015) revealed a similar pattern of sRNA reduction in offspring of heat stressed *Brassica rapa*, which was associated with an upregulation of stress-related genes. Hence, the findings of the *Arabidopsis*, the *Brassica* and my *Taraxacum* studies suggest the importance of sRNA signaling for expressing the transgenerational stress responses in plants.

Absence of heritable DNA methylation effects upon transplantation into different natural habitats

Studies on DNA methylation in natural plant populations have revealed clear environmentally induced DNA methylation variation (Herrera & Bazaga 2011; Richards et al. 2012; Foust et al. 2016). However, little is known about heritability of such epigenetic effects under natural conditions. Therefore, using a reciprocal transplantation experiment (chapter 4), I investigated whether exposing dandelions to natural field stresses triggers heritable DNA methylation changes. However, no DNA methylation differences were detected between offspring of greenhouse and field grown plants. The transplantation into native and non-native field sites did reveal evidence for adaptive divergence between dandelion populations, suggesting that non-native field site are experience as more stressful. But when comparing the offspring of transplants grown at their native and at non-native growth site I discovered only clear DNA methylation clusters by lineage and not by environment. Thus, I suggest that the natural stresses experienced under field conditions might have been a multitude of mild stresses not causing detectable DNA methylations that persist into offspring generations. A single severe stress in an otherwise controlled and stable environment, such as in the greenhouse experiments in the chapters 2 and 3, might more easily trigger detectable DNA

methylation changes. In addition, it might be that the genotypes used in the transplantation experiment were not susceptible to the transplantation stress.

To interpret these findings one has to consider that MS-AFLP-based screenings of DNA methylation variation is quite a rough method and it is possible that relevant DNA methylation changes occurred that went undetected by the method. However, other studies have detected induction-based epigenetic inheritance in plants and in apomictic dandelions using MS-AFLPs. Therefore, the fact that no environmental signal was found with the MS-AFLP screening in chapter 4 is at odds with previous studies on apomictic dandelions. Overall, the results of the reciprocal transplantation experiment does not support that transgenerational induction-based epigenetics is a common phenomenon in natural populations.

Epigenetic differentiation in natural apomictic dandelions populations

The heritability, i.e. proportion of genetically-based phenotype variance in population, cannot be fully explained by whole genome sequencing (referred to as "missing heritability" see Hindorf et al. 2009). Heritable epigenetic variation has been suggested as a possible explanation for this missing heritability (McCarthy & Hirschhorn 2008; Manolio et al. 2009). Heritable epigenetic variation can be induction-based, but also spontaneous epigenetic variation could be transgenerationally stable. However, one study showed that epigenetic effects accumulating across generations are not stable enough to be relevant for selection (Becker et al. 2011), while another study concluded that selection-based epigenetic inheritance can result in mutations that are transient enough to be uncoupled from underlying genetics but stable enough to show a strong response to selection (van der Graaf et al. 2015). I expect when selection acts on induced and/or spontaneously generated epigenetic variation that, over time, epigenetic population differentiation can be detected. Evidence for natural epigenetic differentiation exists, for instance, for the sexually reproducing *Voila cazorlensis* (Herrera and Bazaga 2011). Indications for epigenetic differentiation were also found in several studies on asexually reproducing plants (Richards et al. 2012; Gao et al. 2010; Lira-Medeiros et al. 2010). Asexual plant lineages, due to their limited potential for genetic adaptation, might particularly benefit from additional epigenetic variation for selection to act on.

To test whether there are indications for a unique role of heritable epigenetics in natural populations of apomictic dandelions I screened heritable DNA methylations along a north-south population transect in Europe. This transect follows a historical range expansion, since apomictic dandelion lineages colonized successfully vast areas in Northern Europe following the last retreat of the land ice. Thus dandelion populations may have had to adapt to different ecological and climatological conditions while migrating from the south to the north. In an *Arabidopsis* study on natural DNA methylation variation it was recently suggested that DNA methylation variation is mostly determined by, and not autonomous from, underlying genetic variation (Dubin et al. 2015). Hence, the question arose whether epigenetic differentiation just mimics genetic differentiation or if there is also a unique contribution of DNA methylation to population differentiation that is autonomous from underlying genetic variation. Therefore, I

specifically tested whether patterns of heritable epigenetic variation along the transect deviate from patterns of genetic variation. Any epigenetic differentiation that does not simply reflect genetic variation, whether they arose by induction-based or selection-based effects, could indicate an autonomous role of epigenetic inheritance in population differentiation. The MS-AFLP screening along the north-south transect revealed heritable DNA methylation variation that is associated with the plants' geographical region of origin. I found that much, but not all heritable epi-variation was predicted by underlying genetics. This fraction of heritable epigenetic variation that deviated from genetic variation indicates a potential role of autonomous epigenetic inheritance that could contribute to population differentiation.

Possible mechanism behind epigenetic inheritance across generations

Many studies including my thesis research revealed only the consequences of the transmission of epigenetic information (that is, an epigenetically modified genome) but did not necessarily identify the actual epigenetic signal that carried between generations. It is not clear how exactly epigenetic information is transmitted across generations, but possible epigenetic mechanisms that have been demonstrated to travel between generations are DNA methylations and sRNAs. Most plant studies have shown transgenerational epigenetic changes in DNA methylation, but only few studies (including my work in chapter 3) have looked at transgenerational sRNA effects. Since I found in chapter 3 that sRNA signal was clearly affected by the grandparental stress treatment I propose that future studies should also consider migrating and stress-induced sRNAs as the carrier between the parental generation to the germline or the embryo.

Migration of sRNAs into the gametes or the embryo can affect transgenerational gene regulation possibly through reinforcing TE-silencing by guiding DNA methylations to the TEs (Ibarra et al. 2012; Slotkin et al. 2009). Additionally, sRNA-based gene regulation could be propagated across generations via inherited DNA methylation signals through a feed-forward interaction between sRNAs and DNA methylation via the RdDM (RNA dependent DNA methylation) pathway (Bond and Baulcombe 2014). In *Arabidopsis* sRNA involvement is suggested in transgenerational resistance against herbivores because herbivory resistance is compromised in sRNA biogenesis mutants (Rasmann 2012). And heat-stressed *Brassica* shows persisting sRNA effects from the leaf tissue from the stressed plant to the gametes and finally to the leaves of the offspring (Bilichak 2015). The sRNA screening in chapter 3 suggests as well that sRNAs play a relevant role in transgenerational stress effects but further detailed studies are necessary to unravel if sRNAs are only the consequences of epigenetic inheritance or also the epigenetic carrier of the environmental signal across generations.

With respect to transgenerational stability of DNA methylation changes as carrier of the environmental signal across generations, plants represent good systems to study epigenetic inheritance. This is because DNA methylation resetting, which usually takes place during ontogenesis, is incomplete in plants (Feng et al. 2010). In apomictic dandelions the embryo develops solely from cells in maternal ovule tissues (parthenogenesis), thus the embryo and endosperm develop without fertilization

(Koltunow 1993). It could be that DNA methylations are more easily transmitted to the next generation in apomicts, because during apomictic reproduction normal meiosis is circumvented. However, further studies are needed to investigate how and which DNA methylations are reset in apomicts, and if this differs from resetting in sexually reproducing dandelions.

Methods to detect cytosine methylation and their limitations

MS-AFLP is the method I used throughout my thesis research, which is an AFLP based method that detects cytosine methylation polymorphisms at restriction sites using methylation-sensitive restriction enzymes (*Msp*I, *Hpa*II). When the methylome screening is applied to populations consisting of several genotypes (as the population screening of chapter 5) a combination of both enzymes can be used to distinguish methylation variation from genetic variation. Double absent fragments from these two restriction enzymes (type IV) should be ignored, because they represent an uninformative condition, which indicates either a hyper-methylated state or an absence of the restriction site due to genetic polymorphism. The MS-AFLP approach is especially used in non-model systems since no a priori genome knowledge is necessary. Patterns of DNA methylation variation of a wide range of different species were already successfully described by MS-AFLPs (e.g. Cervera et al. 2002; Salmon et al. 2008; Herrera & Bazaga 2011; Massicotte et al. 2011). However, there are technical limitations of MS-AFLPs, including a relatively low numbers of loci and the lack of information about sequence context, identity and function of the detected loci (Becker et al. 2011; Schrey et al. 2013). A more in-depth screening of the methylome is possible for instance through whole genome bisulfite sequencing (Becker et al. 2011) or through RRBS, reduced representation bisulfite sequencing (Meissner et al. 2005). These methods use bisulfite treatment to convert unmethylated cytosine residues to uracil, providing a basis for recognition of methylated cytosines upon subsequent sequencing. However, these and other sequencing-based methods have not been cost effective for experiments using large sample sizes that are typical for ecological population studies, and have also limited application when no reference genome is available. Promising new approaches are recently published based on RADseq and on Genotyping by Sequencing, which makes RRBS cost effective for large sample sizes as well as for species without a priori knowledge of the genome (Van Gurp et al. 2016; Trucchi et al. 2016). Furthermore, the *Taraxacum* genome is currently being sequenced which will make WGBS, whole-genome bisulfite sequencing, available. For future epigenetic studies it would be interesting to compare MS-AFLP with bisulfite-sequencing approaches within the same experiment, because a full methylome screening with single-nucleotide resolution would reveal how accurate and representative the previous MS-AFLP studies were.

Potential applications for epigenetic inheritance in plants

Previous studies on asexually reproducing plants revealed examples of inherited phenotypes as well as gene expression and epigenetic profiles (Preite & Veroeven 2014). These findings are based mostly on vegetative cloning. My thesis research extends these

previous indications by investigating asexual reproduction via seed formation (apomixis). Since most crop plants are reproducing via seeds the epigenetic research on apomictic plants could be very useful for future applications. However, it remains to be demonstrated how similar or different epigenetic resetting between generations is in apomixis and sexual reproduction. Studies with apomictic plants, in this respect, combine the beneficial aspects of seed production and clonality resulting in a suitable model system to study epigenetic inheritance that is not confounded by genetic variation. Possible applications are for instance the production of cultivars for agriculture and horticulture with desired phenotypes that could not be created by classical genetic breeding alone (Springer 2013). In my thesis I could, however, not detect many stress-induced DNA methylation modifications and it is not clear whether the effects would persist across more than two unstressed generations. However, also transient stress-induced epigenetic modification, as found in previous plant studies including this thesis, can have beneficial consequences in the offspring. Upon stress exposure a molecular stress memory, such as heritable stress-induced DNA methylation changes, could for instance result in a faster and more robust activation of stress defense responses, which is also referred to as priming (Luna et al. 2012; Rasmann 2012; van Hulten et al. 2006; Ahmad et al. 2010). Also stress-inducing plant hormones (JA and BABA) as well as beneficial microbes from the rhizosphere can be used for targeted induction of systemic immunity to aboveground pathogens (Conrath et al. 2015).

The current climate change has wide-ranging impacts, particularly on agriculture and therefore on food safety of crops and livestock. In my opinion, one plausible way to adjust to the challenges of climate change is to critically evaluate conventional agriculture and start implementing sustainable ideas. Most crops to date have been bred for conventional farming, however, the demand for organic farming is currently increasing and new breeding projects have started to provide ‘organic’ crop seeds that are tailored for organic farming. It could be useful to investigate the efficiency of transgenerational priming for stress resistance in such organic crop seed production. For instance, priming crop plants for enhanced disease resistance might contribute to a more sustainable approach for agriculture, reducing the use of pesticides to maintain crop yields.

Conclusions and future perspectives

My PhD research provides new insights into the role of epigenetic inheritance in plant adaptation: Stress can trigger epigenetic changes in the treated generation and, furthermore, these epigenetic changes can persist across more than one offspring generation. Induced epigenetic inheritance was found as transgenerational DNA methylation and sRNA effects. Conclusions that I want to highlight are:

- Small RNAs provided stronger evidence for stress-induced epigenetic changes than the DNA methylation screening, leaving it unclear how relevant DNA methylations are in transgenerational epigenetic effects.

- Overall, the DNA methylation screenings based on MS-AFLPs revealed no ubiquitous evidence for stress-induced epigenetic inheritance. The greenhouse experiments revealed stress- and genotype-dependent treatment-specific epigenetic effects in the stressed plants and in the offspring generation. In the field experiment lineage-specific, but no stress-specific epigenetic patterns were found. It could be that the stress signal needs to be severe and consistent enough to induce DNA methylation changes that are detectable with MS-AFLPs.

- DNA methylation patterns in apomictic dandelions showed clear lineage-specific clusters, indicating the strong relatedness of epigenetic variation with genetic variation.

- In natural apomictic dandelion populations heritable epigenetic differentiation was found that is partly statistically independent, thus possibly autonomous from genetic differentiation. I conclude that epigenetic mechanisms can contribute to population differentiation in a way that is not completely predicted by underlying genetics.

With respect to DNA methylations, the detected context-dependency leaves it unclear whether induction-based inheritance of DNA methylation changes is important under natural conditions. On the other hand, in a clear transgenerational stress response was detected as sRNA changes. I could not establish unambiguously whether induction-based epigenetic inheritance would result in adaptive phenotypic variation in nature and whether selection-based epigenetic inheritance can persist long enough to play a relevant role in adaptation. One can envision that epigenetic variation plays a more facilitating and short-term role by fine-tuning the plant´s phenotype to environmental changes and, in the longer term, by affecting the rate and direction of genetic adaptation (Pal and Miklos 1999; Klironomos et al. 2013). Also heritable epigenetic effects that are stable over thousands of generations could be subjected to selection and play a relevant role in plant adaptation to changing environmental conditions (Kronholm & Collins 2015).

Both induction-based and selection-based epigenetic effects could be linked mechanistically and/or statistically with underlying genetics. However, the heritable epigenetic differentiation found in natural populations of apomictic dandelions showed a fraction that is statistically independent, thus possibly autonomous from genetic differentiation. These new insights offer intriguing contributions to the classical perception of evolution by suggesting that evolution must not be fully blind to environmental changes and that epigenetics could generate additional relevant variation for selection to act on. To further understand stress-induced epigenetic inheritance in plants, different types, combinations and severity of stresses should be tested. A long-term field study with artificially modified conditions (e.g. open top chambers) would bring insight in whether a severe environmental change on otherwise natural conditions could cause transgenerational and epigenetically mediated stress responses. In order to further tackle the generality of epigenetic inheritance, I suggest comparing different plant taxa, for example long-lived vs short-lived, annual vs perennial, and sexual vs asexual species. Especially non-model and non-crop species should be considered in order to increase insight in the ecological and evolutionary role of epigenetic inheritance of stress responses in nature. For non-model species new sequencing-based methods, combined with bisulfite treatments, can improve the detection level at which DNA methylations can be analyzed, and it can be used to pinpoint the actual carriers of epigenetic information that are transmitted between generations.

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Summary

Species have to adapt to changing environments, which has been explained so far by Neo-Darwinism theory that an evolutionary process is based on natural selection acting on heritable genetic variation. However, in a period in which complete genomes of multiple model organisms are known we still have limited understanding of how the information encoded in their genomes is regulated or interpreted. Recent studies revealed that heritable phenotypic effects need not to be based on DNA sequence variation alone. Epigenetic mechanisms, even in the absence of genetic variability, can regulate gene expression which sometimes results in heritable phenotypic variation. Moreover, it has been shown under experimental lab conditions that epigenetic effects can arise through environmental stress induction and some of such stress-induced epigenetic effects were heritable across stress-free generations. Even if only transient, such induction-based epigenetic inheritance might play a facilitating role in the persistence of populations, because environmentally sensitive adjustments might generate adaptive phenotypic variation under fast changing conditions. In addition to induction-based epigenetic inheritance also spontaneously generated epigenetic variation can accumulate across generations and could in principle be subjected to selection. Significant progress in the understanding of induction- and selection-based epigenetic inheritance is, however, based on laboratory strains and these findings should thus be taken as indications of the potential importance of epigenetic mechanisms in nature. Especially the relevance of epigenetic inheritance under natural field conditions is so far largely unexplored. It remained unclear, for instance, how general stress-induced epigenetic inheritance is, whether it is relevant in natural environments, and to what extent heritable epigenetic variation in natural populations can play a role in selection and adaptation that is independent from genetic variation.

To test the role of epigenetic inheritance under natural conditions, I used apomictic dandelion (*Taraxacum officinale* Wig.) as a model system. This perennial plant species consists of apomictic lineages, which reproduce asexually through clonal seeds. Their offspring are genetically uniform due to their asexual reproductive mode and therefore suitable to investigate epigenetic effects that are not confounded with genetic variation. In general plants are convenient systems to study DNA methylation and epigenetic inheritance, because they have incomplete DNA methylation resetting during embryogenesis, allowing part of the methylation variation to be transmitted between generations. In my PhD thesis study, I performed four experiments on apomictic dandelions that aim to unravel environmentally induced and heritable epigenetic effects in this system. Specifically, I investigated DNA methylation and small RNAs in multi-generation experiments using field-collected material from dandelion populations. For plant species without available reference genome, such as dandelions, methylation sensitive AFLPs (MS-AFLPs) are used to screen DNA methylation variation. My PhD research builds on previous findings in apomictic dandelions that showed induction-based (induced through biotic and abiotic stresses) DNA methylation modifications that can be

transmitted faithfully to their offspring, even though the offspring did not directly encounter the initial stress signal.

Chapter 2 is a direct follow-up multi-generation stress experiment that also revealed effects of induction-based DNA methylation changes upon drought and salicylic acid (SA) stress (which mimics pathogen attack). However, the DNA methylation stress effects were not present in all tests and were stress- and lineage-dependent. Drought treatment revealed induction-based DNA methylation change in some lineages that was not inherited, while SA treatment revealed DNA stress-related methylation effects in the two offspring generations, but not in the stressed generation itself. While the underlying causes of variation in the expression of transgenerational DNA methylation effects are not clear, this experiment confirms that environmental stresses can have heritable DNA methylation consequences, at least under the conditions in our study. In addition to specific stress responses this experiment revealed also an increase in undirected DNA methylation variation in the offspring from the control and even stronger from the stressed plants, which suggests that environmental changes can induce random DNA methylation changes. Such random DNA methylation changes could promote heritable differentiation in methylation profiles between experimental groups.

Besides DNA methylations, also sRNAs have the potential to be transmitted between generations by loading maternal sRNAs into the germ line or the embryo. However, the role of sRNAs in transgenerational stress responses has remained unclear. A sRNA screening revealed changes in the production of sRNAs due to the drought and SA stress experienced two generations ago. Furthermore, due to the grandparental stress exposure specific stress-related sRNA showed signature of differentiated abundances and these sRNAs were associated with a drought- and SA-related gene function. The stress-related sRNA effects I detected in the third generation are the consequence of a transgeneration-transmitted epigenetic signal, but it remains to be demonstrated what the actual carrier of the signal is: the signal could be an inherited DNA methylation modification, sRNAs, or some other unknown mechanism. Results of chapters 2 and 3 combined indicate the potential of DNA methylations and sRNAs responding to environmental changes and these effects can lasts for more than one stress-free generation. Since I applied the stresses during the vegetative state of the plant, where the germline is not set apart yet, I can rule out direct environmental effects on the germ cells or on the developing embryo. Thus, my observations exemplify transgenerational epigenetic inheritance.

Insight into heritable epigenetic effects under natural conditions is very limited. Therefore, using a reciprocal transplantation experiment (chapter 4), I investigated whether exposing dandelions to natural field stresses also triggers heritable DNA methylation changes. The reciprocal field transplant experiment revealed adaptive divergence between dandelion populations, suggesting that non-native habitats are experienced as more stressful. I subsequently tested whether transplanting dandelions into non-native growth site compared to native growth site triggers more pronounced DNA methylation changes. However, no induction-based DNA methylations changes were found in these field environments that persisted into offspring. I hypothesize that for

heritable DNA methylation changes to be induced a severe or consistent enough stress signal is required. The natural stresses experienced under field conditions might have been a multitude of different and mild stresses not causing detectable DNA methylations. This finding suggests that induction-based epigenetic inheritance may be generally limited under natural conditions. However, MS-AFLP-based screenings of DNA methylation variation is quite a crude method and it is possible that relevant DNA methylation changes occurred that went undetected by the method.

In studies on natural DNA methylation variation it has been suggested that DNA methylation variation is mostly determined by, and not autonomous from, underlying genetic variation. In dandelions, at the population level, adaptation by selection is presumably mostly based on apomictic lineage sorting, as dandelion populations tend to consist of many different apomictic lineages. However, epigenetic differentiation within lineages might facilitate the adaptation of individual apomictic lineages to changing environments, either through short-term effects (by epigenetics-mediated environmental plasticity) or through long-term effects (by selection on stable epigenetic variants, which can modify the dynamics of genetic adaptation). In chapter 5 the DNA methylation and genetic profiles of natural apomictic dandelion populations were screened across a south-north transect in Europe, reflecting a transect of historical range expansion following the retreat of the land ice after the last ice age. I compared standing genetic and heritable DNA methylation variation, and asked to what extent patterns of MS-AFLP variation along the transect follow patterns of AFLP variation. Any deviations from underlying genetic variation, whether it is caused by induction-based or selection-based epigenetic effects, would suggest a unique contribution of epigenetic inheritance in population differentiation along the transect. The screenings revealed that a large part of the heritable DNA methylation differentiation was correlated with genetic differentiation. However, a fraction of heritable differentiation in DNA methylation along the transect was independent of genetic differentiation. This suggests that, besides genetic effects, also epigenetics plays a unique role in evolution.

Most studies on stress-induced epigenetic inheritance, my thesis research included, detect transgenerational epigenetic effects that are a consequence of the transmission of epigenetic information across generations. However, it is not fully established what the actual epigenetic carrier is that transmits epigenetic information from one generation to the other. One possibility for epigenetic information to persist across generations is that DNA methylations are not reset and thus persist from the somatic gametophyte to the developing embryo. The sRNA screening in chapter 3 suggests that regulatory sRNAs could also be important in epigenetic inheritance across generations. For instance, it is known that sRNAs can migrate from parental cells to germ cells or to the developing embryo. There are only limited indications from plant studies to support the suggested mechanisms, so that further detailed studies are needed to investigate how epigenetic inheritance is achieved between generations. Currently, most plant studies on transgenerational effects focus on DNA methylation only, but I propose that further studies also consider the role of sRNAs in more detail.

Finally, in chapter 6 I discuss the findings presented in this thesis and suggest future research directions. Recent approaches using sequencing-based techniques and bisulfite treatment, which converts unmethylated cytosine residues to uracil, can detect DNA methylation variation in much more detail also in non-model systems. This would replace MS-AFLPs as the method of choice for screening DNA methylation. Additionally, the *Taraxacum* genome is currently being sequenced, making whole-genome bisulfite sequencing possible.

Overall, my results support the hypothesis that epigenetic inheritance exists, arising spontaneously but also due to stress induction. Despite the high correlation between epigenetic variation and genetic variation, my results also revealed some potential of epigenetics to contribute to population differentiation independently from genetics. On the other hand, my results do not provide support for that induction-based epigenetic inheritance is a very common phenomenon under natural conditions. Whether epigenetic variation would result in adaptive phenotypic variation in nature and whether it would persist long enough to play a relevant role in adaptation requires further studies. Nevertheless, my findings on stress-induced epigenetic inheritance show that heritable variation can be triggered by environmental experiences. This challenges the classical perceptions of phenotypic plasticity and adaptation, as the environment not only selects from heritable variation but also generates heritable variation.

Acknowledgements

During my PhD in Wageningen I met so many new people, many had an important impact on me and I found many new friends. When I now think back at that time, it is them that I think about, not really the work or the knowledge I gained, it is the people I met and all the beautiful moments we shared. So, this "Thank you!" is for all of you.

I want to thank the gentlemen that supervised my PhD. First I want to thank Koen, who was my daily supervisor and Wim, my promotor. I got already a great feeling on our first skype talk. Then on my first visit in Wageningen, after I saw the newly built Institute and got a glimpse of the staff working there, I had a beer at the Vlaamse Roes with Koen and my PhD colleague Thomas in the evening. That made my decision easy to start this journey with this group. I had a very good feeling about it (maybe it was the calming effect of the beer though) but I was also very excited and a bit anxious, this was the first time going abroad. But I met such a great and supporting team that there was nothing to worry about and I am very grateful for all the things I learned from them. Dear Koen, alias Dr. Dandelion, you kept me going, always challenging the scientific mind and helping me to accomplish the planned experiments. And to Wim, thank you for supporting me during this time and your help in the writing process of this thesis. And also Arjen, thank you for your input on the experimental design and on the results we found. And Georg (my second/backup supervisor from the Botanical Institute in Basel where I did my Master thesis), your comforting words always kept my moods up.

A big thank you goes to my teammates in the dandelion team. Carla, you made the molecular side of the experiments work. Thank you for teaching me new lab skills, helping me with my experiments and (you and Agaat) for all the sporty runs through the forest. Thomas, we had such a fun time during the crazy field collections across Europe and you always had interesting inputs and ideas for my experiments and data analyses. Julie, in our rather small dandelion group you became my female "compagne". Thank you for being there and helping me with bioinformatics questions, but also for just being a compagne to sit in the grass at the sun and chat. I am very happy to have you as one of my paranymphs. Thanks to our students, Rutger for your support and the challenging table tennis matches, and to Martin for your major addition to the field work in the transplantation experiment. Thanks to Philippine and Maartje for visiting us regularly to discuss the latest literature about epigenetics. Thanks to all the students and technicians who were helping me in the field and in the lab. And last but not least thanks to Gregor and Eke, who are making the greenhouses run smoothly.

I feel deeply grateful to all my colleagues. Your support started with making me feel home, giving me advice in matters of life and science, backing me up, giving me endurance and strengthen my self-confidence. I want to thank my PhD fellows at the Terrestrial Ecology for support and keeping each other motivated: Sabrina, Taia, Roeland, Stijn, Mingang, Moniek, Jasper, Minghui, Marta, Viola and Sigrid. I thank the rest of the department, Tanja, Freddy, Slavica, Carolin, Roel, Ciska V and Ciska R, Martine, Olga, Elly, Kadri, Stefan, Basten, Joke, Kelly, Gerard, Jeff, Pella, Gera, Martijn and Louise for creating such an inspiring atmosphere. To Elly, Gerrie and Gerda thanks for being such

helpful and lovely soles for all my administration questions I had. Especially, Elly, thanks for the Swiss German chats. Furthermore, I enjoyed a lot being a part of the Party Planners organizing NIOO events, writing for the NIOOscoop and leading the Christmas Choir. Thanks to everybody taking part in these groups. Thanks to Paolo, Ruth, Maaike, Sven, Jan, Michiel, Lysanne, Kristin and Thomas L for sharing many fun moments.

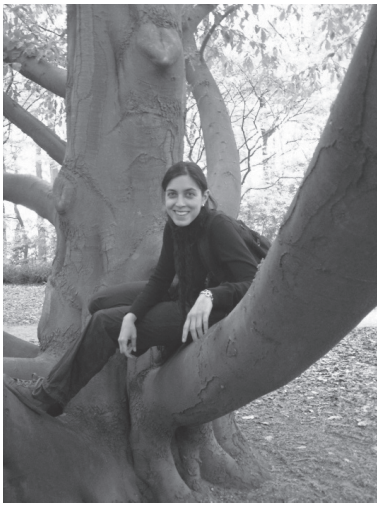
My journey in Wageningen would not have been the same without Droevendaal. For four years these student houses were my home and my housemates were always there, during my good and my bad moods. Organizing everyday life, having dinner, doing gardening and taking care of the pets together made this intermediate home a lovely little place to be. Thank you my Droef-brothers and sisters Noemi, Tobias, Eva, Evelien, Roeland, Jelle, Johan, Merel, Lukas, Michael, JD, Lalo, Giulia, Monica and Thibault for all the laughter, discussions about random stuff, problems and also sad moments we shared.

It is said that friends are like training wheels of bicycle, they are always there to prevent you from falling down. I want to thank these amazing women for their friendship, even when many kilometers lay in between us. Pia and Angela are there with me since primary school and Maria (my Metal-mate) and Rahel are good friends since High school. Then, at the very first day at my biology study, I met Janine. Ever since we kept contact and shared many fun moments, talked and wondered about just everything and traveled together. I am happy that you found your way to the Netherlands too, so I always have a good reason to come back and visit you. When I arrived in Wageningen there was another new PhD at the NIOO. We spent the first week in an abandoned and spooky, but lovely guesthouse in Heteren, cooking Italian food and discovering the area by bike. Then we both found a place in Droevendaal. Dear Lucia, my Droef-neighbor and PhD-mate, thank you for being a precious friend and supporting me at the defense as my paranymph.

My mom always said that you can choose your friends but not your family. Though, I think I was pretty lucky with my family ☺. Liebe Preites und Willis, ein grosses Dankeschön geht an euch. Meinen Entscheid meiner Reise und Abenteuerlust nachzugehen hat mich ein paar Kilometer von euch entfernt. Und trotzdem habt ihr mich immer unterstützt und mich meinen Weg gehen lassen. Grazie per tutte le telefonate con Skype, le vostre visite nei Paesi Bassi, il vostro supporto e la vostra fiducia in me.

Above all people there is one that was most difficult to leave back in Switzerland and I have no words for the luck that I have him at my side. Eleven years it is now and I look very much forward to the years ahead, to keep on going through thick and thin together. Dennis, Schatz, thank you for all the hours of traveling you took to visit me, for your patience with me and for helping and motivating me, especially during the last stretch of accomplishing my PhD.

Curriculum vitae



Veronica Preite was born on the 9th of November 1984 in Grabs, Switzerland. She grew up in the Rhine Valley where she also went to High School. At the University of Basel she studied biology and accomplished her Master's degree in 2010. During her studies she specialized in plant ecology and population genetics. For her Master thesis, supervised by Prof. Jürg Stöcklin and Dr. J. (Niek) Scheepens, she investigated the adaptation of a wide spread herb to its local environmental condition. Next to her studies she also gained work experience at the Institute of Organic Farming (FiBl, Switzerland) and the Institute of Phytopharmaceutical Products (VitaPlant Switzerland). In April 2011 she started as a PhD candidate at the Netherlands Institute of Ecology (NIOO-KNAW) supervised by Dr. Koen Verhoeven and Prof. Wim van der Putten. She investigated heritable and stress induced epigenetic effects using apomictic dandelions and her findings are presented in this thesis. Since summer 2015 Veronica works in the Plant Physiology group at the Ruhr-University of Bochum, Germany, on the metal-hyperaccumulator *Arabidopsis halleri* and its adaptation to metalliferous soils.

Peer reviewed publications

Foust CM, **Preite V**, Schrey AW, Alvarez M, Robertson MH, Verhoeven KJF, Richards CL (2016) Genetic and epigenetic differences associated with environmental gradients in replicate populations of two salt marsh perennials. *Molecular Ecology*, 25(8), 1639-1652, doi: 10.1111/mec.13522.

Huber M, Epping J, Schulze Gronover C, Fricke J, Aziz Z, Brillatz T, Swyers M, Köllner TG, Vogel H, Hammerbacher A, Triebwasser-Freese D, Robert CA, Verhoeven KJF, **Preite V,** Gershenzon J, Erb M (2016) A latex metabolite benefits plant fitness under root herbivore attack. *PloS Biolog*, 14(1), e1002332.

Preite V, Snoek LB, Oplaat C, Biere A, van der Putten WH, Verhoeven KJF (2015) The epigenetic footprint of poleward range-expanding plants in apomictic dandelions. *Molecular Ecology*, 24(17), 4406-4418.

Preite V, Stöcklin J, Armbruster GFJ, Scheepens JF (2015) Adaptation of flowering phenology and fitness-related traits across environmental gradients in the widespread *Campanula rotundifolia*. *Evolutionary Ecology*, 29(2), 249-267

Verhoeven KJF and **Preite V** (2014) Epigenetic variation in asexually reproducing organisms. *Evolution*, 68(3), 644-655.

Publications in preparation

Preite V, Oplaat C, Biere A, Kirschner J, van der Putten WH, Verhoeven KJF; Transgenerational epigenetic inheritance in two widespread apomictic dandelion lineages.

Preite V, Morgado L, Oplaat C, Anava S, Ferreira de Carvalho J, Rechavi O, Johannes F, Verhoeven KJF; Small RNAs reflect grandparental environment in apomictic dandelions.

Preite V, Glabischnig M, Oplaat C, van der Putten WH, Verhoeven KJF; Testing heritable DNA methylation effects upon transplanting apomictic dandelions to native versus non-native field habitats.

Education Statement of the Graduate School

Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: **Veronica Preite**
Date: **7 September 2016**
Group: **Laboratory of Nematology and NIOO department Terrestrial Ecology**
University: **Wageningen University & Research**

1) Start-up phase	*date*
► **First presentation of your project**	
Title: Natural epigenetic variation in apomictic dandelion lineages	Nov 17, 2011
► **Writing a review or book chapter**	
Review: Epigenetic variation in asexually reproducing organisms, EVOLUTION, published: March 2014. DOI: 10.1111/evo.12320	Mar 2014
Subtotal Start-up Phase	*5.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD Student Days**	
EPS PhD Student Days 'Get2Gether 2015', Soest, NL	Jan 29-30, 2015
► **EPS Theme Symposia**	
EPS theme 1 Symposium "Developmental Biology in Plants", Leiden	Jan 08, 2015
EPS theme 3 Symposium "Metabolism and Adaptation", Wageningen	Feb 10, 2015
► **Lunteren days and other National Platforms**	
4rd Ecogenomics Day, Science Parc Amsterdam	Jun 16, 2011
Naem, Netherlands Annual Ecology Meeting 2012, Lunteren	Feb 07-08, 2012
Naem, Netherlands Annual Ecology Meeting 2013, Lunteren	Feb 05-06, 2013
Naem, Netherlands Annual Ecology Meeting 2014, Lunteren	Feb 11-12, 2014
6th Ecogenomics Day, Utrecht University	Jun 24, 2014
Naem, Netherlands Annual Ecology Meeting 2015, Lunteren	Feb 11, 2015
► **Seminars (series), workshops and symposia**	
WEES monthly seminars, Wageningen	2012-2015
► **International symposia and congresses**	
ESF-EMBO Symposium: Epigenetics in Context: From Ecology to Evolution. Sant Feliu de Guixols (Spain)	Sep 18-23, 2011
Plant Epigenetics, Stress and Evolution. CSHA Souzhou Shanghai	Oct 29-Nov 02, 2012
Intecol 2013 British Ecology Society. London	Aug 18-23, 2013

PopBio Meeting 2014. Konstanz	May 29-31, 2014
15th Congress of the European Society for Evolutionary Biology. Lausanne	Aug 09-14, 2015
► **Presentations**	
Poster; ESF-EMBO Symposium Epigenetics in Context: From Ecology to Evolution. Sant Feliu de Guixols (Spain)	Sep 18-23, 2011
Poster; Plant Epigenetics, Stress and Evolution. CSHA Souzhou Shanghai	Oct 29-Nov 02, 2012
Talk; Naem 2014. Lunteren	Feb 11-12, 2014
Talk; PopBio Meeting. Konstanz	May 29-31, 2014
Poster; 15th Congress of the European Society for Evolutionary Biology. Lausanne	Aug 09-14, 2015
► **IAB interview**	
Meeting with a member of the International Advisory Board of EPS	Jan 05, 2015
Subtotal Scientific Exposure	*18.3 credits**
3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Molecular Marker Analysis of plant population structure and processes, University of Copenhagen	Aug 22-26, 2011
Bioinformatics a user approach, WUR Wageningen	Aug 29-Sep 02, 2011
Next Generation Sequencing Workshop, NIOO Wageningen	Sep 05, 2014
► **Journal club**	
PhD Journal Club NIOO (monthly)	2012 - 2013
Epigenetics Journal club (monthly)	2011 - 2014
► **Individual research training**	
Subtotal In-Depth Studies	*6.3 credits**
4) Personal development	*date*
► **Skill training courses**	
Scientific Writing	Oct 18-Dec 06, 2012
Voice and presentation training	Apr 01 & 15, 2014
Presentation Skills	Oct 10, 17, 24, 2014
Subtotal Personal Development	*3.2 credits**
TOTAL NUMBER OF CREDIT POINTS*	**33.3**

* A credit represents a normative study load of 28 hours of study.

Colophon

The research presented in this thesis was conducted at the Department of Terrestrial Ecology at the Netherlands Institute of Ecology (NIOO-KNAW) in Wageningen.
This is NIOO thesis 137.

This research described in this thesis was financially supported by the NWO-ALW grants: NWO-Ecogenomics 884.10.003 and 864.10.008 from the Netherlands Organisation for Scientific Research.

Printed by: GVO drukkers & vormgevers BV (www.gvo.nl)