ECOEPIGENETICS IN CLONAL AND INBREEDING PLANTS: TRANSGENERATIONAL ADAPTATION AND ENVIRONMENTAL VARIATION

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ECOEPIGENETICS IN CLONAL AND INBREEDING PLANTS: TRANSGENERATIONAL ADAPTATION AND ENVIRONMENTAL VARIATION

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"*Trifolium repens* cultivated in the experimental soil" by Vit Latzel, licensed under CC-BY.

Clonality is widespread in plant species, and clonal plants often have a broad geographic range and long lifespan. Clonality can maintain high fitness in the short term, but vegetative reproduction is commonly considered to preclude adaptation to changing conditions. However, an increasing body of empirical and theoretical evidence suggests that epigenetic modifications such as DNA methylation can provide an alternative to gene-driven evolution through natural selection and allow clonal plants to maintain fitness in the long term. To deepen our understanding of clonal ecology, this collection of research papers and reviews focuses on how epigenetic regulation can encode phenotypic plasticity and contribute to the rapid adaptation of clonal plants to accelerating global and regional environmental changes.

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EDITORIAL
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Editorial: Ecoepigenetics in Clonal and Inbreeding Plants: Transgenerational Adaptation and Environmental Variation

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Keywords: clonal growth, environmental change, epigenetics, inbreeding plants, maternal effect, transgenerational plasticity

Editorial on the Research Topic

Ecoepigenetics in Clonal and Inbreeding Plants: Transgenerational Adaptation and Environmental Variation

Accelerating global and regional environmental changes are likely to favor species that can rapidly adapt to new conditions. Long-lived, clonal species whose reproduction is mainly asexual have long been thought to possess a relatively low potential for adaptation. However, the potential for transmitting responses to environmental change between vegetative generations within clones could compensate for lack of natural selection based on sexual reproduction (Latzel and Klimešová, 2010; Douhovnikoff and Dodd, 2015). There are two well-studied mechanisms that underlie transgenerational environmental effects in clonal plants. First, transgenerational environmental effects on clonal (vegetative) offspring may depend on the quality of provisioning, similarly to seeds (Herman and Sultan, 2011; Dong et al., 2018). The relatively large size of clonal offspring may allow for more extensive provisioning with, e.g., carbohydrates or mineral nutrients, thereby obtaining greater fitness. Second, epigenetic changes may encode phenotypic plasticity and allow it to persist between vegetative generations (Dodd and Douhovnikoff, 2016; Richards et al., 2017). Changes such as DNA methylation, chromosome inactivation, and modifications of histones, chromatin, and small non-coding RNAs are now understood to transmit major phenotypic shifts between generations even in the absence of genetically based natural selection. This research topic assembles articles that deal explicitly with the ecological and evolutionary significance of transgenerational environmental effects in clonal plants, and that advance the understanding of the mechanisms of transgenerational effects in clones or inbreeding plants.

Three papers focus on the ecological significance of epigenetic regulation responses for clonal plants to different natural habitats. In a forum paper, Thiebaut et al. proposed how epigenetic regulation such as DNA methylation could cause chromatin dynamics and silencing, and influenced plant phenotypes, contributing to the adaptation of native plants, in the context of environmental variation. Broeck et al. showed the relationship between variability of DNA methylation and bud set phenology of the Lombardy poplar (*Populus nigra* cv. *Italica* Duroi) that is widely introduced in Europe. They suggest that epigenetic-based transgenerational inheritance may be relevant for adaption and evolution of *P. nigra* clones in contrasting or rapidly changing environments. Shi et al. reported that invasive populations of *Alternanthera philoxeroides* in China exhibit extremely low variation in DNA sequence, but high epigenetic diversity. They suggest that epigenetic variation may compensate for

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the loss of genetic variation in this invasive species and thus contribute to their success in novel environments.

Three papers report parental environmental effects on offspring fitness of clonal plants. Dong et al. tested effects of parental soil nutrient environments on offspring performance of the highly invasive, clonal herb *A. philoxeroides* at both the individual ramet level and the level of the whole generation of ramets. They provide novel evidence that the magnitude of parental environmental effects varied at different plant levels, and depended on propagule provisioning. Li et al. examined effects of parental shade environments on growth, morphological and physiological traits of a stoloniferous herb *Centella asiatica*. They found that transgenerational plasticity through both morphological and physiological flexibility was triggered across clonal generations of *C. asiatica* subjected to high/low light treatments, and such effects allowed offspring ramets to present adaptive phenotypes in response to the prevailing light environments. Fan et al. showed that physiological connection with parental ramets of a desert clonal shrub *Calligonum mongolicum* in favorable conditions can alleviate stress on offspring ramets exposed to wind erosion.

Two papers consider the variation in transgenerational environmental effects among genotypes. González et al. examined the generality of transgenerational environmental effects in the clonal plant *Trifolium repens* with five genotypes and five types of parental environments. They found that transgenerational environmental effects were highly genotype-specific and common in some genotypes, and potentially under epigenetic control. Baker et al. set up two glasshouse shade environments for an inbreeding plant *Polygonum persicaria*, and measured ecological important traits of their isogenic offspring in both environments. They found that the adaptive effects of parental shading were pronounced and highly significant for seedlings growing under shade, and such effects were mediated by DNA methylation status of parent plants, rather than changes to propagule provisioning.

Transgenerational environmental effects in sexually reproduced species have received considerable attention, but such effects in clonal plants have begun to attract interest. Researchers are recently attempting to advance understanding of the mechanisms for transgenerational environmental effects between vegetative generations, in the context of environmental variation. Clonal plants are widely distributed in nature and dominate a number of plant communities and ecosystems around the world. Therefore, knowledge of transgenerational environmental effects is important to understand how clonal plants can adapt efficiently to the ongoing, rapid change at both global and regional scales in natural environments. We hope the publication of this research topic will stimulate more studies on this important issue in the coming years.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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A Role for Epigenetic Regulation in the Adaptation and Stress Responses of Non-model Plants

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In recent years enormous progress has been made in understanding the role of epigenetic regulation response to environmental stimuli, especially in response to stresses. Molecular mechanisms involved in chromatin dynamics and silencing have been explained, leading to an appreciation of how new phenotypes can be generated quickly in response to environmental modifications. In some cases, it has also been shown that epigenetic modifications can be stably transmitted to the next generations. Despite this, the vast majority of studies have been carried out with model plants, particularly with Arabidopsis, and very little is known on how native plants in their natural habitat react to changes in their environment. Climate change has been affecting, sometimes drastically, the conditions of numerous ecosystems around the world, forcing populations of native species to adapt quickly. Although part of the adaptation can be explained by the preexisting genetic variation in the populations, recent studies have shown that new stable phenotypes can be generated through epigenetic modifications in few generations, contributing to the stability and survival of the plants in their natural habitat. Here, we review the recent data that suggest that epigenetic variation can help natural populations to cope to with change in their environments.

Keywords: DNA methylation, histone modification, epigenetic variation, stress, environment

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INTRODUCTION OF EPIGENETIC REGULATION

Plants are sessile organisms that are exposed to different environmental conditions. Consequently, plants developed sophisticate mechanisms of gene regulation to ensure the survival upon environmental fluctuations. Plants sense the signals from the environment and transmitted them through a cascade of signal transduction, triggering the accumulation of transcription factors that activate gene expression that can result in adaptation to environmental challenges (Mirouze and Paszkowski, 2011). Another important mechanism of gene regulation in response to stresses is epigenetic regulation, which consists of covalent modifications of DNA and histones, affecting transcriptional activity of chromatin without changing DNA sequence (Iwasaki and Paszkowski, 2014). Chromatin structure is composed of nucleosomes formed by the interaction of histone proteins with DNA, allowing packaging of the DNA in the nucleus (Alberts et al., 2002). Because gene expression is dependent of access to DNA, thus the level of condensation of chromatin is important to this regulation. Euchromatin can be associated with transcriptional active regions, while heterochromatin is normally a transcriptional silenced region, with hypermethylation

of DNA and specific modification of histones (Vaillant and Paszkowski, 2007). Studies have highlighted three epigenetic marks: DNA methylation, histone modifications and small RNAs. Important, in many cases small RNAs can trigger DNA methylation and chromatin modification (Meyer, 2015).

In plants, epigenetic modification by DNA methylation has been thoroughly studied and the mechanisms controlling DNA methylation inheritance is well established (Martienssen and Colot, 2001; Takeda and Paszkowski, 2006). DNA methylation consists mostly in adding a methyl group at the fifth carbon position of a cytosine ring, and, different to what happens in animals, plants have three sites that frequently can suffer methylation: CG, CHG (where H is A, C, or T), and CHH (Law and Jacobsen, 2010). Studies revealed that different enzymes are responsible for methylation in each contexts: MET1 DNA methyltransferase maintains the CG methylation, methyltransferase CHROMOMETHYLASE3 – CMT3 maintains the CHG methylation and DOMAINS REARRANGED METHYLTRANSFERASE – DRM1/DRM2 or CMT2 methyltransferase are responsible for CHH methylation (Ronemus et al., 1996; Chan et al., 2006; Du et al., 2012). In addition, short interfering RNAs (siRNAs) can guide RNA-directed DNA Methylation (RdDM) pathway. In the nucleus, siRNAs are derived from long dsRNAs transcription by RNA Polymerase IV and processed by DICER-LIKE 3 (DCL3). Next, siRNAs are formed and exported to the cytoplasm to be incorporated into the RISC complex containing ARGONAUTE 4 (AGO4). Then, siRNA-AGO4 is transported to the nucleus, where siRNA align with their target, a nascent scaffold transcript from RNA Polymerase V, and recruit DNA methyltransferase to silencing its target (Matzke and Mosher, 2014). Transposons silencing can be due the DNA methylation resulting in a protection of genome integrity (Chomet et al., 1987; Ito, 2013). In addition, DNA methylation is also occurring in gene-coding regions affecting gene expression. Curiously, in Arabidopsis, one-third of methylated genes occur in transcribed regions, and 5% of genes showed methylation in promoter regions, suggesting that many of these are epigenetically regulated by DNA methylation (Zhang et al., 2006).

Modification of DNA methylation profiles in plant can cause phenotypic variation. For instance, demethylation of rice genomic DNA cause an altered pattern of gene expression, inducing dwarf plants (Sano et al., 1990). A 16% reduction in the 5-methylcitosine (m^5C) content was observed in rice plants treated with DNA demethylating agents, and this reduction in DNA methylation leads to phenotypic changes observed in the progeny. According to the above mentioned, stress can also result in changes in DNA methylation. DNA methylation content can also be regulated in response to abiotic stress (Dowen et al., 2012). Experiments in maize and Arabidopsis showed that cold stress might induce modification of the DNA methylation status (Steward et al., 2002; Song et al., 2012). Vernalization treatments result in reduction of levels of DNA methylation and induced the initiation of flowering (Burn et al., 1993). Some stress-induced modifications are reversed to the basal level; however, some of these modifications may be stable and heritable, being named the epigenetic "stress memory" (Kinoshita and Seki, 2014). The knowledge of these stress memories can increase our understanding the processes of plant adaptation to stresses.

An important question is: what is the contribution of epigenetic modification to phenotypic variation in native plants in their environment? Here, we review recent data that suggest that epigenetic variation can contribute to natural populations to cope to with changes in their environments (**Figure 1**). Is important to know that epigenetic can be define as mitotically and/or meiotically heritable variation in phenotype (Niederhuth and Schmitz, 2014). Despite of the importance of other mechanisms of epigenetic modification, DNA methylation is the better studied process in non-model plants. Firstly, we describe epigenetic changes as heritable characteristics. Next, we discuss recent studies performed with non-model plants. Is important to highlight that the knowledge of epigenetic mechanisms from model species is useful in non-model systems, suggesting gene regulation and the components of epigenetic machinery (Richards et al., 2017). However, non-model plants are becoming very attractive study material due their ability to adapt to extreme environments.

INHERITANCE OF EPIGENETIC VARIATION

Epigenetic marks, such as DNA methylation, can be modified and result in an epigenetic response. Transgenerational epigenetic inheritance requires that epigenetic marks can be transmitted to the progeny (Hauser et al., 2011). Thus, we can say that epigenetic marks might be transmitted through mitosis and sometimes also meiosis. The variation in methylation of the same gene between different plants is denominated epialleles. Epialleles differ in the number or distribution of methylated nucleotides at specific gene sequences and it is important to known that different epialleles can result in different phenotypes which are heritable in a new generation. In maize, it has been described the involvement of transposable elements (TE) regulation during plant development and the impact in the inheritance of epialleles (Martienssen et al., 1990). Moreover, a naturally occurring mutant of *Linaria vulgaris* is an example that suggests a transgenerational epigenetic inheritance (Cubas et al., 1999). Authors showed that the levels of DNA methylation of the CYCLOIDEA cause phenotypic alterations in flower symmetry and these are maintained for hundreds of years. Is important to highlight that some epigenetic marks may result in heritable phenotypic variation whereas others are not (Baulcombe and Dean, 2014). Until now, research carried out has not fully explained the mechanisms involved, but data show that DNA methylation is an epigenetic mark easier to pass through generations. In plants some germline cells are descended from somatic cells and they carry epigenetic marks, which can contribute with the heritability of epigenetic marks.

The majority of studies on epigenetic inheritance focused on DNA methylation (Kalisz and Purugganan, 2004). To understand the mechanisms involved in transgenerational epigenetic inheritance is necessary to picture out how of epigenetic marks are propagation during gametophyte development is carried out, which develops through mitotic divisions from the meiotic

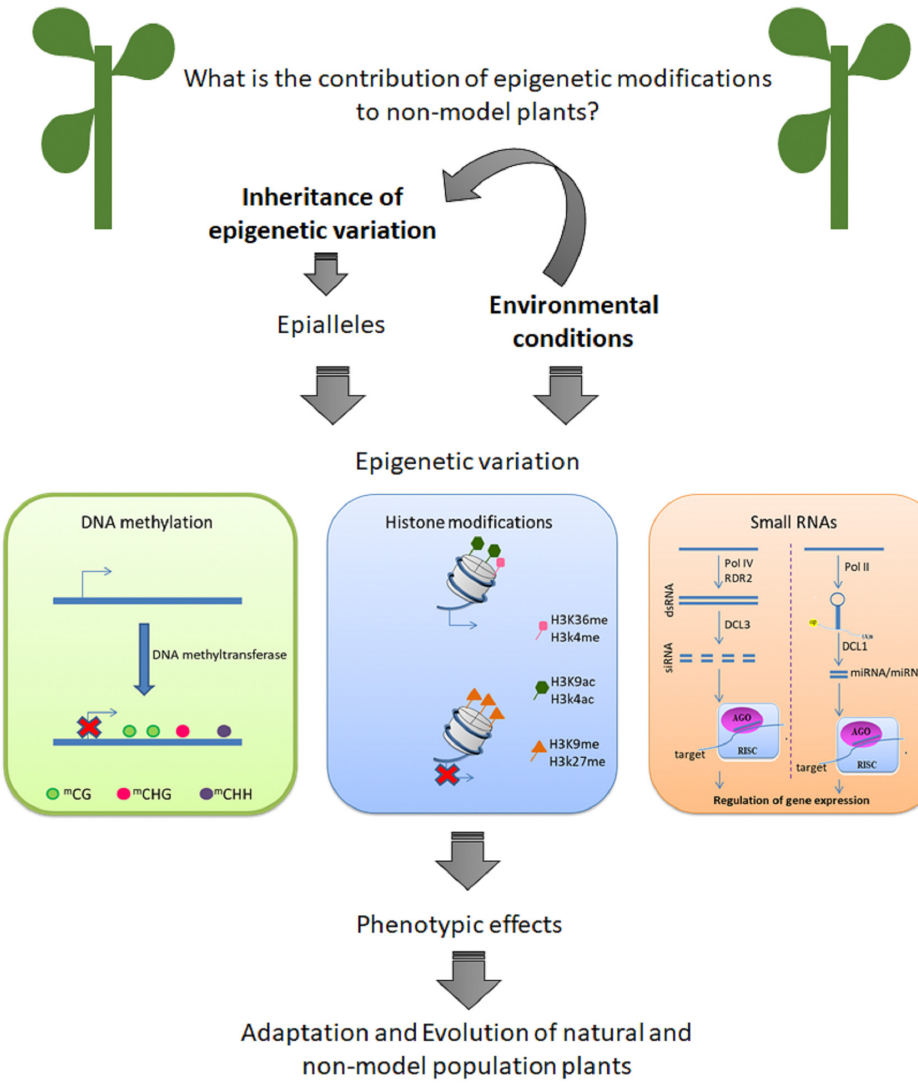

FIGURE 1 | Epigenetic variation can contribute to adaptation and evolution of non-model plants.

products. In Arabidopsis, epigenetic marks are lost in the somatic cells of pollen to activate the transposons, but this RNA can be server as precursor of siRNA production that can silenced this transposon in germ cells and give rise to the next generation (Slotkin and Martienssen, 2007). Thus, studies suggest that heritable epigenetic marks may result in heritable phenotypic variation, influencing fitness, and so be subject to natural selection (Baulcombe and Dean, 2014). Unlike mammals, CG and CHG DNA methylation were kept in three haploid cell types from developing pollen (Calarco et al., 2012). Despite the loss of CHH methylation in retrotransposons in microspores and sperm cells, the action of siRNAs with 24 nucleotides in length can restore methylation by *de novo* DNA methyltransferase activity. This result showed the importance of small RNAs (sRNA) in the methylation process. Moreover, DNA methylation via sRNA is also involved in regulation of TEs and repeats, whose reduction in DNA methylation can result in increased movement of TEs and can also influence genetic variation (Matzke and Mosher, 2014). As replication of methylated DNA sequences results in hemimethylation, where only one strand of the DNA double helix is methylated, plants have a METHYLTRANSFERASE1 (MET1) that is involved in replication of CG methylation and consequently the hemimethylated DNA can server as copy to newly synthesized strand. Interestingly, a study of a mutant for MET1 revealed that the maintenance of methylation in somatic tissues was lost during gametogenesis (Saze et al., 2003).

Is important to recognize that plants can sense the environmental conditions during vegetative growth and this could result in epigenetic modifications in a cell lineage that can generate a germline (Mirouze and Paszkowski, 2011). Studies using the model plant Arabidopsis have showed that stress-induced transgenerational responses depend on changes in DNA methylation (Boyko et al., 2010; Lang-Mladek et al., 2010). Based on this observation, it is possible that phenotypic effects caused

by epialleles are inherited across generations and influenced by environmental conditions also in native plants. Therefore, heritable epialleles will influence plant evolution through their effects on both phenotypic trait distributions and fitness. In addition, many plants are propagated asexually through clonal reproduction, where meiotic epigenetic reset does not occur. The epigenetic information among clonal generations is more effective than in sexual reproduction (Latzel et al., 2016). However, few studies describing epigenetic inheritance in non-model plants have been published. In the next topic, we describe studies showing the role of epigenetic regulation in adaptation of non-model plants and some of this analysis highlighted the roles of putative epigenetic inheritance.

EPIGENETIC REGULATION IN ADAPTATION OF NON-MODEL PLANTS

A number of techniques have been used to identify epigenetic changes in plants, mainly DNA methylation profiling, which is the most studied epigenetic mechanism (Kurdyukov and Bullock, 2016). Recently, a high-resolution method for quantification of DNA methylation was development, the bsRADseq, which combines restriction site associated DNA sequencing with bisulfite sequencing (Trucchi et al., 2016). The technique of bissulfite sequencing, in which genomic DNA is treated with bisulfite to convert unmethylated cytosines to uracil, is usefull to obtain detail of genes methylation sequences, mainly in model plants (Cokus et al., 2008). However, studies of natural plant population have used mainly the Methyl-Sensitive Amplified Polymorphism (MSAP) approach (**Box 1**). MSAP is a technique that allows analyses of epigenetic variation for a high number of individuals (Schulz et al., 2013). In plants, MSAP was first used for identification of patterns of cytosine methylation in rice (Xiong et al., 1999). Given that epigenetic marks can result in changes of plants′ phenotypes, it is important to compare the variation in DNA methylation occuring between different plants in a population. Another method developed for epigenetic studies is a epiGBS, a reduced representation bisulfite method for exploration and comparative analysis of DNA methylation and genetic variation in hundreds of samples *de novo*, which can facilitate the study of plants that no have reference genome available (van Gurp et al., 2016). Here, we describe some studies that showed the variation in epigenetic marks in non-model plants (**Table 1**).

One of the earlier studies using MSAP was performed to examine the epigenetic differences between populations of the southern Spanish violet *Viola cazorlensis* (Herrera and Bazaga, 2010). Interestingly, the same samples used in this study were previously used in other analysis of variation in DNA sequence using AFLP methods (Herrera and Bazaga, 2008). Based on this, it was possible to correlate the genetic and epigenetic variation in *V. cazorlensis* population and methylation-based epigenetic differentiation of populations was associated with adaptive genetic divergence. Thus, the authors highlighted the importance of epigenetic modifications, and consequent phenotypic variation, in adaptation and evolution of natural and non-model population of plants. Analysis in three allotetraploid sibling orchid species, that differ radically in their geographic and ecological context, showed that ecological divergence of Dactylorhiza species is mostly due the epigenetic factors regulating gene expression in response to environmental stimulus (Paun et al., 2010). *D. traunsteineri*, *D. ebudensis,* and *D. majalis* showed species-specific epigenetic patterns that impacted the ecology, distribution, and evolution of these lineages through generations. Curiously, *D. majalis,* the species living in the most diverse environment showed less epigenetic variation than *D. traunsteineri*. However, authors indicate that the epigenetic constitution of an individual or species is sensitive to its environment, and water available in combination with temperature appears to be a key factor causing environmental allopatry in Dactylorhiza. In other words, the environmental conditions, mainly related to water availability and temperature, can result in changes of DNA methylation profiles, resulting in modification of phenotypic evolution and adaptation of plant population.

Genome-wide methylation profiling using MSAP revealed DNA methylation polymorphisms within and between natural populations. A study with two populations of the mangrove plant *Laguncularia racemose* grown in adjacent areas, but with different regimen of exposure to salt water, was performed using MSAP analysis to assess epigenetic variation in CpG methylation (Lira-Medeiros et al., 2010). This study was showed that the mangrove plants living near a salt marsh (SM) were hypomethylated (14.6% of loci had methylated samples) in comparison to the plants that live along a riverside (RS) (32.1% of loci had methylated samples). Is important to mention that those mangrove species can occur naturally in contrasting habitats and have different

BOX 1 | Methyl-Sensitive Amplified Polymorphism – MSAP.

Methyl-Sensitive Amplified Polymorphism (also referred as MS-AFLP) technique is a modification of the amplified fragment length polymorphism method (AFLP) based on the differential sensitivity of isoschizomeric restriction enzymes to site-specific cytosine methylation (Herrera and Bazaga, 2010). Thus, MSAP uses the same rare cutter *EcoRI* substituting the frequent cutter *MseI* by two enzymes that differ in their sensitivity to the methylation state of their recognition site 5′-CCGG, like *MspI* and HpaII (Schulz et al., 2013). For instance, MeCpG sites are recognized by *MspI* only, because *Msp*I does not cut when the inner cytosine is methylated and HemiMeCpCpG sites are recognized by *HpaII* only, because *Hpa*II does not cut when either or both cytosines are fully methylated or hemi-methylated (Schrey et al., 2013). On the other hand, sites hypermethylated and fully methylated are not cut by either enzyme and sites that are free from methlylation are recognized by both (Paun et al., 2010). Among the many benefits of using this technique, we highlighted the fact that this technique is a cost-effective allowing research on non-model systems including those that lack sequenced genomes. However, there are some shortcomings in this technique. One shortcoming is that MSAP cannot specify the region or gene influenced by methylation (Schrey et al., 2013). More recently, the Methylation Sensitive Amplification Polymorphism Sequencing (MSAP-Seq) approach was developed to allow the global sequence-based identification of changes in DNA methylation (Chwialkowska et al., 2017). MSAP-Seq has been validated in *Hordeum vulgare*, and can be used for DNA methylation analysis in crop plants with large and complex genomes and also non-model plants. In relation of technical short-comings of the MSAP technique, a problem is when both *Msp*I and *Hpa*II may fail to cut – in CHG and CHH methylation contexts, some methylated states can be missed (Schrey et al., 2013).

TABLE 1 | Summary of studies with epigenetic in non-model plants.

Plant	Epigenetic modification	Environmental parameter	Heritable	Reference
Viola cazorlensis	DNA methylation	–	–	Herrera and Bazaga, 2010
Dactylorhiza species	DNA methylation	Water available in combination with temperature	–	Paun et al., 2010
Laguncularia racemosa	DNA methylation	Salt	Yes	Lira-Medeiros et al., 2010
Alternanthera philoxeroides	DNA methylation	Water available	–	Gao et al., 2010
Elaeis guineensis	DNA methylation	–	–	Ong-Abdullah et al., 2015
Eucalyptus nitens	DNA methylation	–	Yes	Thumma et al., 2009
Pinus pinea	DNA methylation	–	–	Saéz-Laguna et al., 2014
Ilex aquifolium	DNA methylation	Herbivory	–	Herrera and Bazaga, 2013
Taraxacum officinale	DNA methylation	Low nutrients, salt stress, JA application, SA application	Yes	Verhoeven et al., 2010

phenotype characteristics, for example, SM plants are small and have smaller leaf size compared to the RS plants. In addition, SM also had less epigenetic diversity than RS. Thus, CpG-methylation changes may be associated with environmental heterogeneity suggesting that epigenetic variation in natural plant populations is dependent of different environments. Interesting, AFLP analyzes of the same populations showed very little DNA variation, reinforcing the role of epigenetic variation in their adaptation. Analysis of DNA methylation profile of an invasive weed *Alternanthera philoxeroides* (alligator weed) also showed interpopulation difference in global DNA methylation in field plants (Gao et al., 2010). MSAP analysis revealed distinct DNA methylation patterns between aquatic and terrestrial plants, suggesting the potential of environmental factors to affect the methylation profile. Interestingly, 78.7% of epigenetic variation was observed within populations in response to different habitats. Despites this, 13.4 and 7.9% of epigenetic variation was also observed among geographic sites and between habitats within sites, respectively.

A study addressing phenotypic variation of native *Pinus pinea* plants showed a remarkable degree of phenotypic plasticity, despite having low levels of genetic variation. However, analysis of different vegetatively propagated trees showed a high degree of DNA methylation, suggesting the role of cytosine methylation in the improvement of *P. pinea* fitness under different environmental conditions (Saéz-Laguna et al., 2014). More recently, a study with the oil palm *Elaeis guineensis* revealed the impact of DNA methylation in an important characteristic of the fruit (Ong-Abdullah et al., 2015). Approximately, 75% of hypomethylated loci were transposons and repeats, while less frequent hypermethylated loci included genic sequences regions. This study showed that methylation near the Karma transposon predicts normal fruit and hypomethylation predicts homeotic transformation, parthenocarpy and marked loss of yield. Remarkably, the loss of Karma transposon methylation contributes to the origin of mantled plants, which is a somaclonal variant arising from tissue culture that drastically reduces yield, and has largely halted efforts to clone elite hybrids for oil production. In the tree *Eucalyptus nitens,* methylation of a CpG site in a gene involved in cellulose deposition is heritable, and the methylation pattern in DNA from either xylem or leaf tissues was similar, suggesting that methylation of this site is not tissue specific (Thumma et al., 2009).

Studies revealed that biotic stresses can also trigger an increase of the overall level of genomic methylation. Curiously, the methylation levels of some pathogen response or resistance genes are reduced (Peng and Zhang, 2009). This last profile results in up-regulation of genes involved in fast response to stress, but the increase in genomic DNA methylation may lead to a repression of the transcriptome. Application of jasmonic acid and salicylic acid is often used to experimentally mimic biotic attack and to induce defense pathways. Treatments with those phytohormones in the genetically identical apomictic dandelion (*Taraxacum officinale*) plants promote an increase in methylation changes in each of the treatments when compared with the control group. In addition, the epigenetic marks are largely heritable in the first generation (Verhoeven et al., 2010). In *Ilex aquifolium* (Aquifoliaceae) a link between herbivory, phenotypic plasticity and epigenetic changes was observed (Herrera and Bazaga, 2013). Some plants have leaves prickly and non-prickly, and the presence of this characteristic is a plastic defense response induced by mammalian browsing, which may reduce herbivory (Obeso, 1997). Herrera and Bazaga (2013) used MSAP to analyze the difference in DNA methylation in a heterophyllous tree producing two contrasting leaf, prickly, and non-prickly. Within heterophyllous branchlets, MSAP marker presence was significantly higher for prickly (mean $\pm$ SE = 0.681 $\pm$ 0.072) than for non-prickly (0.632 $\pm$ 0.077) leaves. The genome of prickly leaves was more demethylated in comparison of non-prickly leaf on the same branchlet. Interestingly, the plants that have these two putative leaves can be considered an epigenetic mosaic. Based on knowledge that epigenetic marks are transgenerationally heritable in plants the authors suggest that epigenetic mosaics can be translate into epigenetically heterogeneous progeny.

CONCLUSION

Although part of the plants' adaptation can be explained by the preexisting genetic variation in the populations, recent studies have shown that new stable phenotypes can be generated through epigenetic modifications in a few generations, contributing to the stability and survival of the plants in their natural habitat. The epigenetic regulation can cause dynamic changes, such as the plant hypersensitivity reaction (HR), changes in the structure of chromatin and influence the plant phenotype, contributing to

the adaptation of native plants to stress. Thus, the knowledge of epigenetic contributions in phenotypic plasticity and hereditable variation is important to understand how natural population can adapt in different environmental condition, especially in a world context of climate change. Nevertheless, this is an area of study that clearly asks for additional investigation and the engagement of young scientists.

AUTHOR CONTRIBUTIONS

FT collected and analyzed the data and wrote the manuscript. AH commented and reviewed the article. PF critically reviewed the article and finished the manuscript.

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Variability in DNA Methylation and Generational Plasticity in the Lombardy Poplar, a Single Genotype Worldwide Distributed Since the Eighteenth Century

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In the absence of genetic diversity, plants rely on the capacity of phenotypic plasticity to cope with shifts in environmental conditions. Understanding the mechanisms behind phenotypic plasticity and how local phenotypic adjustments are transferred to clonal offspring, will provide insight into its ecological and evolutionary significance. Epigenetic changes have recently been proposed to play a crucial role in rapid environmental adaptation. While the contribution of epigenetic changes to phenotypic plasticity has been extensively studied in sexual reproducing model organisms, little work has been done on vegetative generations of asexual reproducing plant species. We studied the variability of DNA methylation and bud set phenology of the Lombardy poplar (*Populus nigra* cv. *Italica* Duroi), a cultivated tree representing a single genotype worldwide distributed since the eighteenth century. Bud set observations and CpG methyl polymorphisms were studied on vegetative offspring resulting from cuttings grown for one season in a common glasshouse environment. The cuttings were collected from 60 adult Lombardy poplars growing in different environments. The physiological condition of the cuttings was determined by measuring weight and nutrient condition. Methylation sensitive amplified polymorphisms were used to obtain global patterns of DNA methylation. Using logistic regression models, we investigated correlations among epigenotype, bud phenology, and the climate at the home site of the donor trees, while accounting for physiological effects. We found significant epigenetic variation as well as significant variation in bud phenology, in the absence of genetic variation. Remarkably, phenology of bud set observed at the end of the growing season in the common environment was significantly correlated with climate variables at the home site of the mother trees, specifically the average temperature of January and monthly potential evapotranspiration. Although we could not directly detect significant effects of epigenetic

variation on phenology, our results suggest that, in the Lombardy poplar, epigenetic marks contribute to the variation of phenotypic response that can be transferred onto asexually reproduced offspring resulting in locally adapted ecotypes. This contributes to the growing evidence that epigenetic-based transgenerational inheritance might be relevant for adaptation and evolution in contrasting or rapidly changing environments.

Keywords: bud phenology, DNA methylation, ecological epigenetics, epigenetic variation, Lombardy poplar, *Populus nigra*, transgenerational plasticity, vegetative propagation

INTRODUCTION

In the absence of genetic diversity, plants rely on the capacity of phenotypic plasticity to cope with shifts in environmental conditions (Castonguay and Angers, 2012). Since many tree and shrub species reproduce asexually, resulting in new individuals (i.e., offspring) that are genetically identical to their parents, it is generally recognized that phenotypic plasticity is a favorable feature to respond to changing environmental conditions. For example, in the field of the domestication and breeding of tree species, phenotypic plasticity has been frequently reported as a camouflaging effect on the breeding value of the genotype (e.g., Houtzagers, 1937; Stearns, 1989; Farmer, 1996). However, surprisingly little is known about the mechanisms behind transgenerational plasticity, especially on how phenotypic adjustments to local conditions are passed onto vegetative offspring, the so called transgenerational phenotypic plasticity (Latzel and Klimešová, 2010; Rohde et al., 2011; Münzbergová and Hadincová, 2017).

Although the processes behind transgenerational plastic effects are not yet perfectly understood, it is generally believed that epigenetic inheritance is one of the most important drivers (e.g., Latzel and Klimešová, 2010; Verhoeven et al., 2010; Richards et al., 2017). DNA methylation, the addition of a methyl group to one of the four bases in the DNA molecule (usually cytosine), is recognized as one of the prime epigenetic mechanisms to correlate with gene expression. Moreover, methyl polymorphisms at CpG sites (cytosine-phosphate-guanine sites where a cytosine is directly followed by a guanine in the DNA sequence) have recently been proposed to a play a crucial role in rapid environmental adaptation (Huang et al., 2017) and may provide asexual organisms with additional sources of variation to cope with contrasting or shifting environmental conditions (e.g., Castonguay and Angers, 2012; Richards et al., 2012; Verhoeven and Preite, 2014). Recently, some studies have indeed shown that epigenetic effects can result in novel phenotypes without any variation in the DNA sequence (Cubas et al., 1999), and that epigenetic states may persist after the initiating factor causing the epigenetic effects disappeared (e.g., Verhoeven et al., 2010; Xie et al., 2015; Xu et al., 2016).

Although most studies on epigenetic inheritance in plants have been done in controlled settings and on sexual model organisms such as Arabidopsis (e.g., Zhang et al., 2013; Cortijo et al., 2014; Dubin et al., 2015), some studies also recently documented the occurrence of epigenetic variation in asexually reproducing plant populations (Richards et al., 2012; Preite et al., 2015; Spens and Douhovnikoff, 2016). Nonetheless, insights into the epigenetic stability over generations and its adaptive significance under real environmental conditions remain, largely unknown (Richards et al., 2017).

Here, we report on the variation in DNA methylation and transgenerational phenotypic variation of the Lombardy poplar (*Populus nigra* cv. *Italica* Duroi), a cultivated variety of *P. nigra* L. that is distributed worldwide since the beginning of the eighteenth century. This clonal variety likely originated between 1700 and 1720 (Elwes and Henry, 1913; Henry, 1914) from one single male mutant tree of *P. nigra* located in central Asia from where it was spread to Europe and other continents (Zsuffa, 1974). In the mid-eighteenth century, the Lombardy poplar was spread by cuttings worldwide from Italy, reaching France in 1749, England in 1758, and North America in 1784 (Wood, 1994). It has been widely introduced for use as windbreaks, screens, avenue trees, and landscape plantings all over the temperate regions of the world (in Europe, North and South America, South Africa, Australia, New Zealand, and China) even in subtropical environments where it appears to perform poorly (CABI, 2017). Its clonal origin in combination with its widespread distribution in space and time, makes the Lombardy poplar an excellent study system to investigate how long-lived plant species with a prevailing vegetative reproduction can cope with widely contrasting environmental conditions, without variation at the genetic level. The Lombardy poplar can be easily and inexpensively propagated by cuttings and vegetative propagation is the only way to conserve the typical columnar tree habit and the unusual vertical branching structure. As a result, most Lombardy poplars originate from artificial vegetative reproduction performed by humans, with plant material that has been grown locally for centuries. It can thus be expected, that the large-scale geographic, but artificial expansion of this cultivar may have resulted in the accumulation of lineage-specific, selectively neutral spontaneous epimutations, and in environmental-directed epigenetic effects that are potentially heritable and may have generated different local phenotypes.

In this work, we used Methylation-Sensitive Amplified Fragment Length Polymorphisms (MS-AFLPs) on cuttings grown in a common environment and collected from 60 adult Lombardy poplars representing a single genotype and located in different climates along a north-south distribution of ca. 2120 km (15.2° latitude) and across an east-west distribution of ca. 1700 km (30.1° longitude). We also studied potential transgenerational effects on bud set as a cornerstone of the seasonal growth cycle (Rohde et al., 2011) on ramets collected on

TABLE 1 | Geography and climate data for Lombardy poplar accessions.

Climate variable	Range	
	Min	Max
Latitude (degree)	40.752	55.890
Longitude (degree)	−4.593	25.457
Average January Temp (°C)[a]	−1.400	8.024
Average March Temp (°C)	3.87	10.83
Average July Temp (°C)	14.93	26.20
Average precipitation rate (mm/month)[a]	23.274	101.065
Frost days frequency (days per year)[a]	8.497	26.436
Potential evapotranspiration (mm/month)[b]	22.746	43.879

Variables derived from 1965 to 2015 with the R package RFc version 0.1-2. (Grechka et al., 2016).
[a] *From the climate data-set CRU TS 2.0.*
[b] *From Climate Malmstrom Air Force Base.*

65 Lombardy poplars and grown in the common environment. We considered the adult Lombardy poplars growing in the different environments as the F_0-generation, and their vegetative offspring (cuttings) grown in the common glasshouse environment as the F_1-generation. Specifically, the aims of this study are to test whether; (i) there is significant natural variation in DNA methylation among the widely distributed Lombardy poplar (F_0) that can persists in clonal offspring (F_1) in a common glasshouse environment, (ii) the epigenetic differentiation is associated with the maternal growing environment, (iii) there is adaptive phenotypic variation among the Lombardy poplars in terms of bud set, that can persist among vegetatively reproduced F_1-offspring grown in a common environment, and (iv) the potential variation in bud set is related with the climate of the sampling origin.

MATERIALS AND METHODS

Sampling and Climate Data Collection

Dormant twigs of 94 adult putative Lombardy poplar trees (hereafter called; donor trees, F_0-generation) were collected during the winter of 2016–2017 at in total 37 locations (hereafter called; home sites) in Europe and Asia (**Supplementary Table 1**). After phytosanitary inspection, the twigs were shipped by express mail to the Research Institute for Nature and Forest located in Geraardsbergen, Belgium (lat. 50,77635°, lon. 3,881007°) and upon arrival stored in the fridge at 4°C until the greenhouse experiment was established. We used publically available global climate data sets to characterize the home environment of each donor tree. Climate variables (**Table 1**) were calculated for the period 1965–2015 with the R package RFc version 0.1-2. (Grechka et al., 2016).

Greenhouse Experiment

A greenhouse experiment was set up on 9 and 10 March 2017. Only plant material of good quality (1-year old, fresh shoots) of the collected donor trees was included in the experiment. The collected shoots were divided into cuttings of 22 cm in length. Up to 14 cuttings (mean: 12.8, range: 4–14) per donor tree were planted in trays to a depth of about 19 cm after recording the weight of each individual cutting, resulting in a total of 1133 planted cuttings (further called: ramets, F_1-generation). The trays consisted of 7×4 individual cells and were filled with potting soil (50% white peat / 50% black peat, 0.12% nitrogen, 0.14% phosphorous, 0.24% potassium). Ramets were grouped per donor tree within a tray (generally half a tray per tree), and donor trees were randomly distributed among trays. The trays were placed together under similar light and temperature conditions in the greenhouse and were regularly watered. No fertilizers or other soil supplements were provided during the experiment. On 10 May 2017, a fully expanded, fresh leaf was collected for DNA-analysis from the top of a single ramet per donor tree, except for two donor trees (one from Germany (code: GEB1) and one from Spain (code: SPC1) of which leaves were not yet fully unfolded and collected a few days later. For 14 ramets, a second leaf was collected to serve as a replicate. Sampled leaves were dried in silica gel. A list of the Lombardy poplar accessions is given in the additional **Supplementary Table 1**.

Nutrient Condition of the Ramets

Beside epigenetic mechanisms, other, maternally inherited factors may affect bud phenology like the ramets' nutrient condition (e.g., Marchi et al., 2005) which, in turn, relates to the topsoil mineral condition at the site of the donor tree (Cools et al., 2014). For each sampled donor tree, the mineral nutrition condition was determined by measuring total carbon (C) and total nitrogen (N). Foliage samples (5 to 10 leaves, in total) were collected on 18 May 2017 from one to three ramets per donor tree. They were dried in an oven at 40°C for 1 week and pulverized with a blender. For each of the sampled donor trees a homogenized subsample was analyzed. The total N and total C content was determined using a C/N analyzer (Skalar, FormacsHT, Breda, The Netherlands) and expressed per unit of dry biomass ($g\ kg^{-1}$).

DNA Extraction

Total genomic DNA was extracted from the sampled leaves with the Qiagen Plant DNA kit (Hilden, Germany). The integrity of the DNA was assessed on 1.5% agarose gels, and DNA quantification was performed with Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermo Fisher Scientific, Massachusetts, USA) using a Synergy HT plate reader (BioTek, Vermont, USA).

SSR Analysis

Nuclear microsatellite polymorphisms (SSR) were used to determine the multilocus genotype of the donor trees propagated in the greenhouse experiment. We selected 11 SSRs that were found useful for the identification of *P. nigra* clones in former studies (van der Schoot et al., 2000; Smulders et al., 2001; Liesebach et al., 2010). PCR products were run on an ABI 3500 analyzer with the GeneScan-600 LIZ size standard and analyzed using GeneMapper 4.1 (Thermo Fisher Scientific). The 14 replicated samples were used to calculate the genotyping error rate, calculated as 100 × (number of discordant scores in two

independent analyses)/(number of scored markers × number of individuals analyzed). Details on SSRs and PCR-conditions are given in the additional **Supplementary Table 2**.

MS-AFLP Analysis

The Methylation Sensitive Amplified Length Polymorphism Analysis (MS-AFLP) was performed on vegetative offspring of the donor trees identified as Lombardy poplar based on the results of the SSR-analysis. The MS-AFLP method was adapted from Guarino et al. (2015) using the enzyme combinations *Eco*RI—*Hpa*II and *Eco*RI—*Msp*I. *Hpa*II and *Msp*I cut DNA sequences at the same tetra-nucleotide motif (5′-CCGG-3′), but have different sensitivities to cytosine methylation at the restriction site. This allows the determination of the CpG-methylation status of anonymous regions of the genome. The two MS-AFLP profiles for every sample were compared to identify polymorphic epigenetic loci. We initially tested 32 primer combinations on a subset of 16 samples. Of these primer combinations, seven were selected based on the quality and the reproducibility of amplified bands and the presence of polymorphisms (**Table 2**). Fourteen samples were replicated, starting from a second leaf sample and two different DNA extractions to assess the reproducibility of the analysis. PCR amplicons were fluorescently labeled with one of two dyes: NED or VIC, and were run in simplex on an ABI 3500 analyzer with the GeneScan-600 LIZ size standard (Thermo Fisher Scientific). We used GeneMapper v4.1 (Thermo Fisher Scientific) for the sizing of the DNA fragments. The quality of the electropherograms was visually checked in GeneMapper and electropherograms of low quality (e.g., weaker profiles with unreliable and/or low peak intensities) were removed before importing peak data into RawGeno version 2.0-1 (Arrigo et al., 2009), an R package for automatic scoring of AFLP datasets. Only fragments ≥150 bp in size were considered to reduce the potential impact of size homoplasy (Vekemans et al., 2002). DNA fragment profiles were processed per *Eco*RI/*Hpa*II—*Msp*I primer combination pairs and scored in RawGeno using the scoring parameters given in the additional **Supplementary Table 3**. Singletons were removed from the data. The genotyping error rate was calculated per *Eco*RI/*Hpa*II—*Msp*I primer combination pairs in RawGeno according to Bonin et al. (2004). After removing samples with missing data, the binary data of each of the seven primer combinations were combined resulting in a data matrix of complete *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I fragment profiles for vegetative offspring (F_1-generation) of 60 donor trees (the 14 replicates excluded) and 226 loci. We used the methylation scoring approach described in Herrera and Bazaga (2010) to transform this data matrix into a binary data matrix representing the epigenetic diversity. The absence of fragments of both *Hpa*II and *Msp*I cuts (condition 2 in Herrera and Bazaga, 2010) represents either methylation of both (internal and external) cytosines or absence of the digestion site via mutation (Schulz et al., 2013). We scored the absence of fragments of both *Hpa*II and *Msp*I cuts as uninformative (missing data) to account for somatic mutations. Only loci exceeding a specific methylation threshold were scored. This threshold was specific for each primer combination (**Table 2**) and set equal to the expected per-individual probability of obtaining a mismatch of *Hpa*II and *Msp*I scores owing to technical and/or scoring errors (see Herrera and Bazaga, 2010). We use the term "epigenotypes" to refer to the epigenetically polymorphic CCGG sites resulting from the MS-AFLP-analysis. The R package msap version 1.1.8 (Pérez-Figueroa, 2013) was used to transform the absence and presence of fragments of both *Hpa*II and *Msp*I cuts into epigenotypes. The resulting binary data matrix of polymorphic methylation-sensitive markers was used for analyses of epigenetic data in GenAlEx version 6.4 (Peakall and Smouse, 2012). We identified shared epigenotypes among individuals (considering missing data when finding matches), estimated haplotype diversity, computed the Shannon's Diversity Index and performed a principal coordinate analysis (PCoA) to determine and visualize the epigenetic variation and structure of the analyzed ramets. We then performed an analysis of molecular variance (AMOVA) with samples grouped per country to determine the epigenetic differences among Lombardy poplar ramets sampled in different countries calculated as mean pairwise Φ_{ST} distances. In two countries (Spain and Bosnia Herzegovina), only one tree each was sampled, these were removed prior to the AMOVA approach. The probability for significance of Φ_{ST} was based on 999 permutations across the full data set (Michalakis and Excoffier, 1996). Mantel test analysis (Hutchison and Templeton, 1999) was used to estimate the correlation between the Euclidean epigenetic distance matrix generated by GenAlEx and the geographic distance matrix of sampled trees (km). The significance of the Mantel test was assigned by random permutations tests (based on 999 replicates).

We applied simple logistic regression models to investigate whether the variation in DNA-methylation observed at a particular epilocus, depends on the environmental variables recorded at the location of the donor trees. For this analysis, we ignored possible somatic mutations and scored fragment absence as unmethylated (score: "0"). We included as exploratory variables the average temperature (°C) of the coldest (January) and warmest (July) month in the year and of March (temperature in early spring), the average monthly precipitation rate (mm $month^{-1}$), the average number of frost days per year and the average monthly potential evapotranspiration rate (PET) (mm $month^{-1}$). We also tested if the epigenetic variation is related to the topsoil nutrient availability (carbon-nitrogen ratio or CN) on the location of the donor tree. The analyses were performed in R using the generalized linear model function *glm ()* with a binomial error distribution and a logit link function. The *p*-values were corrected for multiple testing at a false discovery rate of 5% (Benjamini and Hochberg, 2000). All statistical analyses were performed in the open source software R 3.4.3 (R Core Team, 2017).

Bud Set Scoring and Data Analysis

Bud set was scored in late summer 2017, from the beginning of August to the end of September, of the apical bud of the ramets in the greenhouse. We used a seven stage scoring system to cover onset and duration of bud set developed for *P. nigra* by Rohde et al. (2011). Scores go from 3 (growing apical meristem) to 0

TABLE 2 | Characteristics of the primer combinations used in the MS-AFLP analysis of 60 Lombardy poplars grown in a common greenhouse environment.

	Primer combination	Total MS-AFLP markers in the size range 150–600 bp	Scoring error rate[a]	Methylation-susceptible markers[b]	
				N	N Polymorphic (%)
1	*Eco*RI + ACC/*Hpa*II-*Msp*I + TAC	29	0.029	16	9 (56.25%)
2	*Eco*RI + ACC/*Hpa*II-*Msp*I + TAG	22	0.000	21	16 (76.19%)
3	*Eco*RI + AGC/*Hpa*II-*Msp*I + TCC	25	0.024	13	13 (100%)
4	*Eco*RI + AGC/*Hpa*II-*Msp*I + TCT	28	0.033	10	3 (30%)
5	*Eco*RI + AGC/*Hpa*II-*Msp*I + TCG	20	0.038	9	4 (44.44%)
6	*Eco*RI + AGC/*Hpa*II-*Msp*I + TAA	51	0.044	10	10 (100%)
7	*Eco*RI + ACT/*Hpa*II-*Msp*I + TAG	41	0.028	15	10 (66.67%)
	All combined	216	0.028	94	65 (67.65%)

[a]*Calculated by RawGeno according to Bonin et al. (2004) and per EcoRI/HpaII – MspI primer pairs on the DNA fingerprinting profiles from the 14 replicated samples.* [b]*N, number of methylation-susceptible markers. A methylation-susceptible marker was considered polymorphic when both methylated and non-methylated states occurred in the total sample of 60 individuals.*

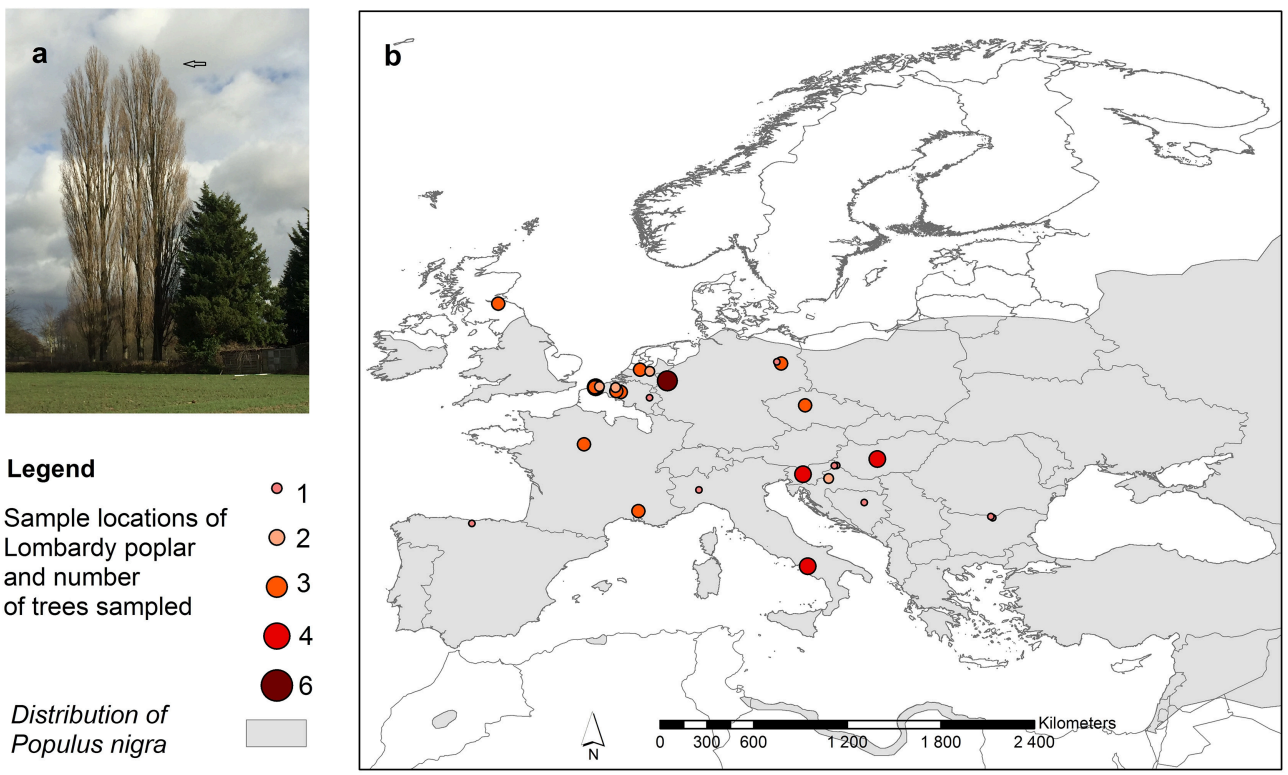

FIGURE 1 | (a) Lombardy poplars, **(b)** Distribution of collection sites of Lombardy poplars and number of trees sampled per location. The map also shows the distribution range of the *Populus nigra* L. in Europe, including natural and naturalized stands (EUFORGEN http://www.euforgen.org/species/populus-nigra/).

(fully developed bud), in 0.5 intervals (**Supplementary Table 5**). Observations were performed once a week resulting in 6 dates of observations for in total 812 individual ramets of 65 donor trees identified as Lombardy poplar based on the results of the SSR analysis (**Supplementary Table 1**). The phenological scores for bud set (Bs) were modeled using cumulative logistic regression using the R package ordinal (Christensen, 2015). Cumulative link mixed models are fitted in this package with the command "*clmm.*" This models the chance (p) to maximally have reached a given level of the ordinal response variable bud set (Bs). The bud set scores were ordered from 3 to 0, so that the chance of maximally reaching e.g., bud set score 1.5 included scores: 3, 2.5, 2, and 1.5. The home site of the donor trees (S) was in the fixed part (categorical variable) of the model. For the ramets, weight data was correlated with total carbon - nitrogen ratio ($r = -0.504$, p-value $< 2.2e\text{-}16$), therefore only CN was

included as a covariate in the model in the fixed part to correct for transgenerational plasticity of bud set due to nutrient condition. Day (D) was also added in the fixed part to account for the different observation days. The random part (random intercept) consisted of a unique donor tree identity (TID) and a unique identity code for each ramet (RID). The latter accounted for the repeated observations on the same ramets. This resulted in the following cumulative link mixed model:

$$\log\left(\frac{P_{Bs}}{1-P_{Bs}}\right) = \alpha_T - \beta_D \times D\,(fixed) - \beta_S \times S\,(fixed) - \beta_{CN} \times CN\,(fixed) - ranef_{TID}\,(random) - ranef_{RID}\,(random) \quad (1)$$

α_T is a threshold value indicating the passing on from one level of the ordinal bud set response variable to the next. β_D, β_S, and β_{CN} are the estimated coefficients for the fixed covariates *D*, *S*, and *CN*. $Ranef_{TID}$ and $ranef_{RID}$ are the random effect coefficients for all levels of the variables TID and RID.

The timing of bud set across the different donor trees was assessed by calculating the DOY (day of the year) when the probability for having reached maximally bud set score 1.5 attained 50% ($D_{50\%}$). A $D_{50\%}$-value for a given donor tree therefore indicated the day that half of the ramets of this tree had reached maximally the given stage of the phenophase, taking into account a mean value for CN (CN = 12).

$$D_{50\%} = \frac{\alpha_T - \beta_{CN} \times 12 - \beta_S - ranef_{TID}\,(random)}{\beta_D}) \quad (2)$$

The $D_{50\%}$ values were used to calculate Pearson correlation coefficients with climate variables from the home sites of the donor trees; the average temperature (°C) of the coldest (January) and warmest (July) month in the year and the average monthly potential evapotranspiration rate (mm month^{-1}). We used Welch's test (Welch, 1938; Ruxton, 2006), a *t*-test for unequal variances, to determine the statistical significance between the methylation state of each epilocus and the $D_{50\%}$ values. For this analysis, we ignored possible somatic mutations and scored fragment absence as unmethylated (score: "0"). Loci scored as present ("1") in only one ramet, or in all ramets except one, were discarded.

RESULTS

Identification of Multilocus Genotypes

The 94 sampled putative Lombardy poplar trees analyzed with 11 SSR markers resulted in 15 different multilocus genotypes (MLG) (**Supplementary Table 4**) of which the most common genotype (G01) was shared by most of the donor trees (65 trees) (**Supplementary Table 1**) and therefore we consider it to be the genotype of the "true" Lombardy poplar. Furthermore, two multilocus genotypes, representing four (G07) and one (G14) samples, respectively, differed from G01 for only one out of the 22 alleles. They were considered as Lombardy poplars representing the identical genotype but with a possible somatic mutation at locus WPMS05 (mismatch of one repeat), resulting in 72 (75%) individual trees identified as Lombardy poplars sampled in 13 different countries at 37 locations (**Figure 1**). Other MLGs showed differences with the most common MLG (G01) for in total 7 to 11 alleles. Remarkably, they share alleles with the "true" Lombardy poplar to a high degree. Three of the latter MLGs showed maximum one difference with the most common MLG at each locus and are likely direct sexual offspring, i.e., the result of a cross between a *P. nigra* female and the Lombardy poplar as the paternal parent. The mean genotyping error rate calculated from the 14 replicates was 0.003 (0.3%).

Variation in DNA Methylation

Complete DNA fragment profiles for both enzyme combinations and the seven primer pairs (7 × 2 =14 DNA-fragment profiles) were obtained for vegetative offspring of 60 out of 65 (92%) "true" Lombardy poplar donor trees grown in the common environment and collected in 13 countries at 25 different geographic locations with a mean number of trees per location of 2.4 (range: 1 to 6) (**Supplementary Table 1**). The mean error rate for all primer pairs calculated based on the 14 replicated leaf samples was 2.8% (**Table 1**), which was within the 2–5% technical error rate range usually found in AFLP studies (Bonin et al., 2004). The transformation of the *Eco*RI/*Hpa*II—*Msp*I fragment profiles into a binary data matrix representing epigenetic differentiations, resulted in 216 epiloci comprising 94 methylation-susceptible epiloci, of which 65 (68%) were polymorphic among the 60 Lombardy poplars (**Table 2**). The number of variable positions in the epigenetic analysis (65) was significantly higher than the estimated scoring error rate of 2.8% (*t*-test, $p < 0.00005$), indicating significant epigenetic differentiation between samples. In contrast to the SSR genotype, all the 60 epigenotypes were unique. Pairs of epigenotypes differed by at least 2 (3%) and up to 23 (56%) epiloci of the 65 epiloci analyzed [mean pairwise differences: 17 (26%), **Supplementary Figure 1**]. Fifteen epiloci with missing values, as a result of scoring fragments of both *Hpa*II and *Msp*I cuts considered as uninformative, were removed prior to further analyses. We estimated a Shannon's Diversity Index of 0.386 (SD: 0.163). The differentiation between country of origin of the donor trees is visible in the PCoA; in fact, ramets obtained from Lombardy poplar trees located in Bosnia, Spain, Croatia, and Hungary clustered apart from the rest of the analyzed samples (**Figure 2**). This was also reflected by the results of the AMOVA analysis. Significant epigenetic differentiation was found between countries [$\Phi_{ST} = 0.078$, $p_{(rand >= data)} = 0.015$]. There was no significant correlation between the pairwise distances calculated for MS-AFLP markers and the geographic distances of sampled trees (km) using a Mantel test [$r^2 = 0.100$, $p_{(rand >= data)} = 0.07$].

Fragment absence scored as unmethylated state resulted in 68 polymorphic methylation-susceptible epiloci. We found significant, simple logistic regressions for 11 out of 68 epiloci with a climate variable as explanatory variable (**Supplementary Table 6**). Several climate variables are correlated with each other (with $r^2 > 0.8$: average January and average March temperature, average January temperature

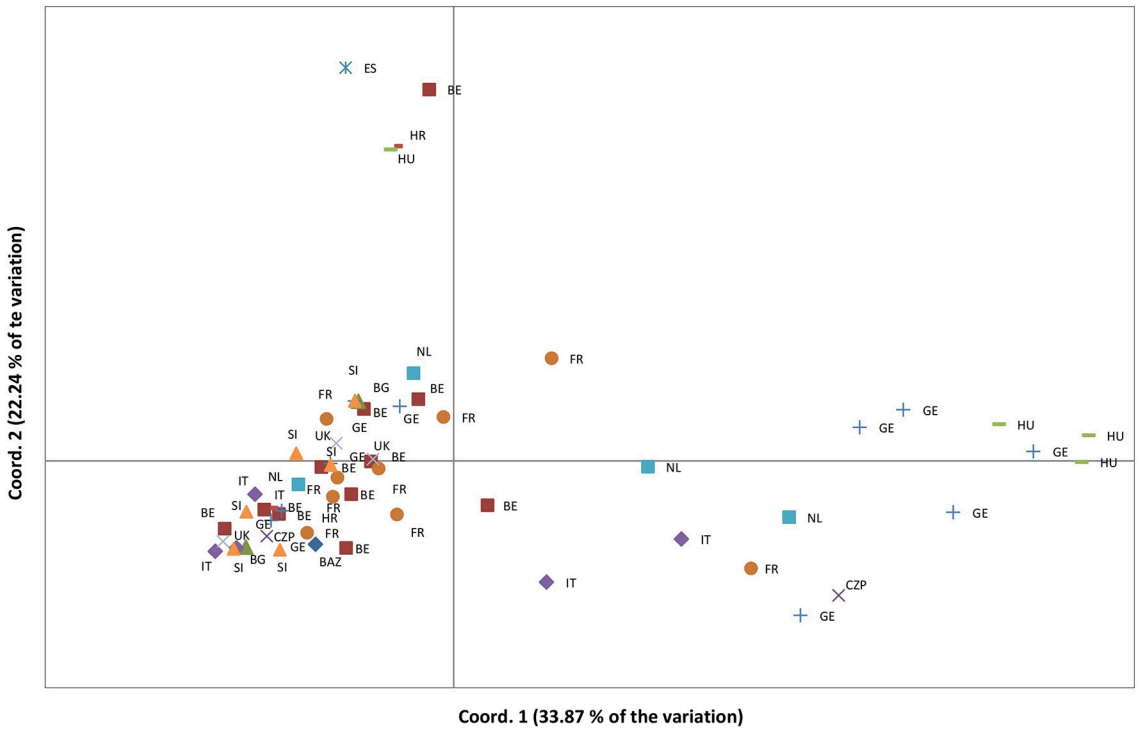

FIGURE 2 | Principal coordinate analyses based on the epigenetic distances calculated on 50 polymorphic MS-AFLP loci and for vegetative offspring grown in a common environment, originating from 60 Lombardy poplar trees collected in 13 countries and representing a single microsatellite genotype. Country codes are explained in **Supplementary Table 1**.

and number of frost days, average January temperature and PET, average March temperature and PET, number of frost days and PET). Therefore, for some epiloci multiple significant associations with climate variables were detected. However, after FDR correction none of these models remained significant meaning that none of the epiloci could significantly be correlated with a climate variable.

Variation in Bud Set

We found significant differences in bud set between ramets originating from 65 "true" Lombardy poplar donor trees located at different sampling locations, while accounting for physiological effects in terms of the ramets' nutrient condition (**Supplementary Table 7**). Furthermore, we found significant correlations between timing of bud set and the mean temperature in January ($r = 0.34$; p-value $= 0.006$) (**Figure 3**) and the average monthly potential evapotranspiration ($r = 0.33$; p-value $= 0.008$), indicating that ramets originating from donor trees at sites with warmer winter temperatures set buds later compared to ramets originating from donor trees at sites with colder winters. No correlation was found with average July temperature ($r = 0.03$; p-value $= 0.80$). Only for one locus (m110; locus amplified with primer combination four and resulting in a fragment of size 186.04 bp), the $D_{50\%}$-values were significantly different for the two methylation states ($t = -2.36$, df $= 43.25$, p-value $= 0.02$). After FDR correction, this relation became non-significant.

DISCUSSION

Patterns of DNA Methylation Variation

In addition to the genetic component, epigenetic variation has been suggested to contribute to the phenotypic plasticity and the adaptive potential of individuals and populations to cope with changing environmental conditions (e.g., Bräutigam et al., 2013; Alsdurf et al., 2016; Whipple and Holeski, 2016). In this context, the Lombardy poplar provides a convenient study system to investigate landscape-level patterns of epigenetic variation along pronounced environmental gradients because it represents a single genotype, it is easy to identify, very widespread and easy to propagate by cuttings. We found high levels of DNA methylation variation [65 (68%) CpG methyl polymorphisms] in asexual reproduced offspring grown in a common greenhouse environment, with a small but significant part of this epigenetic variation (7.8%) distributed among the countries of origin of the donor trees. The observed percentage of polymorphic genome-wide cytosine methylated sites is similar as reported for the genetically depauperate tree species *Pinus pinea* L. (65%) in natural populations covering the entire distribution area of the species in Spain (Sáez-Laguna et al., 2014). Because the Lombardy poplar plants under study represent a single genotype, we assume that the observed methylation variation under the controlled greenhouse conditions in this work is most likely caused by differences in the environment of the donor trees (maternal environment) (Richards et al., 2012;

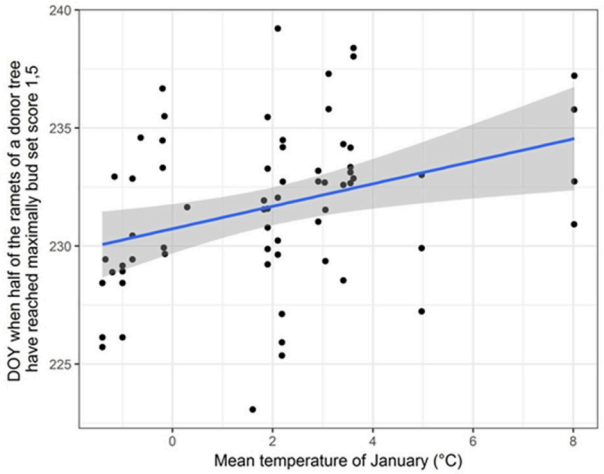

FIGURE 3 | Scatter plot displaying the correlation between the average January temperature at the home-site of the donor trees and the day of the year (DOY) when half of the ramets of a donor tree have reached the probability for having maximally bud set score 1.5 ($D_{50\%}$ -values). A linear regression line with 95% confidence intervals is shown.

Preite et al., 2015; Whipple and Holeski, 2016). Although microenvironmental variation among plants, differences in the ontological status of the ramets, epigenetic mutations and/or differences in storing time of the ramets, may be associated to some extent with methylation variability. Only a few studies have taken advantage of common garden approaches for studying the persistence of environmental-induced epigenetic variation over generations through clonal reproduction. For instance, in the Japanese knotweed [*Fallopia japonica* (Houtt.) Ronse Decr.], Richards et al. (2012) found evidence of the persistence of naturally induced epigenetic marks through clonal reproduction. Comparing the same individuals, Richards et al. (2012) found nearly five times as many variable positions detected in the epigenetic MS-AFLP analysis compared to the genetic AFLP analysis. Verhoeven et al. (2010), on the other hand, triggered stress-induced epigenetic variation in dandelion (*Taraxacum officinale* F. H. Wigg.) by chemical induction of herbivore and pathogen defenses, and found that the majority of artificially induced epigenetic variation was asexually inherited over the next generation. Our results contribute to the evidence that the environment can have an additional role in generating asexual heritable variation through epigenetic marks. Even so, multi-generation common garden experiments across multiple environments will be necessary to provide insights into the stability of the epigenetic variation found beyond the first generation and in the effect of the environment on the phenotype (Whipple and Holeski, 2016).

Epigenetic Associations With Climate Variables

Although our results suggest asexual heritable epigenetic variation, we could not directly link DNA methylation variation with relevant climate variables at the landscape scale (>1,000 km). A number of studies have investigated the role of epigenetics in response to environmental conditions in plants at the local or regional scale (e.g., Richards et al., 2012; Medrano et al., 2014; Dubin et al., 2015; Foust et al., 2016), although only a few have studied genome-wide natural DNA methylation variation in relation to climate at the landscape scale (Preite et al., 2015; Gugger et al., 2016; Keller et al., 2016). Using the MS-AFLP technique, significant correlations could be detected between single-nucleotide methylation polymorphisms and the environment at limited spatial scales (scale length <100 km) (e.g., Richards et al., 2012; Medrano et al., 2014; Foust et al., 2016) while, similar to this study, no or very weak correlations are documented on larger spatial scales (Preite et al., 2015; Foust et al., 2016). A potential explanation for these contra-intuitive observations could be that site-specific conditions may cloud the ability to detect significant correlations with spatial or climate variables over larger distances when using a relative small number of MS-AFLP marks (Foust et al., 2016). Using more powerful whole-genome bisulphite-sequencing methodologies, stronger associations were indeed found at the single-nucleotide methylation level with spatial structure and climate variables, especially temperature, in the long-lived *Quercus lobata* Née 1801 (Gugger et al., 2016) and in *A. thaliana* (Dubin et al., 2015; Keller et al., 2016). These studies documented several climate- and space-associated single methylated variants (SMVs). Many of them were CG-SMVs that tended to occur in or near genes involved in plant's response to environment, suggesting that gene body CG-methylation variation may play an important role in plant's response to adapt to variable climatological conditions (Platt et al., 2015; Gugger et al., 2016; Keller et al., 2016). Moreover, in *A. thaliana*, more strong methylation associations with climate were found at the regional scale (Sweden) compared to the broader geographical scale (Eurasia) (Keller et al., 2016). As discussed by Keller et al. (2016), the epigenetic associations with climate variables appear to depend on the geographic scale as well as the sample size. Mechanisms of local adaptation may be restricted geographically such that global models obscure patterns occurring within regions (Lasky et al., 2012; Keller et al., 2016).

Epigenetic Effects on the Heritable Phenotype

Despite the increasing awareness of the potential role of naturally induced epigenetic changes in an organism's capacity to adapt to its local environment, the contribution of epigenetic effects to the heritable phenotype is largely unexplored (reviewed by Verhoeven et al., 2016; Richards et al., 2017). Here, we found a significant variation in timing of cessation of growth (bud set) in ramets collected from long-lived Lombardy poplars growing over a large geographic range in contrasting environments, after correcting for physiological effects. By growing clonal offspring in a greenhouse experiment, we were able to show that ramets from colder origins set bud slightly quicker than ramets obtained from warmer origins. The average January temperature and the average monthly potential evapotranspiration of the maternal environment were significantly correlated with time of bud set

and thus with the timing of the cessation of growth elongation in the common environment. Although we did not study bud set on the donor trees at the collection sites, former studies in *Populus* have shown that trees from more southern locations indeed ceased shoot growth later in summer than trees from more northern origins (e.g., Farmer, 1996; Rohde et al., 2011; Evans et al., 2016). Moreover, the seasonal growth cycle is known to be genetically controlled in *Populus*, and it is considered ecologically important and even subjected to divergent selection (Rohde et al., 2011; Evans et al., 2016). Different environmental conditions can influence the expression levels of specific genes, resulting in reaction norms, i.e., the specific way that different genotypes respond phenotypically to various environmental signals (e.g., Grenier et al., 2016). However, the contribution of phenotypic plasticity to variation in bud set, or the degree to which the expression of genes is modulated by different environmental conditions, is not well-understood (Rohde et al., 2011). Our results suggest that, in Lombardy poplar, epigenetic marks contribute to variation of phenotypic response that can be transferred to the asexually reproduced offspring resulting in locally adapted ecotypes. So far, effects of DNA methylation on the transgenerational phenotype in the absence of genetic diversity, has only been studied on *A. thaliana* using epigenetic recombinant inbred lines (epiRILs). These studies detected heritable phenotypic effects for root length and flowering time (Zhang et al., 2013; Cortijo et al., 2014). In our study, we could not find direct evidence for the link between epiloci and bud set. The complex genetic architecture behind bud set in *Populus* sp. with many genes of small effects (Rohde et al., 2011; Evans et al., 2016), combined with the low local sample size in our study, may explain why we did not find direct evidence for specific epiloci correlated with time of bud set. However, the results suggest that heritable phenotypes also exist under natural conditions and in the absence of genetic variation. It is therefore plausibly that local adaptive variation, as a result of epigenetic variation and driven by the climate, exists in Lombardy poplar. Although our study system, the Lombardy poplar, does not consist of natural populations, it is likely that similar mechanisms occur in natural populations of the European black poplar.

CONCLUSION

The results of our study support the prediction that epigenetic-based transgenerational inheritance might be relevant for evolution in rapidly changing environments (e.g., Bräutigam et al., 2013; Gugger et al., 2016; Whipple and Holeski, 2016). Whether such transgenerational effects persist over several years and over multiple clonal generations requires further investigation combining epigenomics with common garden experiments over multiple generations.

DATA AVAILABILITY

The datasets for this study (the binary data matrix of the scored MS-AFLP profiles and the data of the bud set observations) are available on Dryad Digital Repository, doi: 10.5061/dryad.2gf700s.

AUTHOR CONTRIBUTIONS

AV, BH, SC, and KC designed the study, AV organized the research and collected the data, AV, KC, and KV analyzed the data. AV, KC, and KV contributed to the interpretation of the data. AV, KC, RB, SC, AC, FG, BH, MS, and KV contributed to the writing of the manuscript and all authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01635/full#supplementary-material

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Transient Stability of Epigenetic Population Differentiation in a Clonal Invader

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Epigenetic variation may play an important role in how plants cope with novel environments. While significant epigenetic differences among plants from contrasting habitats have often been observed in the field, the stability of these differences remains little understood. Here, we combined field monitoring with a multi-generation common garden approach to study the dynamics of DNA methylation variation in invasive Chinese populations of the clonal alligator weed (*Alternanthera philoxeroides*). Using AFLP and MSAP markers, we found little variation in DNA sequence but substantial epigenetic population differentiation. In the field, these differences remained stable across multiple years, whereas in a common environment they were maintained at first but then progressively eroded. However, some epigenetic differentiation remained even after 10 asexual generations. Our data indicate that epigenetic variation in alligator weed most likely results from a combination of environmental induction and spontaneous epimutation, and that much of it is neither rapidly reversible (phenotypic plasticity) nor long-term stable, but instead displays an intermediate level of stability. Such transient epigenetic stability could be a beneficial mechanism in novel and heterogeneous environments, particularly in a genetically impoverished invader.

Keywords: alligator weed, *Alternanthera philoxeroides*, biological invasions, clonal plants, DNA methylation, epigenetic variation, phenotypic plasticity, population differentiation

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INTRODUCTION

Epigenetic modifications can modulate gene expression without altering the underlying DNA sequence (Jones, 2012). Recently, the study of epigenetic modifications, i.e., epigenetics, has attracted increasing attention of ecologists and evolutionary biologists because epigenetic processes may play a role also in the ecology and evolution of natural populations. Specifically, epigenetic variation among individuals can be a source of phenotypic variance within and among plant populations (Herrera and Bazaga, 2011; Medrano et al., 2014; Kooke et al., 2015; Zhang et al., 2018) and can affect their ecological performance, niche breadth, evolutionary potential and invasion

success (Bossdorf et al., 2010; Richards et al., 2010; Herrera et al., 2012; Latzel et al., 2013; Zhang et al., 2013; Hawes et al., 2018). In addition, epigenetic modifications are important for genomic stability during plant hybridization and polyploidization, thus paving the way for genome evolution (Bossdorf et al., 2008; Richards, 2008). These initial observations stimulated a new discipline of ecological epigenetics (Bossdorf et al., 2008; Richards et al., 2017) which focuses on the causes and consequences of epigenetic variation in natural populations. Currently, much of our knowledge on plant epigenetics is from model species like *Arabidopsis thaliana* (Schmitz et al., 2013; Heard and Martienssen, 2014). We know that epigenetic modifications can occur spontaneously or plastically in response to environment stimuli (Richards et al., 2017), that many epigenetic modifications are reset during mitosis or meiosis, but that others are stably maintained throughout the life time of organism, or even transmitted across generations (Richards, 2008; Richards et al., 2010). Such stable epigenetic modifications may provide additional raw material for natural selection to act upon (Bossdorf et al., 2008). For understanding the ecological significance of epigenetics, however, it is important to test whether findings in *Arabidopsis* also hold for wild species, and to what extent natural epigenetic variation is stable enough to play a role in the evolution of plant populations under field conditions (Kalisz and Purugganan, 2004; Richards, 2008; Richards et al., 2017).

The currently most studied epigenetic modification in ecological epigenetics is DNA methylation. It can be investigated easily in large numbers of individuals sampled from natural populations using Methylation Sensitive Amplified Polymorphism (MSAP) markers (Angers et al., 2010), a modification of the AFLP technique. In the past years, MSAP studies often found significant epigenetic population differentiation in wild plant populations, and that epigenetic variation is associated with environment (see Kilvitis et al., 2014 for a recent review). These patterns were confirmed in different plant species (Herrera and Bazaga, 2010; Lira-Medeiros et al., 2010; Richards et al., 2012). However, the origins and stability of epigenetic-environment association often remained unclear. One possibility is that epigenetic differences observed in natural populations are environmentally induced and are therefore reversible when environments change, i.e., they reflect phenotypic plasticity. Another possibility is that these epigenetic differences result from spontaneous epimutation, have been shaped by natural selection and/or (epi-)genetic drift, and are stable across generations (Richards et al., 2017). To test these two contrasting hypotheses, it is necessary to analyze the dynamics of epigenetic population variation both in the field and under common environmental conditions (Bossdorf et al., 2008). Since environmentally induced epigenetic variation could also have a transient stability, i.e., persist across a limited number of generations, a multi-generation common-garden approach is particularly powerful (Whipple and Holeski, 2016).

An important question in ecological epigenetics is how important epigenetic variation is relative to genetic variation. Although many epigenetic modifications may be partly or completely controlled by DNA sequence ("obligatory" or "facilitated" epigenetic variation *sensu* Richards, 2008), others may be independent of DNA sequence ("pure" epigenetic variation). From an evolutionary perspective, pure epigenetic variation is especially interesting because, if heritable and related to phenotype, it provides additional phenotypic variation and thus broadens the potential for evolution and adaptation, even in species lacking DNA sequence variation (Bossdorf et al., 2008). However, the complex interactions between genetic and epigenetic processes make it very difficult to evaluate these questions in natural populations of many species, in which the two factors are often confounded (Bossdorf et al., 2008). One solution to isolate epigenetic processes for more thorough study is to use asexual organisms as research system. In plants, asexual reproduction is widespread, so individuals occurring in different habitats may belong to the same clone lineage, thus providing natural replication of nearly identical genomes across contrasting environments. Moreover, epigenetic variation within the same lineage is necessarily independent of DNA sequence, thus providing opportunities for studying epigenetics-environment relationships in natural populations without the confounding effects of genetic variation (Bossdorf et al., 2008; Richards, 2008; Douhovnikoff and Dodd, 2015).

Some researchers have argued that epigenetic processes may be particularly relevant for the ecological success of asexually reproducing species, because they may generate phenotypic variation even in genetically uniform clonal stands, and thus allow these species to acclimate or adapt to new environments (Castonguay and Angers, 2012; Verhoeven and Preite, 2014; Douhovnikoff and Dodd, 2015). In clonal plants that continuously produce new modules, epigenetic modifications could accumulation over time and result in progressive acclimation (Douhovnikoff and Dodd, 2015).

Some asexually reproducing plants are highly successful invasive species that occur across broad geographic and environmental ranges (Pyšek, 1997; Silvertown, 2008). One of the most dramatic examples is alligator weed (*Alternanthera philoxeroides*), a native to South America which has become invasive in many countries (Holm et al., 1997). Alligator weed can form dense monocultures through clonal growth and cause substantial ecological and economic damage (Li and Xie, 2002). In the native range of alligator weed, both sexual and asexual reproduction are observed (Sosa et al., 2007), but invasive Chinese alligator weed populations are dominated by asexual reproduction, and DNA marker studies found them to be genetically uniform (Xu et al., 2003; Ye et al., 2003). Despite this lack of genetic variation, alligator weed occurs across a broad geographic and climatic range and in highly heterogeneous habitats in China (Pan et al., 2006; Chen et al., 2008; Geng et al., 2016). Previous studies showed that morphological plasticity and clonal integration may contribute to the adjustment of alligator weed to heterogeneous habitats on small spatial scale (e.g., terrestrial vs. aquatic, Geng et al., 2007; Wang et al., 2009; Gao et al., 2010; Xu et al., 2010; You et al., 2014). However, the mechanisms underlying the species' adjustment to large-scale environmental variation, such as climate differences, are not clear yet. In a previous study, we found significant epigenetic differentiation not only among different habitat types but also

among three geographically distinct populations (Gao et al., 2010), suggesting a potential role of epigenetic processes at larger scales. However, the origin and stability of these epigenetic differences are still unknown.

Here, we studied epigenetic variation in genetically uniform invasive Chinese populations of alligator weed. We repeatedly analyzed DNA methylation in populations from different climatic areas, as well as in multiple generations of their offspring grown in a common environment. This allowed us to assess the stability and consistency of epigenetic population differentiation, taking advantage of alligator weed as an excellent model system for studying pure epigenetic variation.

MATERIALS AND METHODS

Study Species

Alligator weed (*Alternanthera philoxeroides*) is a stoloniferous, perennial herb native to South America. In China, the earliest herbarium specimens are from Shanghai in the 1930s, and from other areas of Eastern China in the 1940s (Chen et al., 2008). During the 1950–1970s, the geographic distribution of alligator weed rapidly increased because it was introduced as a fodder crop to many provinces, where it subsequently escaped and established wild populations (Chen et al., 2008). Thus, most Chinese populations have a short history of less than 70 years. At present, alligator weed occupies a geographic range from 20 to 40 degree northern latitude (**Figure 1A**), covering a broad climatic range from tropical to sub-tropical and temperate climate. Alligator weed produces small clover-like white flowers in the summer, but the flowers usually drop before the seeds are mature. Alligator weed has a very vigorous asexual reproduction, with small stem or rhizome fragments rapidly developing into new individuals (Dong et al., 2012; Guo and Hu, 2012). In Northern China, all aboveground biomass of alligator weed dies during the cold season, but belowground roots and rhizomes remain alive and can re-sprout in the spring. In southern areas with mild winter, in contrast, alligator weed grows throughout the year. Asexual reproduction greatly contributes to the species' rapid spread (Guo and Hu, 2012). In aquatic habitats, broken stem fragments can disperse long-distance by water flow, and in terrestrial habitats rhizome fragments of alligator weed are often spread unintentionally over long distance as soil contaminants. Thus, the population regeneration and spread of alligator weed in China is entirely by asexual means. Indeed, molecular marker analyses showed that many invasive populations across large climatic gradients belong to the same clone (Xu et al., 2003; Ye et al., 2003), which make the species an intriguing study system for *in situ* ecological epigenetic studies.

Field Sampling

To encompass the climate range experienced by alligator weed in China, we selected six invasive Chinese populations, ranging from N°22.8 to N°36.5 in latitude (**Figure 1A**), and all from terrestrial habitats. In China, alligator weed occurs in a variety of habitats including aquatic and terrestrial ones (Geng et al., 2007), but in this study we wanted to focus on large-scale variation of alligator weed driven by climate rather than smaller-scale habitat variation, and we therefore collected samples only from terrestrial habitats that are more strongly affected by climate than aquatic ones. In summer 2012, we sampled 10 healthy ramets from each population. To ensure that the ramets were not from the same physiological individual, we kept a minimum distance of 10 m between samples. Moreover, to minimize epigenetic variation caused by differences in plant development, we always sampled the fourth or fifth pair of mature leaves from the stem apex. All leaf samples were dried and stored in plastic bags with silica gel until their use for genetic and epigenetic analyses. In addition to the leaf samples, we also collected the stems (apex to sixth or seventh node) of the same ramets for setting up the common garden experiment (see below). To be able to assess the temporal stability of epigenetic variation in the field, we repeated the leaf sampling again in summer 2014 in the same populations, using the same protocol as in 2012.

Common-Environment Experiments

To assess the heritability of epigenetic variation observed in the field, and thereby disentangle plasticity from heritable variation, we set up a common-garden experiment at Yunnan University in Kunming. All 60 ramets collected from the six invasive populations were planted individually into 4-L plastic pots filled with a 1:1 mixture of vermiculite and sand and placed randomly in an outdoor garden where they were exposed to ambient temperature and precipitation. Each pot was fertilized with 4 g of slow-release fertilizer (N:P:K ratio of 15:11:13; Osmocote controlled release all purpose fertilizer, the Scotts Company, Marysville, OH, United States) and was watered regularly with tap water. The common garden experiment ran for 2 years. After 12 months, we collected leaf material for epigenetic analyses, using the same protocol as in the field sampling. Thereafter we removed all remaining leaves and shoots, leaving only a stem segment with six nodes which was then re-planted into a pot with fresh substrate. The plants regenerating from these stems were considered the second asexual generation. We repeated the procedure in summer 2014, resulting in a third asexual generation (**Figure 1B**).

Since our molecular analyses of the first asexual generations showed a gradual decrease, but not complete loss, of epigenetic population differentiation (see below), we were asking ourselves whether epigenetic differences between populations would eventually completely disappear. Therefore, to fast-forward the asexual generation cycle, we moved the plants to a growth chamber (Percival® E-36L2) in April 2016, where the plants experienced constant benign growth conditions, with a 12/12 h day: night cycle at 30°C/25°C, and a much faster generation time of one asexual generation per month. Because of space limitation, only half of the common-garden plants (5 randomly selected individuals per population) could be moved to the growth chamber. In October 2016, after six additional asexual generations in the growth chamber, we collected a final batch of samples from the altogether tenth asexual generation, for molecular analyses, using the same protocol as in the field and common garden. Altogether, we obtained plant samples from two time points in the field (2012 and 2014) and from three

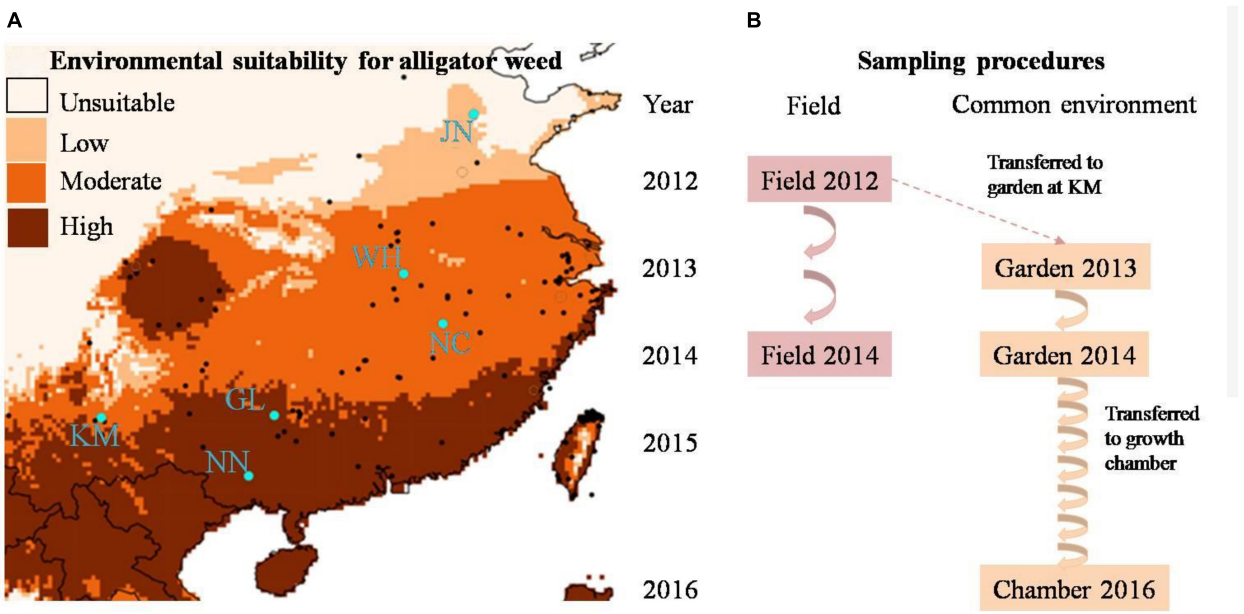

FIGURE 1 | (A) The geographic distribution of alligator weed field sites (blue dots) and **(B)** multi-generation experimental design of our study. In **(B)** the arrows indicate the numbers of generations in the field and common environment, respectively.

time points in a common environment (2013 and 2014 from the common garden and 2016 from the growth chamber, **Figure 1B**) to study the stability of epigenetic population differentiation.

Molecular Lab Work

We isolated total genomic DNA from all silica gel-dried leaf samples using the TIANGAN Plant Genomic DNA kit (Tiangen Biotech, Beijing, China) following the standard manufacturer protocol. The DNA samples were then dissolved in 50 μl TE buffer and stored at −20°C. To assess the genetic variation within and among invasive populations we used amplified fragment length polymorphism (AFLP) fingerprinting, following the standard protocol Vos et al. (1995) with some modifications (Gao et al., 2010). We used nine EcoRI/MseI primer combinations for selective amplification: AGG/CAA, AGC/CAA, AAC/CTT, ACA/CTA, CAA/CAT, AGC/CTT, AGC/CTA, AGG/CTT, and AGG/CAT. The epigenetic variation among and within populations was analyzed with the methylation-sensitive amplified polymorphism (MSAP) technique, which is related to AFLP markers and follows the same protocol as described above, except that the frequent cutter MseI was replaced by methylation-sensitive restriction enzymes HpaII and MspI. HpaII and MspI are a pair of isoschizomers which can both cleave 5′-CCGG sequences but have different sensitivities to the methylation at internal or external cytosine (Schulz et al., 2013). The differences in the final PCR products thus reflect different methylation states at the cytosines of CCGG sites and allow detecting epigenetic differences among plant samples. We conducted MSAP analyses of all alligator weed samples using ten EcoRI+ HpaII/MspI primer combinations each with three selective nucleotides: AAG/TCC, ACA/TCG, ACT/TCT, ACC/TGA, AGA/TTC, AGG/TTG, AAC/TCT, AAG/TTC, AAC/TGA, and AAC/TCAA. The fragments were separated on 6% sequencing gels and silver-stained as described above and scanned for band scoring. To assess the reproducibility of our analyses, we repeated the half of the MSAP analyses with independent DNA isolations. Throughout the molecular analyses, all samples were randomized to avoid any systematic biases or errors.

Data Scoring

To obtain multilocus genotypes and epigenotypes for all plants, we scored all reproducible fragments between 100 and 500 bp as present (1) or absent (0) for AFLP and MSAP data, generally excluding samples of poor visual quality. All fragment scoring was done by the same person unaware of sample identities. The AFLP data was scored as a binary matrix following the standard protocol (Gao et al., 2010), whereas the status of MSAP loci was determined through comparison of the EcoRI/HapII and EcoRI/MspI fragment profiles, with four possible outcomes: (I) fragments were present in both profiles (1/1), (II) fragments were present only with EcoRI/MspI (0/1), (III) fragments were present only with EcoRI/HpaII (1/0), or (IV) fragments were absent with both cutters (0/0). The first three outcomes indicate different methylation status, while the last outcome is uninformative because it can have different causes including methylation variation or DNA sequence mutation (Schulz et al., 2013). Since in our study alligator weed harbored hardly any genetic variation, which was consistent with previous findings (Xu et al., 2003), we considered the fragments of type IV (0/0) as methylated and included them in our dataset. The raw MSAP data was

thus a multi-state matrix containing condition I, II, III and IV. Before further analyses, we transformed this matrix into a binary matrix following the 'Mixed Scoring 2′ method of Schulz et al. (2013), which distinguishes between three types of markers: m-type (full methylation), h-type (hemimethylation) and u-type (no methylation). Monomorphic loci were generally excluded from the data set to avoid biases in parameter estimation (Bonin et al., 2004).

Data Analyses

We analyzed the binary AFLP and MSAP data sets with a band-based strategy (Bonin et al., 2004) and used the R script by Schulz et al. (2013) to calculate genetic and epigenetic diversity within populations, as well as the percentage of polymorphic loci and Shannon's diversity index. Due to the extremely low levels of genetic diversity revealed by the AFLP markers, all further analyses were done only for the MSAP data. First, we visualized patterns of epigenetic variation through principal coordinate analyses (PCoA) based on a matrix of Nei and Li distances calculated with DISTAFLP (Mougel et al., 2002). The distance matrices were square root-transformed to meet the assumptions of PCoA (Legendre and Legendre, 1998). Second, we calculated a hierarchical AMOVA to test for the significance of epigenetic differentiation among populations and groups (five different growth environments and/or years of sampling), with the probability of non-differentiation (PhiPT = 0) estimated over 9,999 permutations. In addition, we also calculated pairwise PhiPT comparisons (an analog of the F_{ST} index) between populations within each of the five groups, plus pairwise comparisons of different groups using Kruskal–Wallis rank sum tests. Last, we ran Mantel tests to test for relationships between AFLP and MSAP distances of individuals, and between genetic, epigenetic and geographic distances at the population level. PCoA, AMOVA, and Mantel test were done with GenAlex 6.5 (Peakall and Smouse, 2012), the Kruskal–Wallis rank sum test in R.

To better understand the dynamics of DNA methylation across generations, we further analyzed the stability of individual epiloci (i.e., conditions I, II, III, and IV) following the method of Herrera et al. (2013), where the stability of an epilocus is defined as the proportion of plants with unchanged DNA methylation status across time, in our case experimental generations. From our technical controls we knew that the error rate of MSAP markers was 1.71%, so we considered loci with stability above 98.29% as 'stable.' We tested the stability of epiloci from the field to the common environment was estimated for three different durations: (1) across two generations, the 2012 field data and 2013 garden data, (2) across three generations, the 2012 field data and both 2013 and 2014 garden data, and (3) across 10 generations, the 2012 field data, 2013 and 2014 garden data and 2016 growth chamber data.

RESULTS

Genetic and Epigenetic Diversity in the Field

We scored a total of 469 AFLP bands and found only six polymorphic AFLP loci (1.28%) and five distinct multi-locus genotypes. One dominant genotype represented 44 of the 60 samples (73.3%) and occurred in all six populations along the geographic gradient. At the population level, the average percentage of polymorphic loci was 0.34% and the average Shannon's diversity was 0.002, indicating extremely low genetic diversity (**Table 1**). There was no significant genetic differentiation among populations (PhiPT = 0.036, $P = 0.171$) and no isolation-by-distance ($r = 0.227$, $P = 0.202$) at the genetic level.

TABLE 1 | Genetic and epigenetic diversity of six populations of *Alternantheraphiloxeroides*in the field, common garden, and growth chamber.

	GL	JN	KM	NC	NN	WH	Mean
%Polymorphic loci							
AFLP loci (469)							
Field 2012	0.42	0.21	0.21	0.83	0.21	0.21	**0.34**
MSAP sub-loci (732)							
Field 2012	9.29	2.60	6.28	7.24	7.79	4.92	**6.35**
Field 2014	8.61	5.74	7.38	7.51	10.93	4.78	**7.49**
Garden 2013	8.88	1.50	4.92	6.01	8.20	7.92	**6.24**
Garden 2014	7.51	2.05	6.97	7.51	7.51	6.15	**6.28**
Chamber 2016	1.23	0.96	1.23	1.09	3.42	1.37	**1.55**
Shannon's diversity							
AFLP loci (469)							
Field 2012	0.003	0.001	0.002	0.002	0.005	0.001	**0.002**
MSAP sub-loci (732)							
Field 2012	0.067	0.017	0.042	0.047	0.057	0.034	**0.044**
Field 2014	0.055	0.036	0.046	0.043	0.079	0.033	**0.049**
Garden 2013	0.064	0.009	0.035	0.039	0.057	0.055	**0.043**
Garden 2014	0.063	0.016	0.057	0.061	0.064	0.051	**0.052**
Chamber 2016	0.011	0.008	0.01	0.01	0.028	0.011	**0.013**

Epigenetic diversity, in contrast, was much higher within and among populations in the field. Out of a total of 510 MSAP multi-state markers (i.e., four possible outcomes with two restriction enzymes), 369 were polymorphic (77.65%). When we re-coded the MSAP multi-state markers into binary data (following Schulz et al., 2013), this resulted in 732 polymorphic subloci (m-, h-, and u-type). At the population level, epigenetic diversity was nearly 20-fold higher than genetic diversity, with an average percentage of polymorphic loci of 6.35% and an average Shannon's diversity of 0.044 (**Table 1**). When these analyses were done separately for the three types of sub-loci, the patterns were similar (**Supplementary Table S1**). AMOVA indicated significant epigenetic differentiation among populations (PhiPT = 0.894, $P < 0.001$) (**Table 2**), and principal coordinates analysis (PCoA) also showed a clear separation of the six populations, based on their epigenetic variation in the field in 2012 (**Figure 2**). Mantel tests showed that epigenetic variation (at the level of individuals) was independent of genetic variation ($r = 0.02$, $P = 0.272$), and that there was (at the population level) a non-significant negative correlation between epigenetic and geographic distance ($r = -0.287$, $P = 0.171$).

Temporal Stability of Epigenetic Variation

Comparison of the epigenetic profiles of the 2 years of field samples showed that the epigenetic population differentiation observed under field conditions was remarkably stable across years. Not only were the levels of epigenetic diversity (**Table 1**) and the overall level of population differentiation (**Table 2**) similar, but the 2012 and 2014 samples from the same populations generally occupied very similar positions in the PCoA space (**Figure 2**). In the common environment, in contrast, the epigenetic variance among populations gradually decreased (**Table 2**), and populations became more similar with increasing numbers of asexual generations (Field 2012 to Garden 2013, Garden 2014 and Chamber 2016 in **Figure 2**). However, even in the growth chamber in 2016, after 10 asexual generations, there was still significant epigenetic population differentiation (**Table 2**). These results were confirmed by the stability analyses of individual epiloci, where around half of the epiloci remained unchanged between two successive generations, but the proportion of changed epiloci increased significantly with increasing numbers of generations (**Table 3**). Nevertheless, even after 10 asexual generations, there were still 38% of the epiloci unchanged. The stability of epiloci also depended on its methylation status: the most stable epiloci were fully methylated ones (type I; 1/1) whereas unmethylated ones (type IV; 0/0) were the least stable. Interestingly, the % epigenetic variance residing among populations did not change much across the different times points in the common environment, indicating that epigenetic differences among and within populations must have decreased at similar rates. Likewise, the overall levels of epigenetic diversity did not change from 2012 in the field to 2013 and 2014 in the common garden, and they only dropped strongly when the plants were grown in the much more homogenous environment of the growth chamber (**Table 1**).

TABLE 2 | Results of AMOVA of six populations of *Alternanthera philoxeroides* in the field, for AFLP data and for MSAP data from different years and growth environments.

	Variance among populations	Variance within populations	*P*-value	Phi-statistics
AMOVA results for AFLP data				
Field 2012	0.009 (4%)	0.248 (96%)	0.171	0.036
AMOVA results for MSAP data				
Field 2012	67.438 (89%)	7.957 (11%)	0.000	0.894
Field 2014	58.120 (87%)	8.724 (13%)	0.000	0.869
Garden 2014	55.817 (88%)	7.819 (12%)	0.000	0.877
Garden 2014	39.011 (78%)	11.200 (22%)	0.000	0.777
Chamber 2016	36.861 (93%)	2.783 (7%)	0.000	0.930

DISCUSSION

Although there is currently much speculation about the potential adaptive significance of natural epigenetic variation, two key questions remain difficult to tackle: the temporal stability of natural epigenetic variation, and its degree of independence from genetic variation. Here, we addressed these questions using the clonal plant invader alligator weed as a model system. We show that invasive alligator weed populations harbor substantial epigenetic but very little genetic variation, so most epigenetic variation is independent. We also show that much of the epigenetic variation is maintained in a common environment and only gradually decreases over multiple generations. This transient epigenetic stability could play a role in environmental adaptation and invasion success.

Genetic and Epigenetic Diversity in the Field

In many plant species, genetic and epigenetic variation co-occur in natural populations and are difficult to disentangle (Bossdorf et al., 2008). Here, we avoided this problem by working with an asexual species. Our MSAP analysis demonstrated abundant epigenetic diversity within and among invasive populations of alligator weed, and much of this epigenetic variation was independent of DNA sequence ("pure epigenetic variation" *sensu* Richards, 2008) as most of the samples (73.3%) shared the same AFLP multi-locus genotype. Similar contrasting levels of genetic vs. epigenetic variation have been reported in other asexual species, including the plants *Fallopia japonica* (Richards et al., 2012), *Pinus pinea* (Saéz-Laguna et al., 2014) and *Taraxacum officinale* (Preite et al., 2015), and the asexually reproducing fish *Chrosomus eos-neogaeus* (Massicotte and Angers, 2012). The high levels of epigenetic variation in genetically depauperate asexual species could have at least two reasons: First, epigenetic variation might be generally larger because of higher spontaneous epimutation rates than genetic mutation rates (van der Graaf et al., 2015), which may uncouple genetic and epigenetic variation. Second, the variation created by spontaneous epimutation can be transmitted and thus accumulated much more easily in asexual

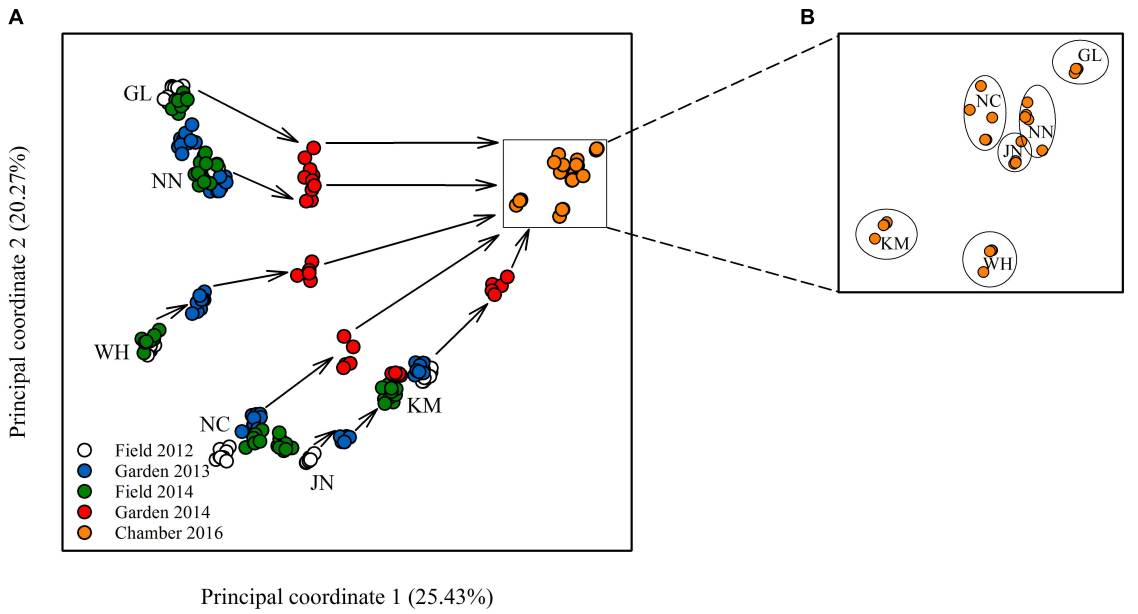

FIGURE 2 | Plot of principal coordinate analysis showing **(A)** the relative epigenetic distances among the different populations in the field and common environments, and, in a zoom-in, **(B)** the population clustering remaining after 10 asexual generations.

species where epigenetic reprogramming (i.e., the resetting of epigenetic modification) during gametogenesis and early embryo development is often circumvented through vegetative reproduction (Verhoeven and Preite, 2014).

Temporal Stability of Epigenetic Variation

We found considerable epigenetic diversity within and among natural populations, which is consistent with previous studies (Kilvitis et al., 2014). However, virtually all previous studies sampled plants at only one point in time, producing a single snapshot of epigenetic dynamics (Lira-Medeiros et al., 2010; Medrano et al., 2014; Schulz et al., 2014; Foust et al., 2016). Still, epigenetic variation is at least partly sensitive to environmental change, and it is therefore generally difficult in such studies to assess the stability and representativeness of observed epigenetic patterns (Verhoeven and Preite, 2014). In our study, we used a repeated sampling strategy also for the field, and we found that the epigenetic diversity within and differentiation among natural populations were largely stable across multiple years, as indicated by the similar Shannon indices and the similar population positions in PCoA space. The differentiation among natural populations was not simply a result of accumulating random epimutations and isolation-by-distance, as some population pairs were epigenetically more similar than others, despite their larger geographic distance.

Most importantly, we found that epigenetic differences among populations were also maintained at first in a common environment, but then progressively eroded over multiple generations. This suggests that a large part of the epigenetic variation observed in the field was environmentally induced, but it did not behave like classic phenotypic plasticity which disappeared quickly (Geng et al., 2007) but instead showed greater inertia and was transiently stable for at least several asexual generations. These results were also confirmed by our locus-by-locus analysis of stability, where the stability of epiloci was a decreasing function of the duration of the experiment. Interestingly, we found some plastic epiloci to be statistically

TABLE 3 | Stability of *Alternantheraphiloxeroides* epiloci across different numbers of generations, and the fractions of stable epiloci residing in the different epiloci types.

	Field 2012 to Garden 2013	Garden 2013 to Garden 2014	Field 2012 to Garden 2014	Field 2012 to Chamber 2016
# Changed epiloci	232 (45.5%)	202 (39.6%)	288 (56.5%)	315 (61.76%)
# Stableepiloci	278 (54.5%)	308 (60.4%)	222 (43.5%)	195 (38.24%)
Types ofstableepiloci				
Type I (1/1)	58.80%	57.40%	64.90%	71.28%
Type II (0/1)	29.00%	26.20%	25.70%	26.07%
Type III (1/0)	4.20%	5.80%	3.20%	2.05%
Type IV (0/0)	8.80%	10.60%	6.20%	0.00%
Observed Instances	278*60 = 16680	308*30 = 9240	222*30 = 6660	195*30=5850

associated with climate variables (unpublished data), indicating that some of the meta-stable epigenetic differences may have been induced by climatic variation. However, the design of our study did not really allow to address the causal drivers of epigenetic population differences, and more research is needed to understand this.

Some epigenetic population differentiation remained significant even after 10 generations of cultivation in a common environment, suggesting that part of the differentiated epiloci were either only very slowly responding to a changing environment, or they might have been permanent, reflecting stably transmitted epigenetic variation, possibly resulting from epimutation and subsequent selection. Altogether, the epigenetic variation observed among invasive alligator weed populations appears to be combination of stable and environmentally induced variation, with the majority of the environmentally induced component showing a transient stability.

It is important to note that the MSAP data in our study was based only on DNA methylation in a CG context, but not in CHH or CHG contexts, and therefore our conclusions only apply to CG context. To understand the dynamics of other DNA methylation contexts, more powerfully, NGS-based methylation analyses are needed, and should be employed in future analyses.

Implication for the Invasiveness of Alien Species

Asexual reproduction is often thought to be beneficial for the establishment and spread of alien plant species, because it provides reproductive assurance at invasion fronts where population densities are often low (Silvertown, 2008). However, the downside of such asexual spread is that the populations are often characterized by extremely low levels of genetic diversity, which may limit their adaptive potential in novel and heterogeneous habitats (Barton and Charlesworth, 1998). Epigenetic processes have been proposed to resolve this 'genetic paradox' of successful invasive species (Hawes et al., 2018): if epigenetic variation is more dynamic and rapidly generated in asexual populations, and it also associated with heritable phenotypic variation, this may significantly alleviate the evolutionary constraints in genetically depauperate invasive populations. In this case, epigenetic variation will have important effects on the invasiveness of alien species. Our results with alligator weed support this hypothesis. Similar results have been found for other invasive species (see Hawes et al., 2018 for a recent review). For example, Richards et al. (2012) found that the invasion of diverse habitats by invasive Japanese knotweed, another prominent clonal plant invader, is more correlated with epigenetic variation than with genetic variation. In a study of an introduced bird, Liebl et al. (2013) found that in invasive populations of house sparrows, genetic diversity decreased because of inbreeding but at the same time epigenetic diversity significantly increased. Thus, epigenetic variation may compensate for the loss of genetic variation in invasive species and a thus contribute to their success in novel environments.

AUTHOR CONTRIBUTIONS

YG, JY, and C-YX designed the research. WS, XC, and LG performed the wet lab work. YG performed the data analysis. WS and C-YX participated in the sampling. YG, JY, XO, OB, and C-YX drafted and revised the manuscript. All authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01851/full#supplementary-material

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Context-Dependent Parental Effects on Clonal Offspring Performance

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Parental environments may potentially affect offspring fitness, and the expression of such parental effects may depend on offspring environments and on whether one considers an individual offspring or all offspring of a parent. Using a well-studied clonal herb, *Alternanthera philoxeroides*, we first grew parent plants in high and low soil-nutrient conditions and obtained 1st generation clonal offspring from these two environments. Then we grew offspring of these two types of 1st generation clonal offspring also in high and low nutrient conditions. We measured and analyzed mean performance and summed performance of the four types of 2nd generation clonal offspring. High nutrient availability of parental environments markedly increased both mean performance (i.e., the average fitness measure across all individual offspring produced by a parent) and summed performance (i.e., the sum of the fitness measure of all offspring produced by a parent) of the 2nd generation clonal offspring. The positive parental effects on summed performance of the 2nd generation clonal offspring were stronger when the 1st generation clonal offspring grew in the high instead of the low nutrient conditions, but the positive parental effects on their mean performance did not depend on the nutrient environments of the 1st generation clonal offspring. The results provide novel evidence that parental environmental effects persist across vegetative generations and strongly depend on offspring environments and levels of plants.

Keywords: *Alternanthera philoxeroides*, clonal plant, individual and whole-generation levels, parental environmental effect, soil nutrients, vegetative offspring

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INTRODUCTION

Vegetative reproduction is a life-history trait that contributes to the wide distribution of clonal plants in natural habitats (Sosnová et al., 2011). Some clonal plants occupying large geographical areas exhibit distinct phenotypes that are in some cases derived from only one genotype or several closely related genotypes (Poulin et al., 2005; Barrett et al., 2008; Gao et al., 2010; Zhang et al., 2010). Clonal (vegetative) offspring ramets are repeatedly produced by parent ramets during the life cycle of clonal plants, and environmental effects experienced by parents may influence performance of clonal offspring. Such parental (environmental) effects have been increasingly considered an important life-history property, acting as an environmental link across generations and influencing the rapid adaptation of offspring to new environments (Schwaegerle et al., 2000; Donohue, 2009; Mousseau et al., 2009; Latzel and Klimešová, 2010; González et al., 2016).

Like genetic effects, parental effects have ecological and evolutionary significance, especially when they can pre-adapt offspring to local conditions that the parents experienced, and which the

offspring are also likely to experience (Pigliucci, 2005). Parental effects may modify propagule size to match offspring environments if they are predictable (Allen et al., 2008; Charpentier et al., 2012; Huber et al., 2014). For instance, plants in favorable habitats may produce larger but fewer seeds (or clonal offspring) to shorten time to establishment, resulting in an early competitive advantage in the next generation. By contrast, plants in unfavorable habitats may produce smaller but more offspring to potentially increase offspring dispersal away from the unfavorable habitat but at the cost of individual offspring fitness (Dong et al., 2012; Wang et al., 2014). To achieve a long-term fitness benefit, parental effects may also trigger phenotypic similarity between parents and offspring. For instance, drought-stressed plants may develop longer root systems and produce sexual offspring that also develop longer root systems (Herman et al., 2012), and plants exposed to insect herbivory produce sexual offspring with a strong herbivory-resistant phenotype (Agrawal, 1999, 2002). While many studies have tested parental effects on performance of sexual offspring, few have tested those on performance of clonal offspring and thus little is known about whether parental effects can persist across clonal generations (Latzel and Klimešová, 2010; Huber et al., 2014; González et al., 2016).

Parental effects on offspring fitness can be categorized into four predictable scenarios (**Figure 1**). In the predictable scenarios, parental effects are assumed to be caused by two types of parental environments, i.e., favorable and unfavorable environments. First, parental effects are independent of offspring environments (**Figure 1A**; Schwaegerle et al., 2000; Dong et al., 2017, 2018), i.e., offspring of parent plants grown in favorable environments always perform better than offspring of parents grown in unfavorable environments (Uller et al., 2013; Engqvist and Reinhold, 2016). Second, parental effects are context-dependent and adaptive (**Figure 1B**). Parental effects are advantageous if offspring grow in an environment similar to the one that their parents have encountered (Mousseau and Fox, 1998; Galloway, 2005), and disadvantageous if they grow in an environment dissimilar to the one that their parents have encountered (Dyer et al., 2010). Third, parental effects are advantageous only when offspring grow under favorable environments (**Figure 1C**). In *Plantago lanceolata*, for example, offspring of parents growing in nutrient-rich soils accumulated more carbohydrates in roots than offspring of parents growing in nutrient-poor soils (Latzel et al., 2014). However, such parental effects were detected only for offspring growing in nutrient-rich soils, and not for offspring in nutrient-poor soils (Latzel et al., 2014). Fourth, parental effects are advantageous only when offspring grow under unfavorable environments (**Figure 1D**). In animals, for instance, the positive effect of egg size (an indication of maternal nutritional provisioning) is often more pronounced in stressful environments (Fox, 2000; Dziminski and Roberts, 2006).

Although parental effects have been increasingly documented at the individual level (i.e., mean fitness of individual offspring; Huber et al., 2014; González et al., 2016; Groot et al., 2016), they have rarely been explored at the level of the whole offspring generation (i.e., the sum of the fitness measure of all offspring produced by a parent during, e.g., one growing season; Beckerman et al., 2002; Plaistow and Benton, 2009; Molofsky et al., 2014). From an offspring-generation perspective, parental environments may interact with, e.g., offspring survival, size and number, so that the pattern of parental effects at the offspring-generation level is more complex and unpredictable than that at the individual level (Crone, 1997; Charpentier et al., 2012). For instance, due to a potential trade-off between offspring size and number (Stuefer et al., 2010; Dong et al., 2012; Wang et al., 2014), parental effects that are adaptive at individual offspring level may not necessarily be so when fitness of all offspring of a parent are considered (i.e., at the level of the whole offspring generation), and *vice versa*. Given that parental effects have an impact on performance of the offspring generation, they may play an important role in population dynamics (Molofsky et al., 2014). Therefore, it is important to understand parental effects also at the offspring-generation level.

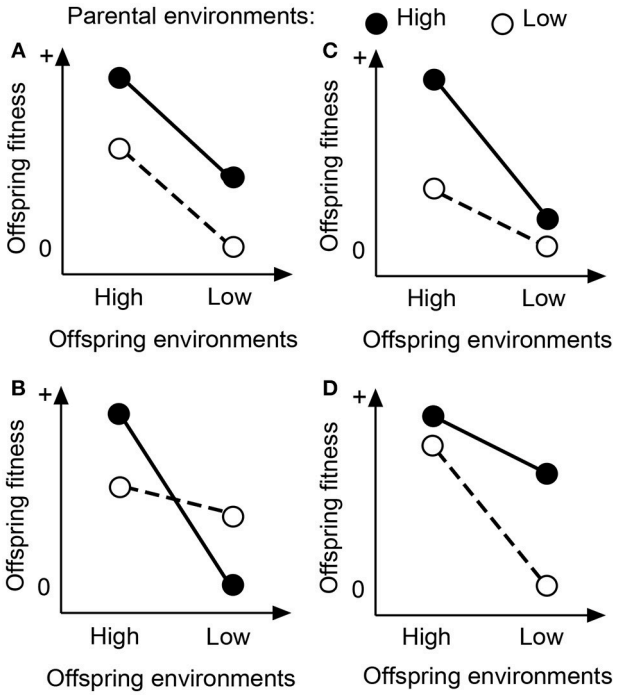

FIGURE 1 | Schematic diagram for parental environmental effects that are not related to offspring environments **(A)**, or that are related to offspring environments **(B–D)**. See the text for details.

We investigated effects of nutrient environments experienced by parent plants on performance of clonal offspring of a well-studied clonal herb, *Alternanthera philoxeroides*, both at the level of individual offspring and offspring generation. Specifically, we tested the following hypotheses. (1) Parental nutrient effects can persist across vegetative generations in clonal species. One prediction is that clonal offspring produced by parent plants subjected to high soil nutrients will perform better than do the offspring produced by parent plants subjected to low soil nutrients, since providing parent plants with high soil nutrients

may allow them to produce high-quality clonal offspring. (2) The magnitude of parental nutrient effects depends on the nutrient environments of clonal offspring. One prediction is that providing clonal offspring with high nutrient levels amplifies parental effect as shown in **Figure 1C**. (3) Parental nutrient effects at the offspring-generation level are inconsistent with the effects at the individual-offspring level. This is because parental effects at the individual level are determined only by average offspring size, while parental effects at the generation level are determined jointly by the survival, size and number of offspring.

MATERIALS AND METHODS

Study Species and Plant Material

Alternanthera philoxeroides (Mart.) Griseb. is a creeping perennial herb of the Amaranthaceae family, native to South America (Holm et al., 1997). It is listed as one of the most noxious invasive plants in China and other regions around the world (Julien et al., 1995; Sainty et al., 1998). In China, for lack of viable seeds, *A. philoxeroides* mainly relies on clonal growth by producing creeping stems and/or root fragments to achieve offspring recruitment (Wang et al., 2008, 2009). Each stem node of *A. philoxeroides* is considered an asexual individual (i.e., ramet), because it has the potential to root and develop into a physiologically independent plant (Dong et al., 2010, 2012). This species can rapidly disperse and colonize both aquatic and terrestrial habitats, causing severe economic and environmental problems (Wang et al., 2008, 2009).

For our study, original plants of *A. philoxeroides* were collected on 18–19 May 2011, from several locations in a riparian agricultural area in Zhejiang province (28.87° N, 121.01° E), in the south of China. The sampling site did not belong to any farms or national parks, so that we did not need any relevant permissions for collecting plant samples. To reduced potential phenotypic differences among the plants due to variation in parental environments, the plants had been propagated vegetatively for 4 years in a heated greenhouse at Forest Science Co., Ltd., of Beijing Forestry University. In China and Australia, *A. philoxeroides* does not produce viable seeds (Sainty et al., 1998; Zhu et al., 2015). Studies using molecular markers showed that populations sampled in South China derived from a single genotype (Xu et al., 2003; Wang et al., 2005; Li and Ye, 2006). Thus, it is very likely that the plants we collected and their clonal offspring share the same genotype.

Experimental Design

The experiment consisted of two steps (**Figure 2**). In brief, we first grew 42 parent plants each with a stem of about 15 cm long in high and low soil nutrient conditions (i.e., 21 replicates per parental treatment) and obtained seven replicates of two types of the 1st generation clonal offspring ramets for growth measurements and the remaining 14 replicates for subsequent experiment. Then we grew seven replicates of 1st generation clonal offspring ramets of each type also in high and low nutrient conditions and measured the 2nd generation clonal offspring ramets (**Figure 2**).

In more detail, on 28 June 2014, 100 stem fragments of *A. philoxeroides*, each consisting of one node bearing two opposite leaves and 3-cm-long proximal and distal internodes, were cut off from the stock plants. In the same greenhouse as the pre-cultivation, fragments were grown in planting trays filled with an 1:1 volume mixture of quartz sand (0.5–1.0 mm particle size) and peat (Pindstrup Seedling, Pindstrup Mosebrug A/S, Pindstrup, Denmark). After 3 weeks when most fragments had produced a new stem of ∼15 cm long, we selected 60 fragments (plants) of similar sizes. Of the 60 plants, 18 were harvested to measure initial dry mass (mean: 111.5 mg; 95% confidence interval: 100.1–122.4 mg; $N = 18$). The remaining 42 plants were used as parent plants, and were transplanted into pots that were 14 cm in diameter and 12 cm in depth and filled with the soil mixture described above.

We randomly assigned the 42 parent plants to two soil nutrient treatments, and thus each treatment had 21 replicates. For the high-nutrient treatment, 2 g L^{-1} of slow-release fertilizer (16 N: 9 P: 12 K: 2 Mg; Osmocote Standard, Scotts, Marysville, Ohio, USA) was mixed into the soil of each pot. For the low-nutrient treatment, no fertilizer was added. The two nutrient treatments are the nutrient conditions commonly experienced by the species. Pots were randomly repositioned once a month to minimize possible effects of environmental heterogeneity in the greenhouse. Tap water was supplied daily to keep the soil moist. The treatments lasted for 13 weeks, during which the mean air temperature (± SE) in the greenhouse was 23.1 ± 0.4°C, as measured by a Hygrochron temperature logger (iButton DS1923; Maxim Integrated Products, USA).

On 18 October 2014, we randomly chose seven replicate plants (parent ramets with clonal offspring ramets) in each treatment and counted the number of offspring ramets. The plants were then subdivided into the aboveground part (the assembly of single-node offspring ramets attached with two opposite leaves and half of both the proximal and distal internodes) and belowground part (roots), and dried at 70°C for 48 h.

For each of the remaining 14 replicate plants in each of the two nutrient treatments, we obtained single-node offspring ramets (a stem node attached with two opposite leaves and a half of proximal and distal internodes) by cutting off the nodes along the newly produced stems of each parent plant. Each of these single-node ramets (i.e., the 1st generation clonal offspring) was labeled to mark its position along the stems produced by the parent plants, and weighed to obtain initial fresh mass. The parent plants in the high-nutrient treatment each produced 10–47 offspring ramets, and those in the low-nutrient treatment each produced 6–19 offspring ramets. Each of the 1st generation offspring ramets taken from seven randomly selected plants of each of the two nutrient treatments was grown in the high nutrient treatment (adding 2 g L^{-1} of slow-release fertilizer to the soil), and each of the 1st generation offspring ramets taken from the remaining seven plants of each of the two nutrient treatments was grown in the low nutrient treatment (no fertilizer added). The soil mixture

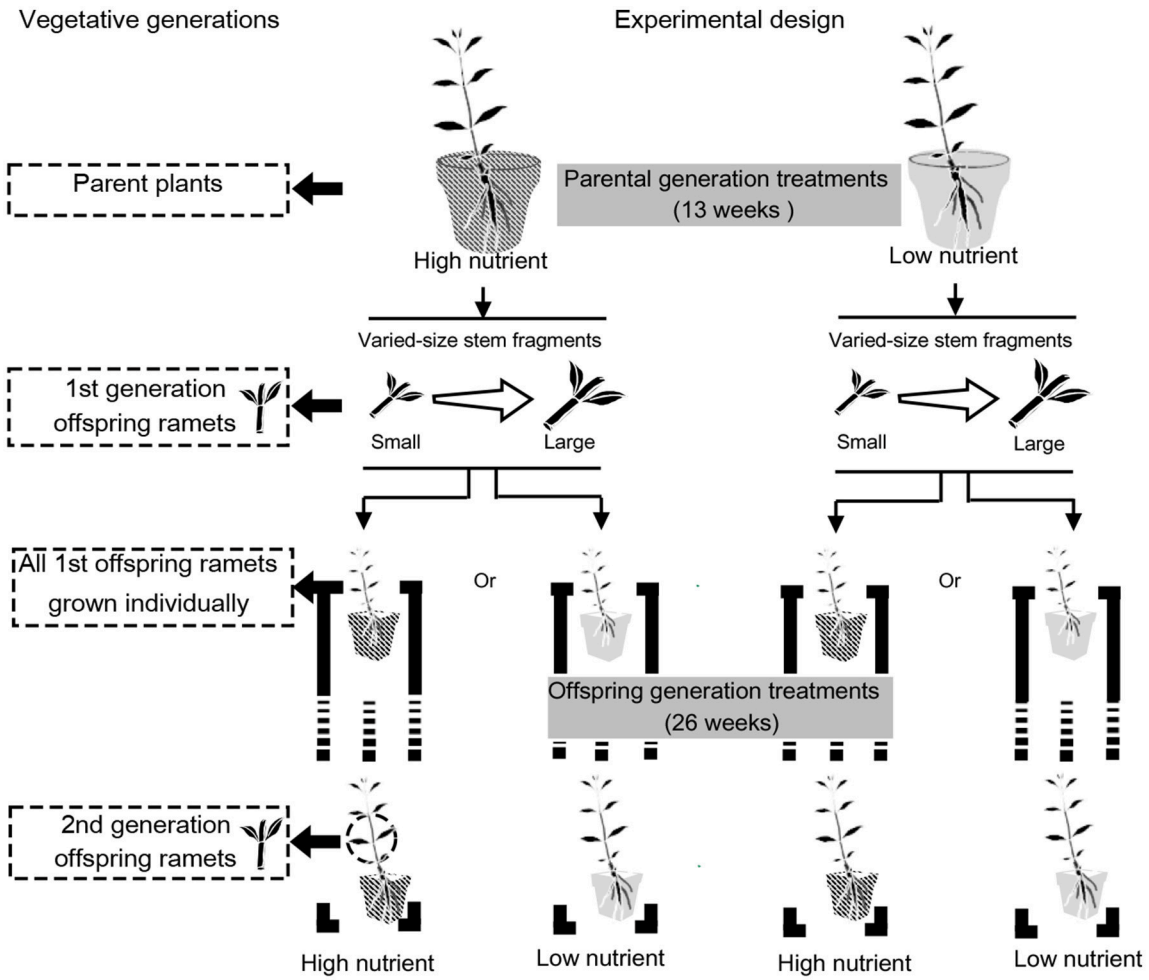

FIGURE 2 | Experimental design. The experiment consisted of two steps. First, we grew parent plants in high and low soil nutrient conditions and obtained two types of the 1st generation clonal offspring ramets. Then we grew the 1st generation clonal offspring ramets of each type also in high and low nutrient conditions and measured the 2nd generation clonal offspring ramets.

used for the 1st generation offspring ramets was the same as that for the parent plants, and all offspring ramets taken from one parent plant were grown in different cells (each 4.6 cm long × 4.6 cm wide × 11 cm deep) within the same planting tray, and subjected to one nutrient treatment. There were seven replicate trays for each of the four parent-offspring treatment combinations. Trays were randomly repositioned every month.

The treatments for the 1st generation offspring lasted for 26 weeks, from 18 October 2014 to 18 April 2015. They were conducted in the same greenhouse (mean temperature ± SE was 15.4 ± 0.2°C). At harvest, we recorded the survival status of the originally planted offspring ramets. We counted the number of the 2nd generation ramets originated from each of the 1st generation offspring ramets and also measured biomass of the 2nd generation ramets that originated from each of the 1st generation offspring ramets by drying them at 70°C for 48 h. Based on these data, we calculated the summed mass and summed number of the 2nd generation ramets produced by all the 1st generation offspring ramets from each parent plant. We also calculated mean mass and mean number of the 2nd generation ramets per 1st generation offspring from each parent plant (summed mass or number of the 2nd generation ramets divided by number of the 1st generation offspring ramets from each parent plant).

Data Analyses

We used *t*-tests to examine the effects of soil-nutrient treatments on total mass, number of ramets and mean ramet mass (shoot mass divided by number of ramets) of the 1st generation offspring (i.e., the ramets produced by the parent plants). We used two-way ANOVAs to test the effects of parental nutrient conditions (fixed effect), offspring nutrient conditions (fixed effect) and their interaction (fixed effect) on performance of *A. philoxeroides* at both the offspring generation level (summed mass and summed number of the 2nd generation ramets produced by all the 1st generation offspring from one parent plant) and the individual level (mean mass and mean number of the 2nd generation ramets

across the 1st generation offspring ramets from a parent plant). We also used two-way ANOVAs to test the effects of parental and offspring nutrient treatments on initial fresh mass and number of the surviving 1st generation offspring ramets as well as survival rate of the ramets. These data met the assumptions of homoscedasticity and normality. The analyses were conducted using SPSS 22.0 (SPSS, Chicago, IL, USA).

A three-parameter lognormal distribution $\left(Y = \frac{a}{X} * \exp\left(-0.5 * \left(\frac{\ln\left(\frac{X}{X0}\right)}{b}\right)^2\right)\right)$ was employed to fit the frequency distribution of initial fresh mass of the pooled 1st generation offspring ramets produced by parent plants grown in the high nutrient or the low nutrient treatments. In the equation, X is the initial mass of each 1st generation offspring ramet; X_0, a, and b are the location parameter, the scale parameter, and the shape parameter of distribution, respectively. The regression analyses were performed using Sigmaplot 12.5 (Systat Software Inc., San Jose, CA, USA).

RESULTS

Performance of the Parental Generation

Total mass, number of ramets and mean ramet mass of the 1st generation offspring ramets produced by the parent plants were all significantly lower in the low nutrient than in the high nutrient treatment (**Figure 3**).

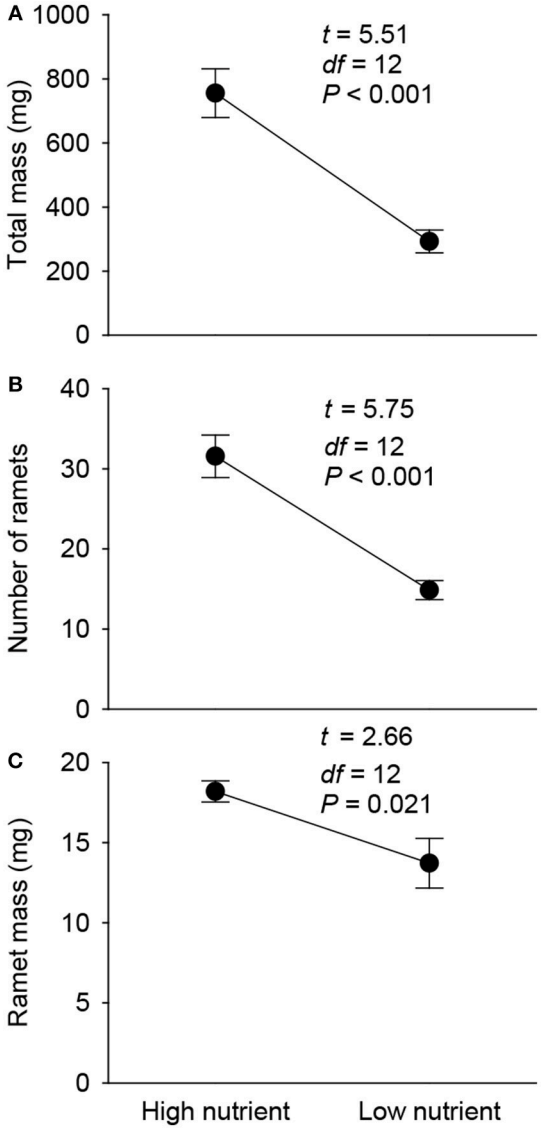

FIGURE 3 | Effects of parent nutrient treatment on total mass **(A)**, number of ramets **(B),** and mean ramet mass **(C)** of the 1st generation offspring of *Alternanthera philoxeroides*. Means + SE and *t*, *df*, and *P* values of *t*-test are given.

Performance of the Offspring Generation

Summed mass and number of the 2nd generation offspring ramets were significantly affected by parental and offspring environments, as well as by their interaction (**Table 1**, **Figure 4**). High nutrient availability of parental environments markedly increased summed performance of all 2nd generation offspring produced in the offspring generation (**Figures 4A,B**). However, these positive parental effects were stronger when the 1st generation offspring grew in the high than in the low nutrient conditions (**Figures 4A,B**). By contrast, mean mass and mean number of the 2nd generation offspring ramets were independently affected by parental and offspring environments (**Table 1**). High nutrient availability of parental and offspring environments both increased mean mass and mean number of the 2nd generation offspring ramets, but the positive parental effects did not depend on the nutrient environments of the 1st generation offspring (**Figures 4C,D**).

Initial fresh mass of the surviving 1st generation offspring was independently affected by parental and offspring environments (**Table 2**). High nutrient availability of parental environments increased initial fresh mass, and high nutrient availability of offspring environments allowed the smaller 1st generation offspring ramets to survive during the experiment (**Figure 5A**). The number of the surviving 1st generation offspring ramets was only affected by parental environments, rather than by offspring environments (**Table 2**). High nutrient availability of the parental environments increased number of the surviving 1st generation offspring ramets (**Figure 5B**). By contrast, survival rate of the 1st generation offspring was affected by neither parental nor offspring environments (**Table 2**, **Figure 5C**).

Frequency distribution of the pooled 1st generation offspring ramets subjected to each of parental nutrient treatments fitted well to the lognormal distribution (**Figure A1**). The distribution of 1st generation offspring ramets produced by parent plants growing in the high nutrient treatment was positively skewed [spanning a broad range of 2.5–66.1 mg; $R^2 = 0.886$, $F_{(2, 26)} = 83.65$, $P < 0.001$]. By contrast, the distribution of 1st generation offspring ramets produced by parent plants growing in the low nutrient condition tended to be platykurtic (flat) and symmetrical [spanning a narrow range of 1.8–34.3 mg; $R^2 = 0.924$, $F_{(2, 15)} = 79.37$, $P < 0.001$; **Figure A1**].

DISCUSSION

For the parent generation of *A. philoxeroides*, limited soil nutrients reduced biomass accumulation and new ramet production by ∼50% and mean ramet mass (or vegetative offspring size) by 20%. These results were consistent with the negative responses of *A. philoxeroides* to low resource availability (e.g., Li et al., 2014). Interestingly, parental nutrient environments exerted a strong effect on performance across vegetative generations. One apparent reason is that the offspring from parents growing in the nutrient-rich environment were relatively larger, having about 2-fold greater initial mass than did offspring taken from parents growing in the nutrient-poor environment. Such a size advantage of offspring benefited the subsequent growth of offspring both in the high and in the low nutrient environment. The variation in offspring size, and the corresponding provisioning of internal resources (e.g., non-structural carbohydrates and nitrogen) may be one of potential mechanisms that triggered the observed variation in fitness between offspring ramets taken from parents growing in contrasting habitats (Herman and Sultan, 2011; Latzel et al., 2014).

We also found that the magnitude of parental effects depended on the environment of the offspring, i.e., the positive effect of the parental high-nutrient treatment was amplified when the offspring were also in a high-nutrient environment (**Figure 1C**). To some degree, parental effects could facilitate the pre-adaptation of offspring of *A. philoxeroides* to their parental environment by modifying offspring size, thereby helping to gradually accumulate a size advantage over previous generations in favorable habitats. Such a life history may possibly contribute to the abundance and invasiveness of *A. philoxeroides* in the environments where resource availability is high, e.g., crop fields and irrigation ditches (Pan et al., 2006). The ecological significance of parental effects have also been reported in many sexually propagated species (Miao and Primack, 1991; Miao et al., 1991; Herman et al., 2012; Jacobs and Lesmeister, 2012; Latzel et al., 2014). For example, parental effects could maximize biomass and root carbohydrate storage accumulation in *P. lanceolata*, seedling yield in *Campanulastrum americanum*

TABLE 1 | Effects of parent and offspring (1st generation) nutrient treatments on summed and mean performance of the 2nd generation offspring across the 1st generation offspring of a parent plant.

Measure	Parent (P)		Offspring (O)		P × O	
	$F_{(1, 24)}$	*P*	$F_{(1, 24)}$	*P*	$F_{(1, 24)}$	*P*
Summed mass	**21.83**	**<0.001**	**10.34**	**0.004**	**5.67**	**0.026**
Summed ramet number	**29.83**	**<0.001**	**17.65**	**<0.001**	**7.20**	**0.013**
Mean mass	**12.62**	**0.002**	**38.77**	**<0.001**	2.20	0.151
Mean ramet number	**6.68**	**0.016**	**79.83**	**<0.001**	0.09	0.765

Degrees of freedom (df), F, and P of ANOVA are given. Values for which P < 0.05 are shown in bold.

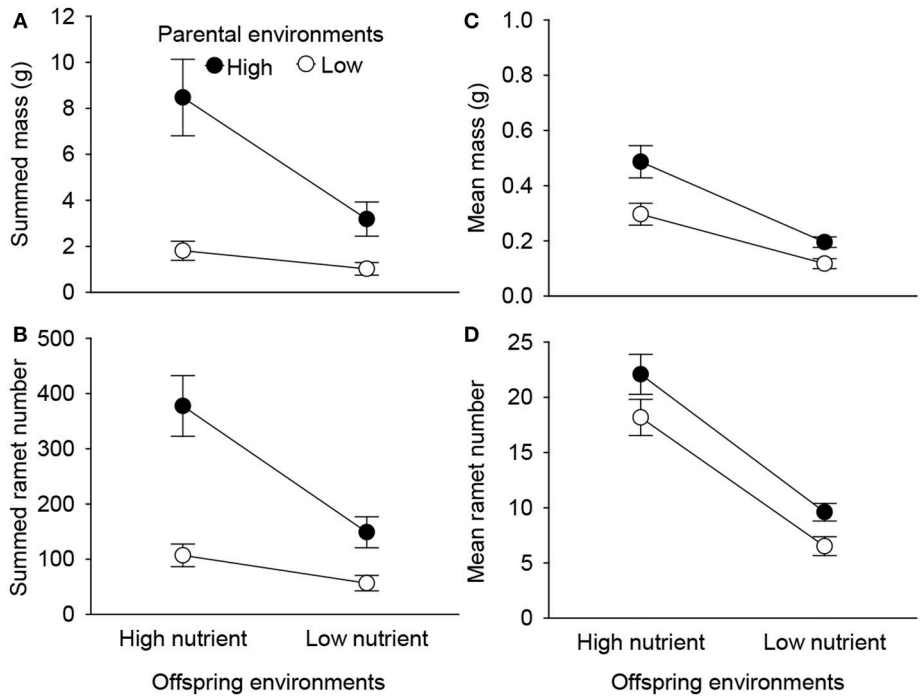

FIGURE 4 | Effects of parent and offspring (1st generation) nutrient treatments on summed mass **(A)**, summed ramet number **(B)**, mean mass **(C),** and mean ramet number **(D)** of the 2nd generation offspring of *Alternanthera philoxeroides* across the 1st generation offspring of a parent plant. Means + SE are given.

TABLE 2 | Effects of parent and offspring (1st generation) nutrient treatments on initial fresh mass and number of the surviving 1st generation offspring ramets and survival rate.

Measure	Parent (P)		Offspring (O)		P × O	
	$F_{(1, 24)}$	*P*	$F_{(1, 24)}$	*P*	$F_{(1, 24)}$	*P*
Initial fresh mass	**34.31**	**<0.001**	**4.88**	**0.037**	0.02	0.894
Number	**18.06**	**<0.001**	0.34	0.567	0.27	0.608
Survival rate	0.10	0.758	0.07	0.791	0.14	0.705

Degrees of freedom (df), F, and P of ANOVA are given. Values for which P < 0.05 are shown in bold.

and drought tolerance in *Polygonum persicaria*, when the offspring grew in the environments similar to their parental environments (Galloway and Etterson, 2007; Herman et al., 2012; Latzel et al., 2014).

While positive parental nutrient effects were detected at both individual and whole-generation levels, the patterns of these parental effects differed. Context-dependent parental effects in *A. philoxeroides* were detected at the offspring generation level (summed performance of the 2nd generation offspring across all the 1st generation offspring ramets from a parent plant), but not with respect to individual ramet performance (mean performance of the 2nd generation offspring across the 1st generation offspring ramets of a parent plant). One possible reason is that parental effects at the offspring-generation scale were jointly influenced by offspring size and offspring number, while parental effects at the individual offspring scale were only determined by mean offspring size (Hopper et al., 2003; Charpentier et al., 2012; Dong et al., 2012). Our results detected that parental nutrient environments significantly altered the survival and production of offspring, so we speculated that such variation in number of surviving 1st generation individuals may facilitate the parental effects on the summed fitness of offspring in the nutrient-rich environment, but contributed less to the parental effects on the summed offspring fitness in the nutrient-poor environment (Schwaegerle et al., 2000). Besides, parent plants growing in the high nutrient condition produced a positively skewed size distribution of offspring ramets, while parent plants growing in the low nutrient condition only produced a relatively platykurtic (flat) and symmetrical size distribution. To some extent, the changes in size distributions of offspring ramets caused by parental environments may potentially result in a difference in parental effects at individual and whole-generation levels. However, the ecological significance of the offspring-size distribution with regard to parental effects should be further explored in future studies.

We thus conclude that parental nutrient effects can persist across clonal generations of *A. philoxeroides* in terms of offspring size and number. Such positive parental effects may contribute to the colonization of *A. philoxeroides* in resource-rich habitats because parental effects lead to a gradual increase in the size advantage across clonal generations in such habitats (Marshall and Uller, 2007; Dyer et al., 2010; Gao et al., 2010). Our study also provides novel evidence that the ecological significance of parental environmental effects vary at different levels, implying

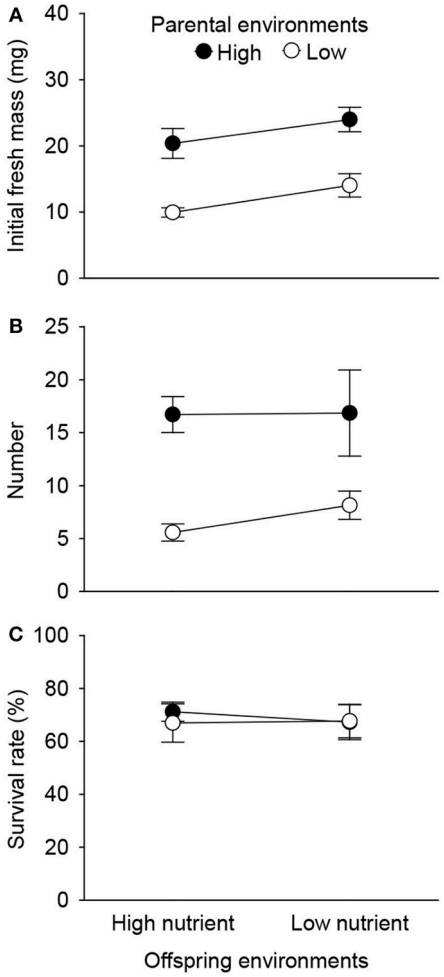

FIGURE 5 | Effects of parent and offspring (1st generation) nutrient treatments on initial fresh mass **(A)** and number **(B)** of the surviving 1st generation offspring ramets, and survival rate **(C)**. Means + SE are given.

that these effects cannot simply be extrapolated from the individual to the whole-generation level. Apart from the variation in offspring size (or resource provisioning) and offspring number, parental environmental effects may also be closely related to multiple external or internal factors, including morphological and physiological changes (e.g., plant vigor) and epigenetically based variation (e.g., DNA methylation; Bossdorf et al., 2008; Herman and Sultan, 2011; Zhang et al., 2013; Douhovnikoff and Dodd, 2015; Dodd and Douhovnikoff, 2016). Therefore, future studies that integrate morphological, physiological and molecular evidence should be necessary to better understand the mechanisms of parental environmental effects in clonal species.

AUTHOR CONTRIBUTIONS

B-CD and F-HY designed the experiment. B-CD performed the experiment. B-CD and F-HY did the statistical analysis. B-CD, MvK, and F-HY wrote the first draft of the

manuscript. B-CD and F-HY contributed substantially to the revisions.

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APPENDIX

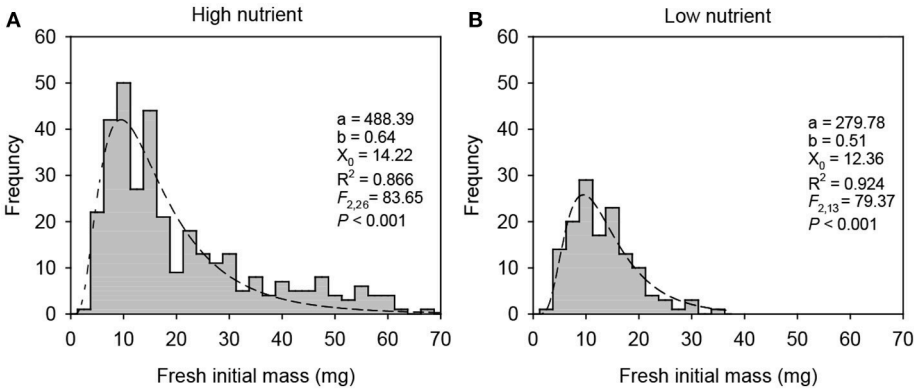

FIGURE A1 | The frequency distribution of initial fresh mass of the 1st generation offspring ramets produced by parent plants grown in **(A)** the high nutrient or **(B)** the low nutrient treatments. Each frequency distribution of the pooled 1st generation ramets subject to one of parent nutrient treatments fits well to the lognormal distribution. The equation of 3-parameter lognormal distribution is $\left(Y = \frac{a}{X} * \exp\left(-0.5 * \left(\frac{\ln\left(\frac{X}{X0}\right)}{b}\right)^2\right)\right)$, where X is the initial mass of each 1st generation offspring ramet; X_0, a, and b are the location parameter, the scale parameter, and the shape parameter of distribution, respectively. The fitted parameters of size distribution (a, b, and X_0), R^2, *F* and *P* values are given.

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ORIGINAL RESEARCH
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Effects of Transgenerational Plasticity on Morphological and Physiological Properties of Stoloniferous Herb *Centella asiatica* Subjected to High/Low Light

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Environmentally induced transgenerational plasticity can increase success of progeny and thereby be adaptive if progeny experiences the similarly parental environment. The ecological and evolutionary significance of transgenerational plasticity in plant has been studied mainly in the context of sexual generations. A pot experiment using the stoloniferous herb *Centella asiatica* was conducted to investigate the effects of high/low light treatment experienced by parental ramets (F_0 generation) on morphological and physiological properties of offspring ramets (F_2 generation) as well as growth performance. Light environment experienced by parental ramets (F_0 generation) significantly influenced petiole length, specific petiole length, internode length of stolon, leaf area, specific leaf area (SLA), leaf nitrogen and chlorophyll contents, potential maximum net photosynthetic rate (P_{max}) in offspring ramets subjected to parental or non-parental environments even after they were detached from the parental ramets. Potential maximum net photosynthetic rate (P_{max}) of offspring ramets (F_2 generation) from parental ramets (F_0 generation) subjected to low light treatment was significantly greater than that of offspring ramets (F_2 generation) from parental ramets (F_0 generation) subjected to high light treatment. Potential maximum net photosynthetic rate (P_{max}) of offspring ramets (F_2 generation) subjected to parental light environment was greater than that of offspring ramets (F_2 generation) subjected to non-parental light environment. The greatest biomass accumulation and total stolon length were observed in offspring ramets (F_2 generation) subjected to low light treatment as parental ramets (F_0 generation) experienced. When parental ramets (F_0 generation) were subjected to low light treatment, biomass accumulation and total stolon length of offspring ramets (F_2 generation) experiencing parental light environment were significantly greater than those of offspring ramets (F_2 generation) experiencing non-parental light environment. Opposite pattern was observed in offspring ramets (F_2 generation) from parental ramets subjected to high light treatment. Our work provides evidence that transgenerational plasticity through both morphological and physiological flexibility was triggered across vegetative generations for stoloniferous herb *C. asiatica* subjected to high/low light

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treatment. The transgenerational plasticity can allow offspring ramets to present adaptive phenotype early without lag time in response to the current environment. Thus, it is very important for clonal plants in adapting temporally and spatially heterogeneous habitats.

Keywords: maternal effects, potential maximum net photosynthetic rate (P_{max}), leaf nitrogen content, internode length of stolon, leaf area

INTRODUCTION

The environmental cues experienced by parents, may influence the phenotype of their progeny. This phenomenon is termed as transgenerational plasticity (Dyer et al., 2010; Fenesi et al., 2014). Transgenerational plasticity elicits phenotypic adjustments to environmental conditions experienced by sexually produced progeny. For example, soil nutrient conditions encountered by parent, affects size and germination of progeny in *Senecio* sp (Aarssen and Burton, 1990); the competitive ability in *Plantago major* and *P. rugelii* is related to the environmental conditions experienced by both parent and progeny (Miao et al., 1991). The parental light environment influences the life history schedule of progeny in *Campanulastrum americanum* (Galloway and Etterson, 2007). The defensive resistance of progeny is induced by herbivory in the parental generation of *Raphanus raphanistrum* (Agrawal, 2002). Transgenerational plasticity can be mediated by altered DNA methylation (Rossiter, 1996; Douhovnikoff and Dodd, 2015) or seed quality (Roach and Wulff, 1987). Thereby, transgenerational plasticity may be potentially important for evolutionary dynamics of plant population (Riska, 1989; Räsänen and Kruuk, 2007).

Transgenerational plasticity may be adaptive in progeny grown under the same environmental conditions as experienced by parent (Galloway, 2005; Galloway and Etterson, 2007; Chen et al., 2014; Latzel et al., 2014). For the monocarpic herb *C. americanum*, fitness of progeny grown under a parental light environment is significantly greater than that of progeny grown under a non-parental light environment (Galloway and Etterson, 2007). Similarly, nutrient conditions experienced by parent, significantly affects biomass and carbon storage of progeny in *Plantago lanceolata* (Latzel et al., 2014). In addition, transgenerational plasticity may be more important for plant grown under limited resource conditions (such as in soil with low water level and nutrient or in shaded habitat) than one grown under ample resource conditions (Sultan, 1996). However, the ecological and evolutionary significance of transgenerational plasticity mainly focus on studies across sexual generations.

Clonal plant can reproduce a large number of interconnected, potentially independent and genetically identical offspring ramets. Transgenerational plasticity may influence phenotype of offspring ramets (Dong et al., 2017; González et al., 2017). For stoloniferous herb *Trifolium repens*, greater compensatory growth was observed in offspring ramets propagated from parental ramets subjected to repeated application of jasmonic acid compared to ones from parental ramets subjected to the same volume distilled water without application of jasmonic acid (González et al., 2017); similar pattern was still observed in offspring ramets of *Alternanthera philoxeroides* propagated from populations suffering from long-time herbivory disturbance (Lu and Ding, 2012). As an alternative to the slower mechanisms of adaptation through natural selection, transgenerational plasticity may confer ecological advantages to clonal plants against the challenges of current and future rapid environmental changes (Verhoeven and Preite, 2014; Douhovnikoff and Dodd, 2015; Dong et al., 2017).

A greenhouse experiment was conducted to explicitly investigate effects of transgenerational plasticity across vegetative generations on morphological and physiological properties of stoloniferous herb *Centella asiatica* subjected to high/low light treatment. Our first hypothesis is that effects of transgenerational plasticity on morphological and physiological properties persist across vegetative generations. Light may be an important resource for growth, development and reproduction of plants (Madsen and Sand-Jensen, 1994; Wagner et al., 2005; Glover et al., 2015). Morphological plasticity is an adaptive strategy of clonal plants to heterogeneous light conditions. For example, internode extension of stolon and petiole elongation may allow clonal ramets to escape from low light patches and lift leaf blades to higher light zones (Oborny, 1994). The ramets subjected to low light condition can intercept more light by enlarging leaf area (Dong, 1995). So, flexible responses in the internode length of stolon, petiole length and leaf area are crucial for clonal plant in capturing light (Hutchings and Kroon, 1994). As a component of chlorophyll, leaf N content is positively correlated with photosynthetic capacity in plant (Feng et al., 2007; Chen et al., 2015). We predicted that high/low light treatment experienced by parental ramets significantly influenced internode length of stolon, specific internode length of stolon, petiole length, specific petiole length, leaf area, specific leaf area (SLA), leaf nitrogen and chlorophyll contents, potential maximum net photosynthetic rate(P_{max}) in offspring ramets subjected to parental or non-parental light environments.

Our second hypothesis is that effects of transgenerational plasticity on growth performance are context-dependent. Then, we predicted that biomass accumulation and total stolon length of offspring ramets experiencing parental light environment significantly increased than those of offspring ramets experiencing non-parental light environment. Our third hypothesis is that offspring ramets reproduced from parental ramets subjected to low resource level environment should be favored in parental or non-parental environments. So, we predicted that whether in parental or non-parental light environment, biomass accumulation and total stolon length of offspring ramets from parental ramets subjected to low light

treatment significantly increased than those of offspring ramets from parental ramets subjected to high light treatment.

MATERIALS AND METHODS

Plant Material

Centella asiatica (Umbelliferae) is a stoloniferous perennial herb, which is generally distributed in ditches, margins of ponds, lawns and roadsides. Each ramet is composed of two zygomorphic leaves with slender petiole. The axillary bud on the vertical stem may grow out and form stolon (Chinese Academy of Sciences, 2004). The stolon usually take roots when in contact with moist substratum, forming a network of stolon above the ground.

Eight original plants of *Centella asiatica* were collected in Chengdu, Sichuan Province, China (30°05′ 31°26′N; 102°54′ 104°53′E) (**Table 1**). The original plants were at least 1 km away each other. They may or may not differ in genotype.

In April 2016, they were cultivated in a greenhouse, located in Sichuan Normal University. All pots were filled with substrate (3:1 mixture of humus soil and sand). During the experiment, fertilizer (20% N, 20% P, 20% K; The Scotts Company, United States) was applied to each pot once per week. Tap water was supplied to keep the substrate moist. After 4 months, offspring ramets of each original plant formed a "ramet bank" (EI-Keblawy and Bhatt, 2015).

Experimental Design

F_0 generation August 2016, two parental ramets with similar size from each "ramet bank" were grown into plastic pots (42 cm × 34 cm × 11 cm) respectively. We standardized size of the ramets by removing extra leaves and cutting the roots (Wang et al., 2013; Dong et al., 2017). One ramet was subjected to high light treatment (full light) and the other was subjected to low light treatment (50% full light). All reproduced ramets in each pot were named as F_0 generation during 10 weeks period.

F_1 generation Two ramets with similar size were chosen from each F_0 generation and grown in new pots, respectively. One ramet was subjected to high light treatment and the other was subjected to low light treatment. All reproduced ramets in each pot were named as F_1 generation during another 10 weeks period.

F_2 generation One ramet was chosen from each F_1 generation and grown in a new pot. The ramet was subjected to light treatment as its F_1 generation experienced. All reproduced ramets in each pot were named as F_2 generation during another 10 weeks period. Four treatments were included for F_2 generation: F_0 generation high light + F_1 generation high light + F_2 generation high light (HHH); F_0 generation high light + F_1generation low light + F_2 generation low light (HLL); F_0 generation low light + F_1 generation high light + F_2 generation high light (LHH); F_0 generation low light + F_1 generation low light + F_2 generation low light (LLL) (**Figure 1**). There were eight replicates per treatment. The pots were re-randomized to avoid potential effects of environmental heterogeneity. Offspring ramets from each original plant underwent all treatments.

Morphological Properties

After harvesting, offspring ramets (F_2 generation) were separated into root, leaf, petiole and stolon. Internode length of stolon and petiole length were measured by ruler. Specific internode length of stolon (internode length of stolon / dry weight) and specific petiole length (petiole length/ dry weight) were counted after drying to constant weight. Leaf area was measured according to the method described by Dong et al. (2015). Specific leaf area (SLA) was counted as follows:

$$\text{Specific leaf area (SLA)} = \frac{\text{leaf area}}{\text{leaf dry weight}}$$

Photosynthetic Properties

A portable photosynthesis system GFS-3000 (Heinz Walz GmbH, Effeltrich, Germany) was used for measurement of photosynthesis during the last week of growth. Eight offspring ramets (F_2 generation) with similar size were chosen from each treatment. A fully expanded and mature leaf from each ramet was selected for photosynthetic measurement.

Under a CO_2 pressure of 400 $\mu molmol^{-1}$, a light–response curve [net photosynthesis rate (P_n)–photosynthetic photon flux density ($PPFD$) curve] was generated according to the method described by Chen et al. (2015). The P_{max} was calculated according to the P_n–$PPFD$ curves which were fitted with a non-rectangular hyperbola model using the plotting software Origin (Origin Lab, United States) (Gomes et al., 2006; Sorrell et al., 2012):

$$Pn = \frac{\phi PPFD + P_{\max} - \sqrt{(\phi PPFD + P_{\max}) - 4\phi\theta P_{\max} PPFD}}{2\theta} - R_d$$

Where Ø was the apparent quantum efficiency, θ was the convexity of the curve and R_d was the dark respiration rate.

Leaf Properties

The leaf for measurement of photosynthetic parameters was then finely ground to determine the nitrogen content with an elemental analyser (vario MACRO CUBE, Elementar Analysensysteme, Hanau, Germany). At the same time, the other zygomorphic leaf originating from the each ramet was selected to measure the absolute chlorophyll content using the dimethylsulphoxide (DMSO) chlorophyll extraction technique (Richardson et al., 2002).

Growth Performance

After harvesting, dry weights of stolon, petiole, root and leaf were recorded after oven drying at 60°C until constant weight was obtained.

Statistical Analysis

Prior to analysis, a square root transformation was used for total stolon length and a logarithmic transformation applied to petiole length. Two-way ANOVA was used to

TABLE 1 | Basic information on original plants of *Centella asiatica* in the experiment.

No	Location	Habitat type	Dominant species of community	Community transmittance	Size of original plant	
					Height (cm)	Leaf area (cm^2)
1	30°56′N;104°18′E	lawn	*Dichondra repens*	98%	4.2	9.5
2	30°57′N; 104°19′E	lawn	*Cynodon dactylon*	96%	4.5	9.8
3	30°59′N; 104°18′E	lawn	*Centella asiatica*	98%	4.3	9.7
4	30°57′N; 104°18′E	roadsides	*Trifolium repens*	97%	4.4	9.7
5	30°59′N; 104°19′E	roadsides	*Poa annua*	95%	4.7	10.0
6	30°57′N; 104°17′E	roadsides	*Buchloe dactyloides*	96%	4.6	9.9
7	30°99′N; 103°54′E	ditches	*Poa annua* + *Zoysia japonica*	92%	4.8	10.2
8	30°92′N; 103°56′E	ditches	*Buchloe dactyloides* + *Cynodon dactylon*	91%	4.9	10.1

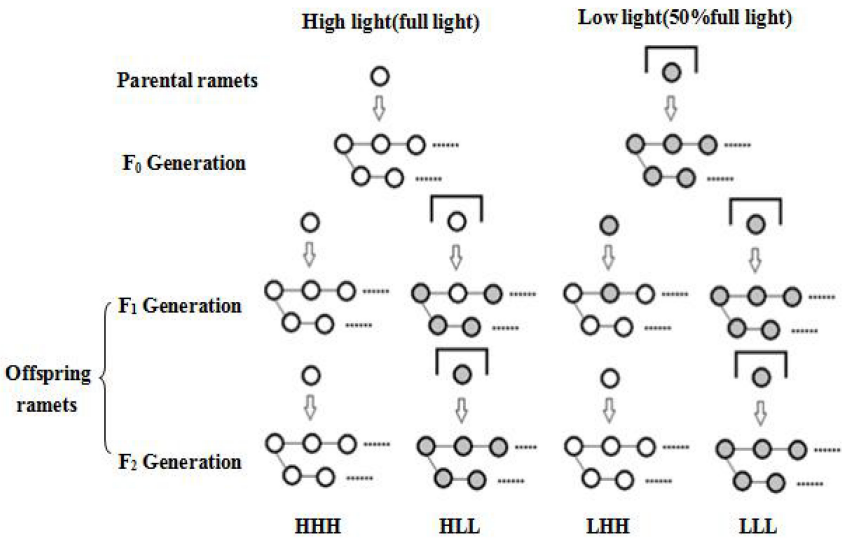

FIGURE 1 | Schematic diagram of the experimental design. The experiment is across three vegetative generations. Four treatments were included for F_2 generation: F_0 generation high light + F_1 generation high light + F_2 generation high light (HHH); F_0 generation high light + F_1generation low light + F_2 generation low light (HLL); F_0 generation low light + F_1 generation high light + F_2 generation high light (LHH); F_0 generation low light + F_1 generation low light + F_2 generation low light (LLL).

investigate the effects of light treatment experienced by F_0 *generation* (F_0), light treatment experienced by F_2 *generation* (F_2) and their interaction ($F_0 \times F_2$) on morphological, leaf and photosynthetic properties of offspring ramets (F_2 generation) as well as growth performance. Tukey HSD *post hoc* test was empolyed to compare difference among different treatments experienced by F_2 generation. All analyses were conducted with SPSS 20.0 software (SPSS, Chicago, IL, United States).

RESULTS

Morphological Properties

Specific petiole length and internode length of stolon of offspring ramets (F_2 generation) were significantly affected by light treatment experienced by F_0 generation (F_0), light treatment experienced by F_2 generation (F_2) and their interaction ($F_0 \times F_2$) (**Table 2**). Petiole length and leaf area of offspring ramets (F_2 generation) were significantly affected by light treatment experienced by F_0 generation (F_0) and light treatment experienced by F_2 generation (F_2) (**Table 2**). However, specific leaf area (SLA) was significantly affected by light treatment experienced by F_0 generation (F_0) (**Table 2**). We did not detect significant effects of the different treatments on specific internode length of stolon of offspring ramets (F_2 generation) (**Table 2** and **Figure 2D**).

When parental ramets were subject to low light treatment, petiole length and internode length of stolon of offspring ramets (F_2 generation) experiencing parental light environment significantly increased than those of offspring ramets (F_2 generation) experiencing non-parental light environment (**Figures 2A,C**). However, opposite pattern was observed in petiole length and internode length of stolon of offspring ramets (F_2 generation) from parental ramets subjected to high light treatment (**Figures 2A,C**). When parental ramets

TABLE 2 | Two-way ANOVA results for effects of light treatment experienced by F_0 generation (F_0), light treatment experienced by F_2 generation (F_2) and their interaction on the morphological, leaf and photosynthetic properties of of offspring ramets (F_2 generation) as well as growth performance.

Source	df	Morphological properties					Leaf properties			Photosynthetic property	Growth performance	
		Petiole length	Specific petiole length	Internode length of stolon	Specific internode length of stolon	Leaf area	Specific leaf area (SLA)	Area-based leaf chlorophyll content (ACC_a)	Mass-based leaf chlorophyll content (ACC_m)	Leaf nitrogen content per unit mass(N_A)	Leaf nitrogen content per unit area(N_M)	Potential maximum net photosynthetic rate(P_{max})	Biomass accumulation	Total stolon length
F_2	1	40.71***	7.79*	410.16***	0.18^{ns}	135.80**	2.41^{ns}	28.87**	52.671***	11.98**	9.89*	0.008^{ns}	6.25*	13.96**
F_0	1	31.09**	5.46*	231.13***	0.25^{ns}	13.94**	30.68**	22.09**	37.44***	27.82**	0.71^{ns}	38.18***	8.05*	18.27**
$F_0 \times F_2$	1	0.31^{ns}	9.23*	9.74*	0.001^{ns}	3.62^{ns}	0.33^{ns}	1.77^{ns}	5.40*	6.83*	0.05^{ns}	12.32**	0.016^{ns}	0.03^{ns}
Error	28													

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, *ns, non-significant*, $P > 0.05$.

were subject to high light treatment, specific petiole length of offspring ramets (F_2 generation) experiencing low light environment significantly increased (**Figure 2B**). Leaf area of offspring ramets (F_2 generation) experiencing low light treatment significantly increased than that of offspring ramets (F_2 generation) experiencing high light treatment (**Figure 2E**). When parental ramets were subject to low light treatment, leaf area of offspring ramets (F_2 generation) experiencing parental light environment significantly increased than that of offspring ramets (F_2 generation) experiencing non-parental light environment (**Figure 2E**). Compared to offspring ramets from parental ramets subjected to high light treatment, specific leaf area (SLA) of offspring ramets (F_2 generation) from parental ramets subjected to low light treatment significantly decreased (**Figure 2F**).

Leaf Properties

Area-based leaf chlorophyll content (ACC_a) of offspring ramets (F_2 generation) was significantly affected by light treatment experienced by F_0 generation and light treatment experienced by F_2 generation (**Table 2**). Mass-based leaf chlorophyll content (ACC_m) of offspring ramets (F_2 generation) was significantly affected by light treatment experienced by F_0 generation, light treatment experienced by F_2 generation and their interaction ($F_0 \times F_2$) (**Table 2**). When parental ramets (F_0 generation) were subjected to low light treatment, area-based leaf chlorophyll content (ACC_a) and mass-based leaf chlorophyll content (ACC_m) of offspring ramets (F_2 generation) experiencing parental light environment significantly increased than those of offspring ramets (F_2 generation) experiencing non-parental light environment (**Figures 3A,B**). Opposite pattern was observed in area-based leaf chlorophyll content (ACC_a) and mass-based leaf chlorophyll content (ACC_m) of offspring ramets (F_2 generation) from parental ramets subjected to high light treatment (**Figures 3A,B**).

Leaf nitrogen content per unit area (N_A) of offspring ramets (F_2 generation) was significantly affected by light treatment experienced by F_0 generation, light treatment experienced by F_2 generation and their interaction ($F_0 \times F_2$) (**Table 2**). However, leaf nitrogen content per unit mass (N_M) of offspring ramets (F_2 generation) was significantly affected by light treatment experienced by F_2 generation (F_2) (**Table 2**). When parental ramets (F_0 generation) were subjected to low light treatment, leaf nitrogen content per unit area (N_A) of offspring ramets (F_2 generation) experiencing parental light environment was significantly greater than that of offspring ramets (F_2 generation) experiencing non-parental light environment (**Figure 3D**). Leaf nitrogen content per unit mass (N_M) of offspring ramets (F_2 generation) subjected to low light treatment was significantly greater than that of offspring ramets subjected to high light treatment (**Figure 3C**).

Photosynthetic Property

Potential maximum net photosynthetic rate (P_{max}) of offspring ramets (F_2 generation) was significantly affected by light treatment experienced by F_0 generation and interaction between light treatment experienced by F_0 generation and light treatment

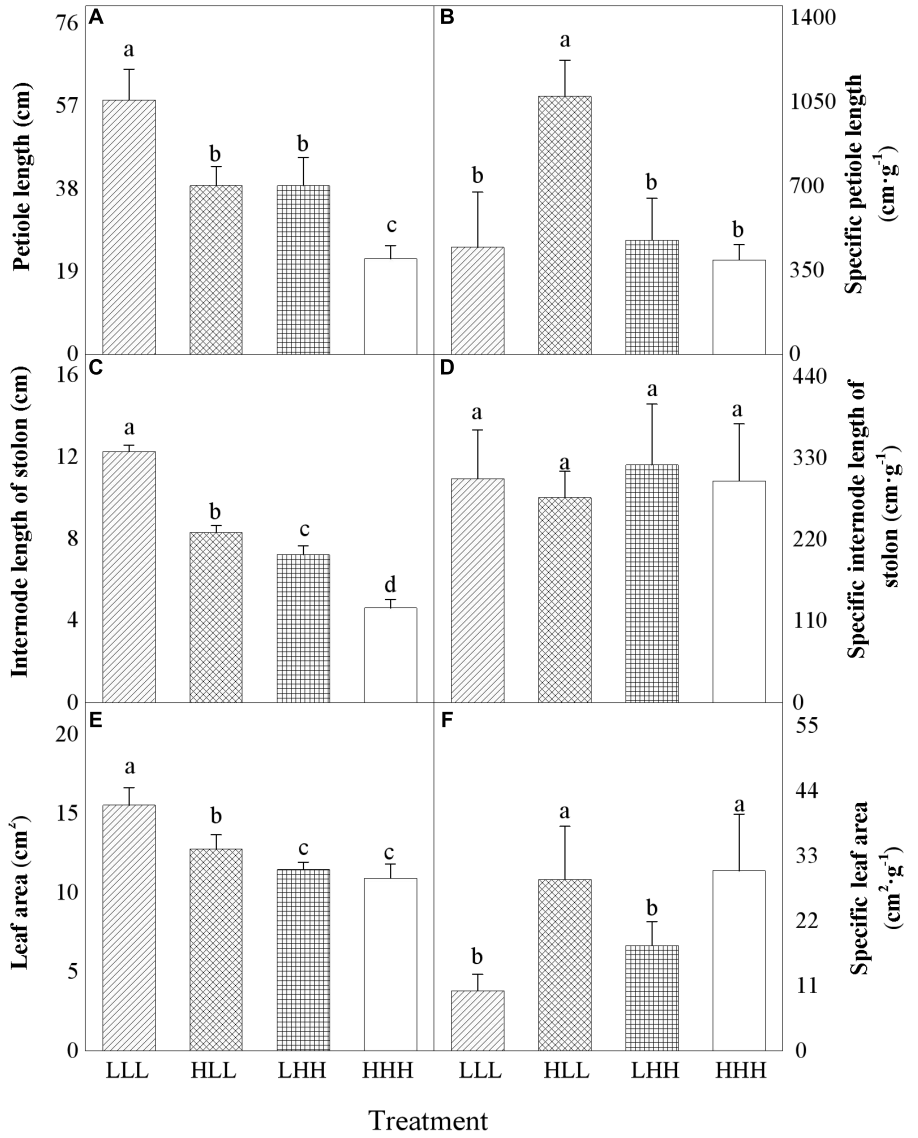

FIGURE 2 | Effects of transgenerational plasticity on morphological properties of offspring ranets (F_2 generations). The same lower case letters are not significantly different at the $P = 0.05$ level. Values are means ± s.e. (standard errors), $n = 8$. HHH: F_0 generation high light+ F_1 generation high light+ F_2 generation high light; HLL: F_0 generation high light+ F_1 generation low light+ F_2 generation low light; LHH: F_0 generation low light+ F_1 generation high light+ F_2 generation high light; LLL: F_0 generation low light+ F_1 generation low light+ F_2 generation low light.

experienced by F_2 generation ($F_0 \times F_2$) (**Table 2**). Potential maximum net photosynthetic rate (P_{max}) of offspring ramets (F_2 generation) from parental ramets subjected to low light treatment was greater than that of offspring ramets (F_2 generation) from parental ramets subjected to high light treatment (**Figure 4**). Potential maximum net photosynthetic rate (P_{max}) of offspring ramets (F_2 generation) subjected to parental light environment was greater than that of offspring ramets subjected to non-parental light environment (**Figure 4**).

Growth Performance

Biomass accumulation and total stolon length of offspring ramets were significantly affected by light treatment experienced by F_0 generation (F_0) and light treatment experienced by F_2 generation (F_2) (**Table 2**). The greatest biomass accumulation and total stolon length were observed in offspring ramets (F_2 generation) subjected to low light treatment as parental ramets (F_0 generation) experienced (**Figures 5A,B**). When parental ramets (F_0 generation) were subjected to low light treatment, biomass accumulation and total length of stolon of offspring ramets (F_2 generation) experiencing parental light environment were significantly greater than those of offspring ramets (F_2 generation) experiencing non-parental light environment (**Figures 5A,B**). Opposite pattern was observed in offspring ramets (F_2 generation) from parental ramets subjected to high light treatment (**Figures 5A,B**).

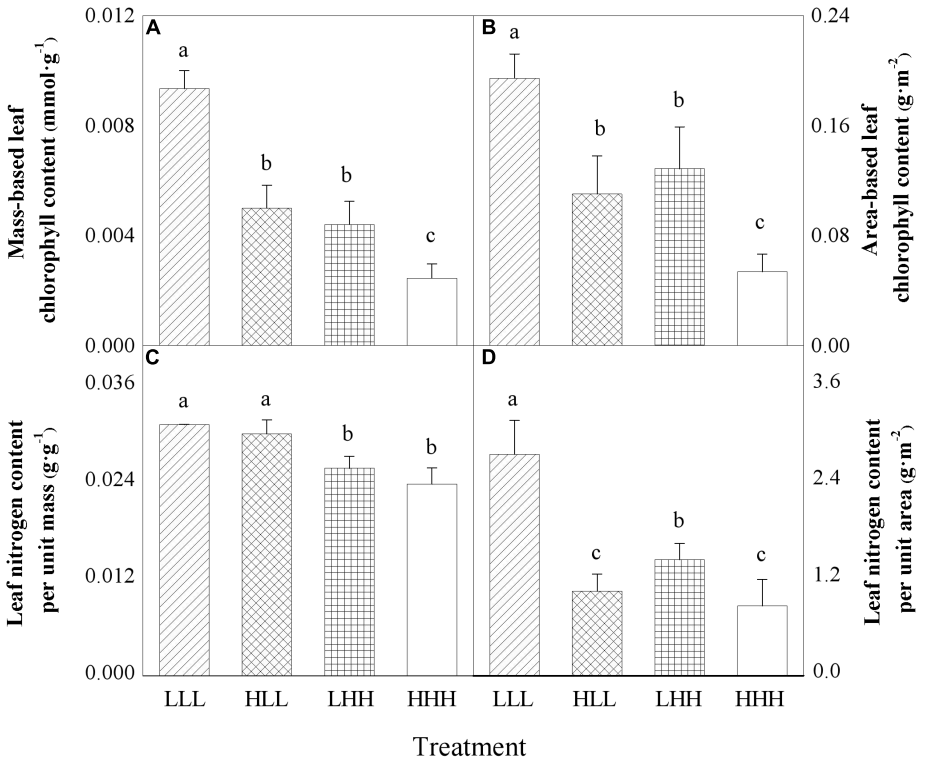

FIGURE 3 | Effects of transgenerational plasticity on leaf properties of offspring ramets (F_2 generation). The same lower case letters are not significantly different at the $P = 0.05$ level. Values are means ± s.e. (standard errors), $n = 8$. HHH: F_0 generation high light+ F_1 generation high light+ F_2 generation high light; HLL: F_0 generation high light+ F_1 generation low light+ F_2 generation low light; LHH: F_0 generation low light+ F_1 generation high light+ F_2 generation high light; LLL: F_0 generation low light+ F1 generation low light+ F_2 generation low light.

DISCUSSION

For stoloniferous herb *C.asiatica*, the experiment demonstrated transgenerational plasticity triggered by high/low light treatment. Light environment experienced by parental ramets (F_0 generation) significantly influenced morphological and physiological properties of offspring ramets (F_2 generation) as well as growth performance even after they were detached from the parental ramets. The results supported our first hypothesis that effects of transgenerational plasticity on morphological and physiological properties can transmit across vegetative generations of *C. asiatica*. Due to limited opportunities to adapt to environmental changes genetically, transgenerational plasticity can impose substantial impact on population dynamics (Benton et al., 2005; Plaistow et al., 2006) and evolution of clonal plants in response to environmental changes (Wade, 1998; Räsänen and Kruuk, 2007). So, transgenerational plasticity would have consequences for population dynamics, and ultimately, evolution, especially given the limited levels of genotypic variation in clonal plants (González et al., 2017).

When parental ramets (F_0 generation) were subjected to low light treatment, offspring ramets (F_2 generation) experiencing parental light environment presented better growth performance than offspring ramets (F_2 generation) experiencing non-parental light environment. This is consistent with previous study that if offspring ramets spread into a new environment as experienced by parental ramets, transgenerational plasticity may facilitate establishment of their populations by enabling adaptation to the new environment more rapidly than natural selection (Latzel and Klimešová, 2010). In addition, opposite pattern was observed in offspring ramets (F_2 generation) from parental ramets subjected to high light treatment. The results supported our second hypothesis that effects of transgenerational plasticity on growth performance are context-dependent. Such transgenerational plasticity spanning across vegetative generations is likely adaptive in clonal species (Herman and Sultan, 2011; Holeski et al., 2012).

Compared to offspring ramets (F_2 generation) from parental ramets (F_0 generation) subjected to high light treatment, growth performance of offspring ramets (F_2 generation) from parental ramets (F_0 generation) subjected to low light treatment was favored in parental or non-parental light environment. The results supported our third hypothesis. Habitat-specific DNA methylation of clonal genotypes from natural populations may result in locally specialized ecotypes (Verhoeven and Preite, 2014). Further, transgenerational plasticity can affect the evolutionary rate and direction of clonal plants (Latzel et al., 2016; González et al., 2017).

Clonal species often adopted morphological response such as stolon elongation or petiole expansion to escape from environmental stress such as flooding (Luo et al., 2009), low light

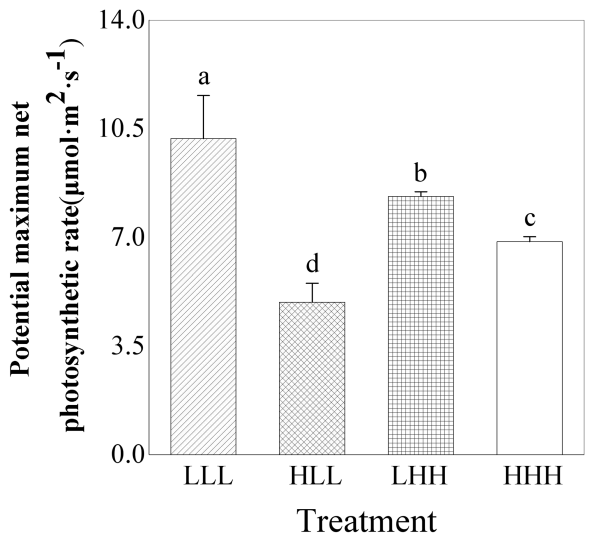

FIGURE 4 | Effects of transgenerational plasticity on potential maximum net photosynthetic rate of offspring ramets (F_2 generations). The same lower case letters are not significantly different at the $P = 0.05$ level. Values are means ± s.e. (standard errors), $n = 8$. HHH: F_0 generation high light+ F_1 generation high light+ F_2 generation high light; HLL: F_0 generation high light+ F_1 generation low light+ F_2 generation low light; LHH: F_0 generation low light+ F_1 generation high light+ F_2 generation high light; LLL: F_0 generation low light+ F_1 generation low light+ F_2 generation low light.

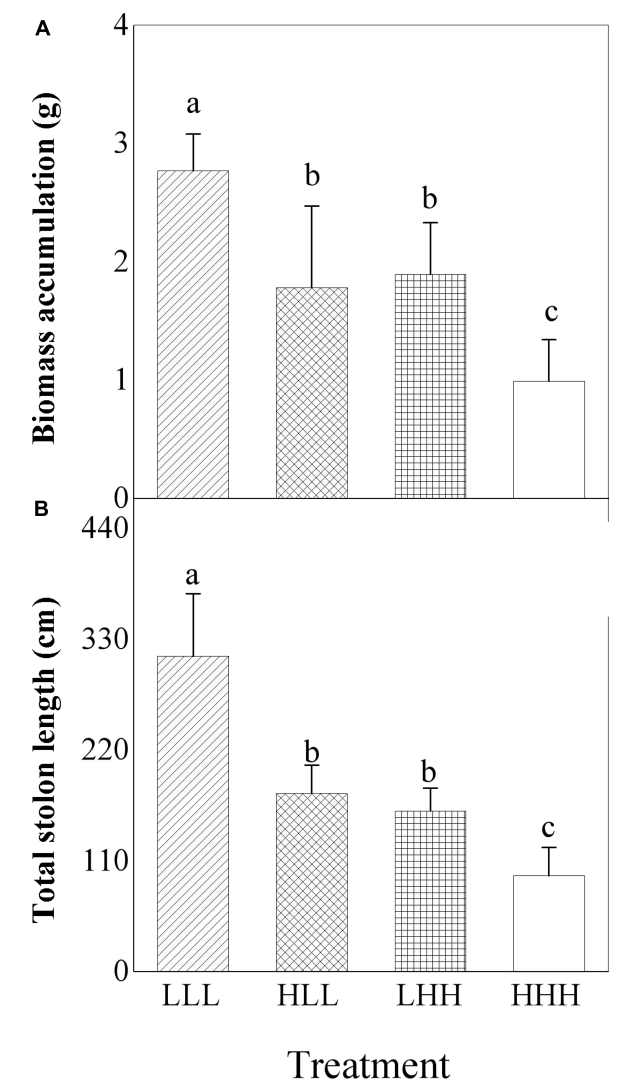

FIGURE 5 | Effects of transgenerational plasticity on growth performance of offspring ramets (F_2 generation). The same lower case letters are not significantly different at the $P = 0.05$ level. Values are means ± s.e. (standard errors), $n = 8$. HHH: F_0 generation high light+ F_1 generation high light+ F_2 generation high light; HLL: F_0 generation high light+ F_1 generation low light+ F_2 generation low light; LHH: F_0 generation low light+ F_1 generation high light+ F_2 generation high light; LLL: F_0 generation low light+ F_1 generation low light+ F_2 generation low light.

(González et al., 2017), metal pollutions (Roiloa and Retuerto, 2012) and interspecific competition from neighbor species (Evans and Cain, 1995). Seedlings from parents grown in a CO_2-elevated environment reduced photosynthesis compared to seedlings from parents grown in ambient CO_2 conditions (Huxman et al., 2001). Parents may enable their offspring to adapt to environmental changes through morphological and photosynthetic adjustment. With plastic changes of morphological properties, photosynthetic capacity and growth performance were significantly improved in offspring ramtes (F_2 generation) experiencing parental light environment than in offspring ramtes (F_2 generation) experiencing non-parental light environment when parental ramets (F_0 generation) were subjected to low light treatment. We tentatively concluded that in response to low light treatment, variation of morphological and physiological properties in parental ramets was transmited to their offspring ramets (Latzel et al., 2009). When parental ramets (F_0 generation) were subjected to low light treatment, the greatest leaf nitrogen content per unit area (N_A) was observed in offspring ramtes (F_2 generation) experiencing parental light environment with a decrease of specific leaf area (SLA). The results implied that effects of transgenerational plasticity on photosynthesis of offspring ramets might be mediated by alternation of resources allocation toward the photosynthetic apparatus (Latzel et al., 2009). It is suggested that environmentally induced epigenetic change and/or inherited resource allocation pattern toward photosynthesis may be responsible for effects of transgenerational plasticity on photosynthesis of offspring ramets.

Furthermore, clonal plants have the potential to selectively place ramets and to avoid unfavorable conditions through morphological plasticity of spacer or branching intensity (Hutchings and Kroon, 1994; Kleunen and Fischer, 2001). For *Polygonum persicaria*, progeny from parent experiencing drought environment produced longer, more rapidly extending root systems and greater biomass in parental environment than those of progeny in non-parental environment (Galloway and Etterson, 2007). In addition, root-shoot biomass ratio and specific root length of progeny subjected to drought environment experienced by parent significantly increased than those of progeny in ample

water environment (Sultan et al., 2009). In our experiment, effects of transgenerational plasticity on morphological and physiological properties of offspring ramets (F_2 generation) depended on environmental characteristics experienced by their parent and themselves. Clonal plants thus have the potential to reflect past and current environmental conditions even anticipate future conditions (Latzel et al., 2016). So, the dynamics and genetics of clonal populations may be affected by the interaction of genotypes to phenotypes.

To the best of our knowledge, there has been rare study directly examining the effects of transgenerational plasticity on clonal plants through both morphological and physiological properties. Our work provides evidence that transgenerational plasticity through both morphological and physiological flexibility was triggered across vegetative generations for stoloniferous herb *C. asiatica* subjected to high/low light treatment. Life-history traits such as clonal integration, intraclonal division of labor and clonal architecture et al may be advantageous to exploitation and colonization of clonal plants in heterogeneous habitats (Latzel and Klimešová, 2010; Martina and Ende, 2013; Chen et al., 2015). The transgenerational plasticity can allow offspring ramets to present adaptive phenotype early without lag time in response to the current environment. Thus, it is very important for clonal plants in adapting temporally and spatially heterogeneous habitats. A wider range of species are needed to understand the generality of this pattern and to assess fully the ecological advantages afforded by these features.

AUTHOR CONTRIBUTIONS

All authors conceived, designed, and performed the experiments and wrote the paper.

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Impacts of Sand Burial and Wind Erosion on Regeneration and Growth of a Desert Clonal Shrub

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Sand burial and wind erosion caused by sand movement are common phenomena in desert environments, but the effects on clonal shrub have rarely been investigated. Here, we assessed how sand movements affect the population regeneration capacity of juvenile clonal fragments of the shrub *Calligonum mongolicum* growing in mobile desert sand dunes. We investigated the population status and natural regeneration capacity in three types of mobile dunes (heavy wind erosion, heavy sand burial and moderate sand burial). Clonal propagation of *C. mongolicum* was markedly different across sites. Moderate sand burial sites had the largest ramet density and bud number per unit length of rhizome, and the overwinter survival rate was significantly higher at sand burial sites than at wind erosion sites, suggesting that *C. mongolicum* may have well adapted to the moderate sand burial environment. We further examined the effects of clonal integration on clonal regeneration of this species. Physiological, biochemical and morphological characteristics of parent and daughter ramets growing in heterogeneous sandy habitats (sand burial or wind erosion) were measured. The results showed that being connected or severed from the maternal plant critically determined survival of daughter ramets on wind eroded rhizomes. When eroded rhizomes remained connected, the mother ramets had the highest chlorophyll a, b and a + b contents. However, both the mother plant and the daughter ramets undergoing erosion had higher proline and soluble protein levels than sand buried ramets. Meanwhile, the daughter ramets undergoing sand burial had higher photosynthetic rates (P_n), chlorophyll fluorescence parameters (F_m and F_o), and phenotypic traits of assimilating shoots, i.e., node number, length and volume than wind-eroded ramets. However, significant differences with mother plants, whether connected or severed, were very limited. It was concluded that moderate sand burial environments promoted clonal reproduction and growth of *C. mongolicum*. Additionally, physiological integration with mother raments in favorable conditions can alleviate stress on daughter ramets exposed to wind erosion. This physiological effect may do not occur for sand buried daughter ramets. These survival strategies and phenotypic responses should be carefully considered in shrub and sand dune management in sand fixation plantations of *C. mongolicum*.

Keywords: *Calligonum mongolicum*, clonal fragment, clonal integration, physiological and biochemical, sand burial, wind erosion

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INTRODUCTION

Windblown sand movement is a common phenomenon in deserts (Maun, 1996; Liu et al., 2014). It can either bury vegetation, or conversely, denude the plant and roots through erosion (Xu et al., 2013). Individual plants and plant parts in deserts often experience heterogeneity in sand coverage (Liu et al., 2014). Previous studies have found that there is considerable small-scale spatial variation in the degree of sand movement and the associated degree of burial or denudation of desert plants (Maestre and Reynolds, 2006; Okayasu et al., 2012; Xu et al., 2013). To cope with adverse environments, the majority of indigenous plants in arid regions have evolved different strategies (Su et al., 2009). One of these strategies is to reproduce asexually by means of clonal growth (Maun, 1996). For clonal plants growing in arid dune environments, rare and irregular seedling recruitment is common even when seeds are regularly produced (Eriksson, 1989; Mandujano et al., 2001; Li et al., 2015). This is because frequent sand movement and unpredictable rainfall often lead to failure in seedling recruitment as long-lived perennials often have extended juvenile stages. In contrast, prior to establishment vegetative offspring receive support including water, carbohydrates, and other nutrients from the parent plant at least until it is established (Li et al., 2015). In addition, clonal reproduction permits the effects of deleterious genetic alleles to be masked at heterozygous states, thereby increasing overall fitness per plant (Zhou et al., 2017). Clonal integration has also been shown to enhance plant survival under sand burial (Yu et al., 2001, 2004, 2010), promotes colonization in resource-poor or stressful habitats (Oborny et al., 2000; Song et al., 2013; Xu et al., 2013; Lechuga-Lago et al., 2016). In heterogeneous environments, due to the ability of clonal plants to share parental resources gives them a competitive advantage over non-clonal plants, (Golubski et al., 2008; Oborny et al., 2012; Dickson et al., 2014), thus daughter ramets have a better chance of survival if they remain attached to the parent plant or mother ramet (Balestri and Lardicci, 2013).

Bud count is an important indicator of regeneration potential, and research on clonal and bud bank traits and performances in European and high altitude settings are well described by Klimeš and Klimešová (2000), Pausas and Bradstock (2007), Klimešová and Klimeš (2008) and Klimešová et al. (2011) after fire exposure in Australia. Numerous studies have examined the effects of sand burial on the survival and growth of clonal plant fragments (Dong et al., 2010; Li et al., 2013; Luo and Zhao, 2015a,b). Several studies have also tested the regeneration capacity and subsequent growth of clonal fragments after burial or wind erosion in natural desert environments (Dong et al., 2011; West et al., 2012; Luo and Zhao, 2015b). However, to date, little is known regarding the ability of clonal rhizomatous shrubs to adapt to harsh desert environments characterized by the exposure of clonal plant. Many long-lived shrubs that survive adverse conditions regenerate naturally in mobile sand dunes and these shrubs play a more important role as windbreaks and sand fixation, especially in spring, when sand movement is frequent. Thus, it is important to improve our understanding about the capacity of clonal regeneration of those shrubs that show strong natural regeneration in mobile sand dunes (Huang et al., 2015). Filling these knowledge gaps require field studies that both consider the capacity for clonal regeneration on the population level and assesses the parental effects on the clones themselves.

Calligonum mongolicum, a windbreak and sand-fixation pioneer species, occurs naturally in mobile dunes and plays an important role in protecting ecological security in western China. Knowledge of the processes responsible for the natural regeneration of pioneer species during of sand dune stabilization is surprisingly rare (Fan et al., 2018), but is necessary for effective desert control. *C. mongolicum* displays strong clonal regeneration ability in mobile sand dunes, however, available data on the clonal growth pattern of this species are scarce and little is known on the impact of physiological integration between parents and offspring. This study focused on the effects of sand movement on population regeneration and the generative capacity of the bud bank of *C. mongolicum* juvenile shrubs. The study also examined the effects of wind erosion and sand burial on the physiological, biochemical and morphological characteristics of the parental and offspring ramets. To our knowledge, this work is the first to examine clonal regeneration and clonal integration of *C. mongolicum* in a heterogeneous mobile sand dune environment.

MATERIALS AND METHODS

Plant Species and Site Description

Calligonum mongolicum is a dominant native perennial shrub in active sand dunes in the arid deserts of northern China (Fan et al., 2018). Well adapted to harsh climate, the foliage of *C. mongolicum* consists of slender, highly branched green to gray–green branchlets that bear small minute scale-leaves. Although *C. mongolicum* populations can propagate sexually and asexually in mobile sand dunes, seedlings appear to suffer high mortality, and therefore clonal reproduction and growth seem to play a major role in the natural regeneration and maintenance of populations in mobile dune habitats. This species is capable of forming several horizontal rhizomes from each node sited along the principal root. The principal roots of *C. mongolicum* are rather short compared to its vertical shoots, i.e., the root length to shoot length ratio is around 0.65 ± 0.08 (mean $\pm$ SE), the minimum is 0.39, and the maximum is 0.83. Following sand burial, daughter ramets are formed as new root branches emerge from vegetative buds located at the nodes of buried horizontal roots or shoots.

This study was carried out in mobile sand dunes near the Minqin meteorological station (101°05′E, 38°38′N), in Gansu Province, northwest China. Minqin is adjacent to the Badain Jaran Desert in the northwest and the Tengger Desert to the east. The area has an arid desert climate with an average annual temperature of 7.8°C. Precipitation is usually the only source of water for desert plant growth, and the average annual precipitation is 116.5 mm, with average annual potential evaporation of 2383.7 mm (Fan et al., 2018). The mean wind speed is 2.4 $m.s^{-1}$ and the average number of days with gales (i.e.,

a wind velocity $\geq$ 17 m.s^{-1}) is 27.4 days per year. The fertility of all soil types in this area is very low due to the harsh climate and sparse desert vegetation.

Experimental Design

Our research consisted of two sequential field experiments. The first experiment investigated the effects of plot type (comparing heavy wind erosion, heavy sand burial, and moderate sand burial plots) on the population growth and clonal regeneration of *C. mongolicum*. However, almost no horizontal rhizomes were observed in the heavy sand burial microhabitats, thus this treatment (site) was dropped from subsequent investigations. The second experiment assessed survival and effects of clonal integration of *C. mongolicum* in two heterogeneous sand microhabitats (moderate sand burial and heavy wind erosion).

Experiment 1

Vegetation Survey

During early September, 2015, we conducted a vegetation survey in three distinct microhabitats, including the windward sides of dunes (referred to hereafter as 'heavy wind erosion plots'), plots suffering from heavy sand burial ('heavy sand burial plots'), and plots that alternated from wind erosion to sand burial ('moderate sand burial plots'). There were three 20 m $\times$ 20 m replicates of each of these microhabitats. We recorded dead shoot percentage of mature mother ramets in each plot and dead shoot percentage were recorded. We assessed the density of mature, seed seedling and clonal juvenile (<30 cm in height) shrubs, mature shrub height and basal diameter of *C. mongolicum* in each of nine 20 m $\times$ 20 m plots. Shrub basal diameter and height were measured from where the main root initiated, not at ground level. On heavy wind eroded plots, basal diameter was measured aboveground level, while in heavy and moderate sand burial plots, we excavated the trunk to measure basal diameter at the point where we found an obvious color change, which marked the start of the main root.

Horizontal Rhizome Condition

To measure the condition of horizontal rhizomes over time, in early spring 2015, we selected and marked 20 horizontal roots found on plants in the moderate sand burial and wind erosion microhabitats. Horizontal rhizomes existing in heavy sand burial plots were not easily excavated, and no buds emerge from the heavily buried horizontal rhizomes, our observations did not include data from the heavy sand burial plots. Due to the considerable sand movement in some plots over the monitoring period some roots died after they were severed from their mother shrubs. Consequently, we were only able to monitor 11 roots in the erosion plots and 15 roots in the moderate sand burial plots. On all roots, the number of buds and the number of clonal offspring (i.e., ramets) were counted at three points: in the early spring of 2015, in later autumn 2015, and in spring 2016. We then calculated the bud survival percentage and the overwinter survival rate at each plot.

Experiment 2

Clonal Integration

To assess the effects of maternal plant survival on the growth of daughter ramets in different wind and sand environments, we chose fragments that including mother ramets attached with two horizontal rhizomes, of which one rhizome lived in a moderate sand buried microhabitat, and the other one was totally eroded and exposed to the air (**Figure 1A**). We manually standardized the growing conditions of clonal fragments according to its microhabitat; for instance, we ensured that rhizomes buried in the sand were entirely buried, and that the wind eroded rhizomes were entirely eroded (**Figure 1A**), each treatment with three replicates. All mother ramets were similar in growth and condition, as were the daughter ramets. The distance between the mother and daughter ramets was between 20 and 40 cm. In late spring 2016, in each plot measurements were taken on all (mother and daughter) ramets. Connections between ramets were then severed (**Figure 1B**) and the same measurements were retaken 4 weeks later. The data collection included: the morphology of assimilating shoots (i.e., length, diameter, assimilating shoots number per cluster, cluster number per branch and node number of shoots), chlorophyll content concentration, gas exchange parameters and chlorophyll fluorescence. The *C. mongolicum* leaf is a branchlet with reduced leaves (assimilating shoots), therefore the length and diameter of these branchlets were measured with a micrometer. Thus, the volume of assimilating shoots was estimated by: $LA = L_n \times D^2 \times \pi/4$, where L_n is the length of assimilating shoots, and D is the diameter of assimilating shoots. The physiological and biochemical parameters measured are included in the following section.

Physiological and Biochemical Parameters of Assimilating Shoots

The gas exchange parameters of mature assimilating shoots were recorded using a portable open-path gas exchange system with a CO_2 control (Li-6400, LI-COR Biosciences, Inc., Lincoln, United States). On July 1, 2016, measurements were taken between 10:00 and 12:30 am in full sun on five replicates from each ramet type. The net photosynthetic (P_n) rate, stomatal conductance (Cond), and transpiration (Trmmol) rate were determined for the branchlets of 3 plants of each ramet type under an artificial light source with a photosynthetic photon flux density (PPFD) of 1800 μmol m^{-2} s^{-1} (provided by a Li-6400-02 LED light source) and an ambient concentration of CO_2 concentration. Assimilating shoots used for photosynthetic measurements were marked and sampled at the end of the experiment, and the surface area of each marked assimilating shoots was determined using a LI-3000A planimeter (LI-COR). Surface area measurements were then used to calculate the net photosynthetic rate per unit area of the assimilating shoots.

Following the gas exchange measurements on each clonal fragment, mature leaves were selected from the south side of the crowns. These were placed in opaque plastic bags, cooled by liquid nitrogen and transported to the laboratory. Proline, total soluble sugars and soluble protein content were determined and

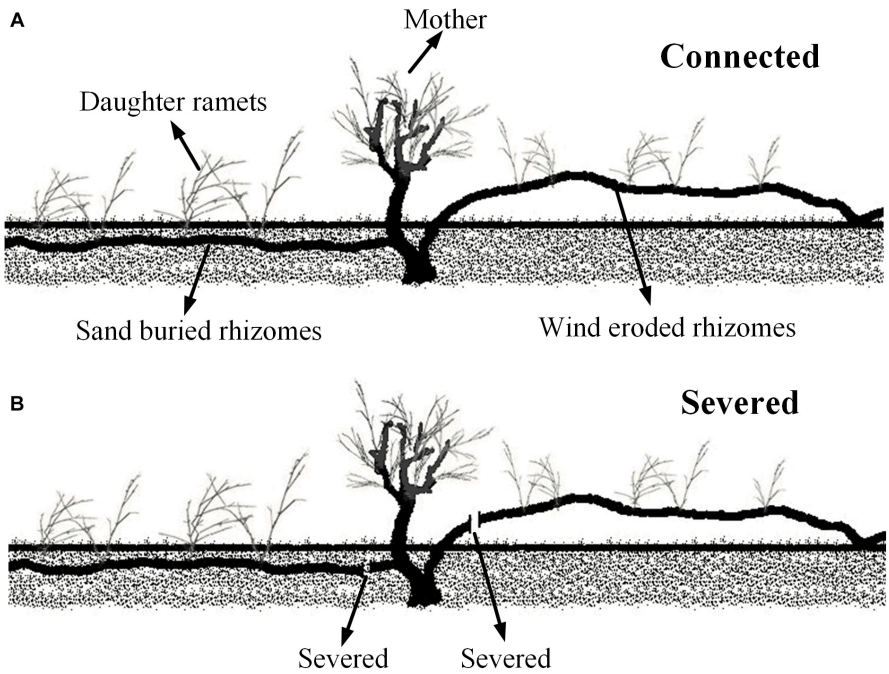

FIGURE 1 | Diagram of Experiment 2. **(A)** Connected treatment; **(B)** severed treatment.

measured following the methods of Troll and Lindsley (1955); Spiro (1966), and Bradford (1976), respectively.

Chlorophyll Fluorescence

In parallel with the gas exchange parameters, chlorophyll fluorescence was measured by a pulse amplitude modulated portable fluorometer (PAM 2100. Walz, Germany). Assimilating shoots were dark adapted for 30 min, after which the minimal fluorescence level (F_o) was measured by low modulated light and the maximal fluorescence level (F_m) was determined by a saturating pulse on the dark-adapted branchlets. The maximum quantum yield of PSII (F_v/F_m) was then calculated using the equation $F_v/F_m = (F_m - F_o)/F_m$ after (Genty et al., 1989). The steady-state fluorescence (F_s) was recorded after 6 min of light adaptation. Maximal fluorescence level in a light-adapted state (using a saturating pulse, F_m') and the minimal fluorescence level (using far-red light, F_o') was determined. The effective quantum yield of PSII (Φ_{PSII}), photochemical quenching (qP) and electron transport rate (ETR) were then calculated using the equation: $\Phi_{PSII} = (F_m' - F_s)/F_m'$; $qP = (F_m' - F_s)/(F_m' - F_o')$; $ETR = PAR \times 0.5 \times \Phi_{PSII} \times 0.84$, after Genty et al., 1989).

Statistical Analyses

One-way ANOVA was used to compare the differences in the population of *C. mongolicum*, clonal regeneration features, clonal fragment under different sand environments, leaf morphology and physiological parameters of mother and daughter ramets. Where significant differences were found, multiple comparisons using LSD tests at $P < 0.05$ were performed. Data were tested for homogeneity prior to determining ANOVAs or conducting multiple comparisons. All statistical tests were performed using SPSS 16.0 software. Data means ± SE and figures were calculated using Origin 8.0.

RESULTS

Population Features and Clonal Regeneration in Three Mobile Sand Dune Habitats

Population of *C. mongolicum* in eroded, heavily buried and moderate burial sites expressed distinctly different morphologies. At the wind-eroded site, mature shrubs were flattened, the top branches were dead, and many horizontal rhizomes were exposed to the air. At the heavy sand burial sites, only the very top of the exposed shrub survived. The dead shoot percentage at both the eroded and heavily sand buried plots were significantly higher than at the moderate sand burial plot (**Table 1**).

TABLE 1 | Description of populations of *Calligonum mongolicum* under the three sand dune conditions.

Sand dune conditions	Mother ramets status	Percentage of flattened plants	Dead shoot percentage (%)
Heavy wind erosion	100% wind eroded	100%	63.28 ± 7.72^a
Heavy sand burial	100% sand buried	No	63.76 ± 8.24^a
Moderate sand burial	100%	No	5.80 ± 2.91^b

Different lowercase letters denote significant difference ($p < 0.01$) among sand dune environments.

Shrub height and basal diameter were significantly larger at the moderate sand burial site compared to the heavy sand burial site (**Figures 2A,B**), but no significant changes were found in the crown area or in the mother ramet population density among the three environments (**Figures 2C,E**). Seedlings from asexual and sexual reproduction both occurred at the moderate sand burial sites, while seed propagation did not occur at the heavy sand burial or wind eroded sites; at these sites we found only clonal regeneration (**Figure 2F**). The rate of clonal propagation of *C. mongolicum* was markedly different among these three different environments. Ramet density at the moderate sand burial sites was 431% greater than that at eroded sites, and 241% greater than that at the heavy sand burial sites (**Figure 2F**).

Effects of Wind Erosion and Sand Burial on Horizontal Rhizome Fragments

Calligonum mongolicum on mobile sand dunes in the experimental site had well developed horizontal rhizomes up to several meters in length that gave rise to ramets (**Table 2**). Length of horizontal rhizomes in eroded sites was much longer than in sand buried sites (**Table 2**). Although buds were abundant on all rhizomes, the bud number per unit length of rhizome was 2.23 times greater on plants from sand burial plots than from those at eroded sites. In both environments, ramets had very high mortality rates, with only 10% of juvenile ramets surviving at the end of summer. However, the overwinter survival of rate at sand burial sites was 60%, while it was <30% at eroded sites (**Table 2**). Moreover, the number of assimilating shoots per unit branch under sand burial was significantly larger than that at eroded sites.

Clonal Fragments in Different Sand Environments

Clonal ramets that sprouted from sand-buried and wind-eroded horizontal rhizomes had significantly different clonal growth characters. Ramet density was much greater from sand-buried than from wind-eroded rhizomes ($P < 0.05$), although spacer length showed a contrary result ($P < 0.05$) (**Table 3**).

Sand movement significantly affected the number of assimilating shoot nodes, the length of assimilating shoots and the volume of assimilating shoots of daughter ramets when connected with mother ramets. Each of these parameters was less

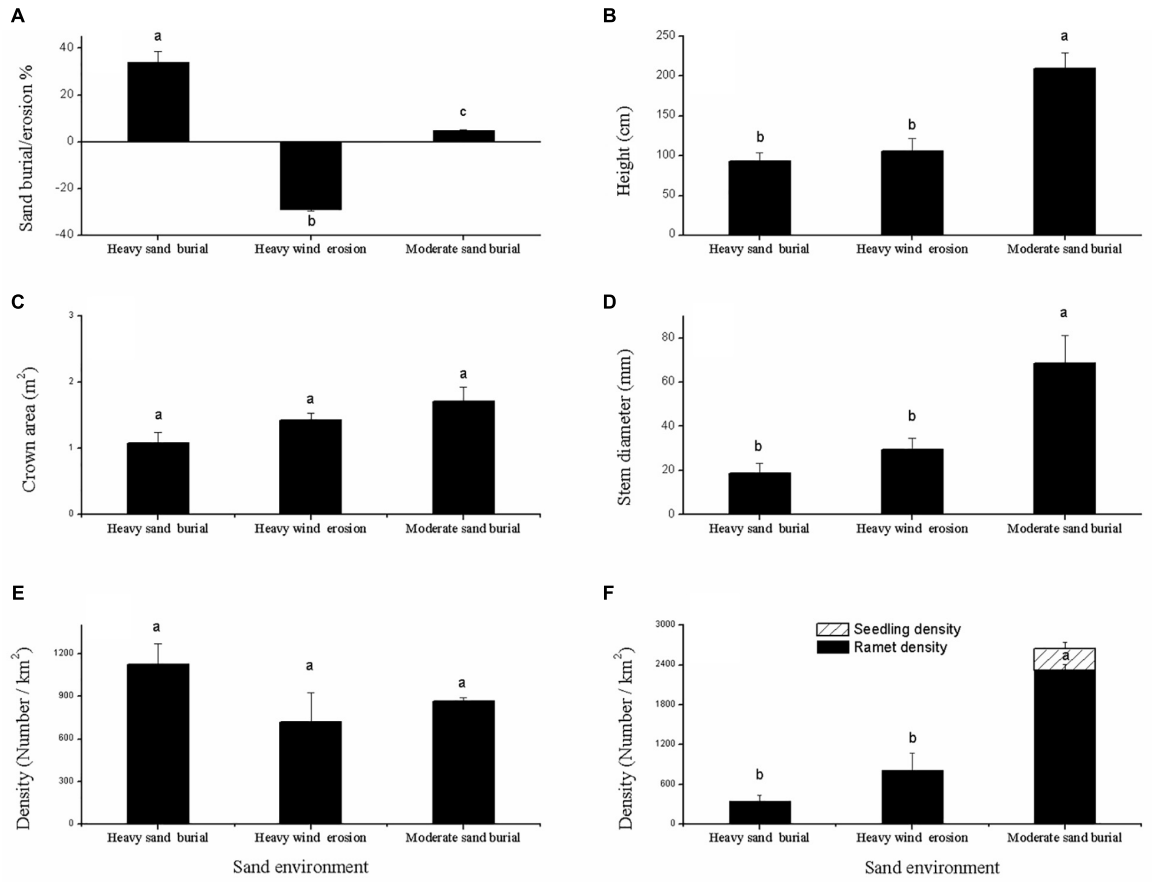

FIGURE 2 | Description of the population dynamics of *Calligonum mongolicum* in various sand dune environments, including heavily buried, moderately buried, and wind-eroded sites. **(A)** Sand buried or wind eroded depth of mature shrub; **(B)** height of mature shrub; **(C)** crown area of mature shrub; **(D)** stem diameter of mature shrub; **(E)** density of mature shrub; **(F)** density of seed seedlings and clonal ramets. [Different lowercase letters denote significant differences ($p < 0.05$) among sand dune environments].

TABLE 2 | Clonal growth of horizontal rhizomes and bud banks in natural sand burial and wind erosion environments.

Parameters	Status of horizontal rhizomes		F	*P*
	Sand buried	Wind eroded		
Length of horizontal rhizome (cm) (Minimum ~ Maximum)	32 ~ 370	80 ~ 713	1.33	0.26
Diameter of horizontal root (mm) (Minimum ~ Maximum)	13.7 ~ 23.9	7.9 ~ 17.6	20.85	<0.001
Bud number per unit length of rhizome	1.68 ± 0.21	0.52 ± 0.08	21.64	<0.001
Bud survival percentage (%)	10.88 ± 2.36	10.18 ± 2.9	0.035	0.85
Overwinter survival rates (%)	58.63 ± 5.17	28.44 ± 4.38	15.63	0.001
Number of assimilating shoots per unit branch	0.18 ± 0.04	0.05 ± 0.02	7.24	0.013

TABLE 3 | Description of mother and daughter ramets of *C. mongolicum* in different sand burial and wind erosion environments.

	Mother ramet	Sand-buried rhizome	Wind-eroded rhizome	*F*(*P*)
Burial depth (cm)	13 ~ 21	7.8 ~ 13	/	/
Wind erosion (cm)	/	Nd	8 ~ 28	/
Ramets density (number per unit length)	/	0.085 ± 0.007	0.031 ± 0.005	38.11*
Ramet spacing length (cm)	/	11.92 ± 1.94	33.82 ± 5.59	14.76*

**Means sig < 0.05; Nd, not determined due to high mortality rates.*

in wind eroded conditions, although they were not significantly different from mother ramets (**Figure 2**). Being connected or severed critically determined the survival of daughter ramets at wind-eroded rhizomes. Severing rhizomes at wind-eroded sites caused the total senescence of daughter ramets within 1 week, thus data on these ramets was not available. In contrast, severing rhizomes did not affect the survival of daughter ramets at sand burial sites. However, at sand burial sites the length of assimilating shoots length ($F = 14.334$, $P < 0.05$) and the volume of assimilating shoots ($F = 190.86$, $P < 0.001$) were greater than these in mother ramets following severing. The difference in diameter of assimilating shoots of *C. mongolicum* for all surviving mother and daughter ramets did not significantly differ, while the number of assimilating shoots per cluster in mother ramets was larger than in the sand buried daughter ramets, no matter whether they were connected or severed ($F = 69.99$, $P = 0.004$) (**Figure 3**).

When rhizomes remained connected, both mother plant and the daughter ramets under eroded conditions had greater proline and soluble protein content than sand buried ramets (**Figures 4A,B**). However, when rhizomes were severed the proline content of mother ramets significantly declined from 705 to 245 μg/g (**Figure 4A**). Severed or connected, proline and protein content at sand burial sites did not significantly differ. Meanwhile, reducing and soluble sugars did not differ between severed and connected rhizome treatments or among clonal fragments before or after being severed with the exception of reducing sugar content between mother and wind eroded daughter ramets (**Figures 4C,D**).

Net Photosynthetic Rate and Chlorophyll Content

When ramets remained connected, P_n values in both the mother and daughter ramets were greater at sand burial treatments than at wind-eroded sites (**Figure 5**). After rhizomes were severed P_n values were greater in sand burial daughter ramets than mother ramets (**Figure 5**).

Chlorophyll a, b and total chlorophyll contents of mother ramets were higher at sand-buried sites before severing, while ramets at wind-eroded sites had the least chlorophyll a, b and a + b contents. However, no significant differences in chlorophyll content between ramets in either connection condition were apparent (**Figure 6**).

Chlorophyll Fluorescence Parameters

The potential quantum efficiency of PSII (F_v/F_m) did not differ among the three types of ramets and was unaffected when the rhizomes were severed. When rhizomes remained intact, F_m and F_o were considerable greater in sand buried ramets than in mother or wind eroded ramets ($P < 0.001$). In addition, there were no significant difference between mother and wind-eroded ramets ($P > 0.05$). Moreover, F_m and F_o values did not significantly differ between mother and sand-buried ramets after rhizomes were severed ($P > 0.05$). However, the F_m and F_o values in the mother ramets themselves were significantly different after rhizomes were severed (both $P < 0.05$) (**Table 4**).

The effective quantum yield of PSII (Φ_{PSII}), photochemical quenching (qP) and electron transport rate (ETR) values for the three types of ramets remained constant between the connected and severed conditions. Under connected conditions, Φ_{PSII}, qP, and ETR were greatest in mother ramets and not significantly different in sand-buried ramets, but were significantly different for wind eroded ramets ($P < 0.001$). Under severed conditions, these three indicators were not significantly different between mother and sand buried ramets (**Table 4**).

DISCUSSION

The shrub *C. mongolicum* is well adapted to desert environments where populations experiencing fast expansion mainly by clonal

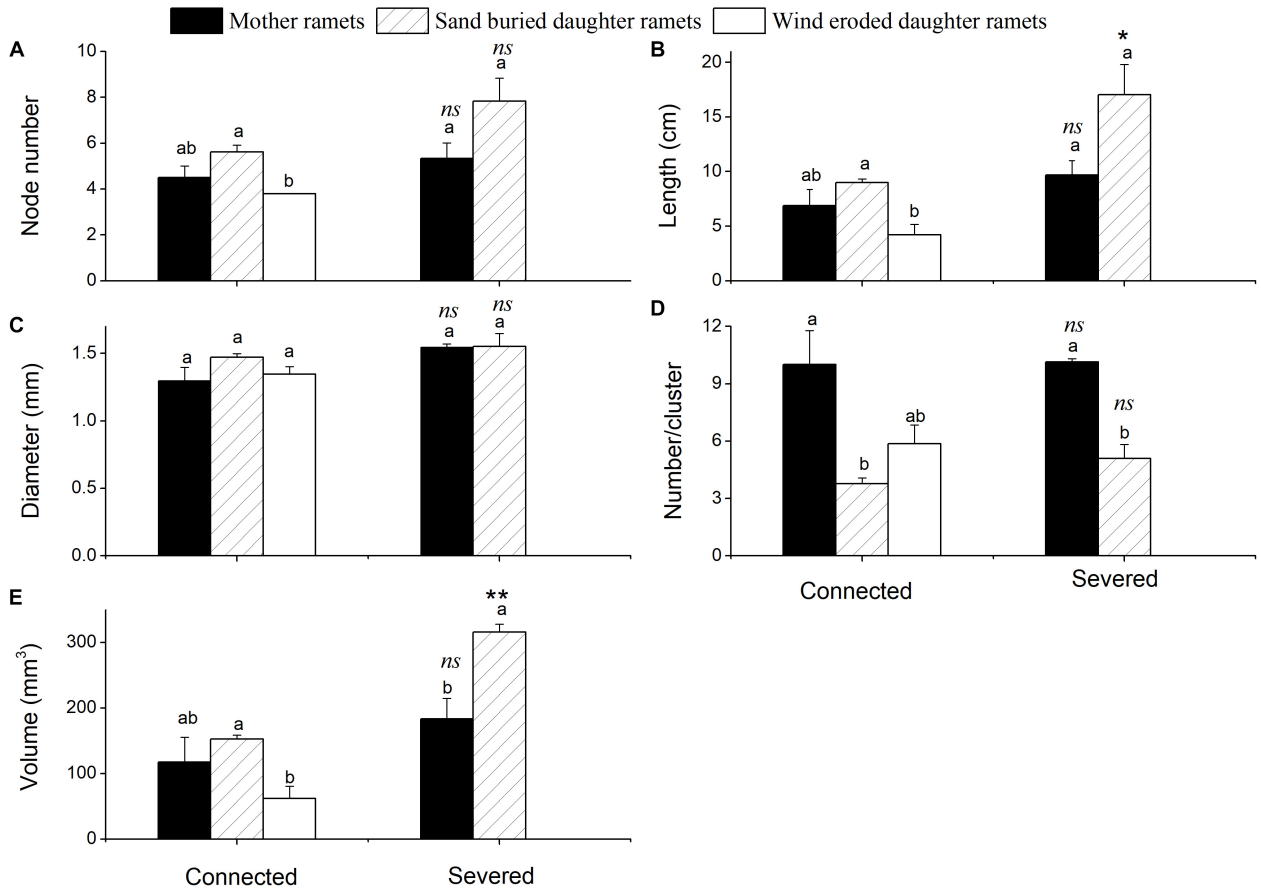

FIGURE 3 | Phenotypic changes of assimilative branches of mother and daughter ramets in *C. mongolicum* at wind eroded and sand accumulated environments under different treatments (mean ± SE). **(A)** Assimilating shoots node number; **(B)** assimilating shoots length; **(C)** assimilating shoot diameter; **(D)** assimilating shoot number per cluster; **(E)** assimilating shoot volume. [Different lowercase letters denote significant differences ($p < 0.05$) among ramets before or after being severed, * denotes a significant difference ($p < 0.05$) between the same type of ramets before and after being severed]; ** denotes a significant difference ($p < 0.01$) between the same type of ramets before and after being severed; ns denotes $p > 0.05$].

regeneration and clonal growth (Fan et al., 2018). However, for continual population recruitment, increased colonization capacity depends on juvenile ramet survival, especially when occupying stressful (wind eroded) environments.

Effects of Sand Movement on the Population Status and Capacity of Clonal Regeneration

Both heavy sand burial and heavy wind erosion can greatly impair the growth and physiology of a plant population (Yu et al., 2008; Lechuga-Lago et al., 2016). Plants growing on the windward side of dunes commonly lose water from their root system due to root exposure by wind erosion (Yu et al., 2008). In addition, plants on the leeward side of the dune are prone to being buried by sand (Li et al., 2013). Only moderate sand burial formed by the interaction of wind erosion and sand burial provides an ideal microhabitat, where plants can propagate (by ramets) quickly and without inhibition. In addition, we found that the proportion of dead shoots at both wind-eroded and sand-buried sites were significantly higher than at the moderately sand-buried plot. This indicates that this was the most favorable environment for growth of *C. mongolicum* shrubs (**Table 1**). Clonal regeneration also differed among the three different mobile sand environments. In the severe sand burial and wind erosion sites, many fewer daughter ramets were found than at the moderate sand burial site. Moreover, new ramets initialized on rhizomes failed to emerge through the physical barrier of deep sand burial sites and thus increased mortality, as reserves stored in the plant organs were depleted (Yu et al., 2001, 2004). Ramets on rhizomes exposed to serious wind erosion suffered extreme senescence from the lack of moisture (Luo and Zhao, 2015b). Our results demonstrated that the effects of sand burial and wind erosion on clonal plants may have significant effects on ecological succession patterns over several years, as was reported by Mandujano et al. (2007).

Horizontal rhizomes play a critical ecological and physiological role for *C. mongolicum*. They are important clonal organs that produce and maintain belowground buds, which are capable of forming daughter ramets from each

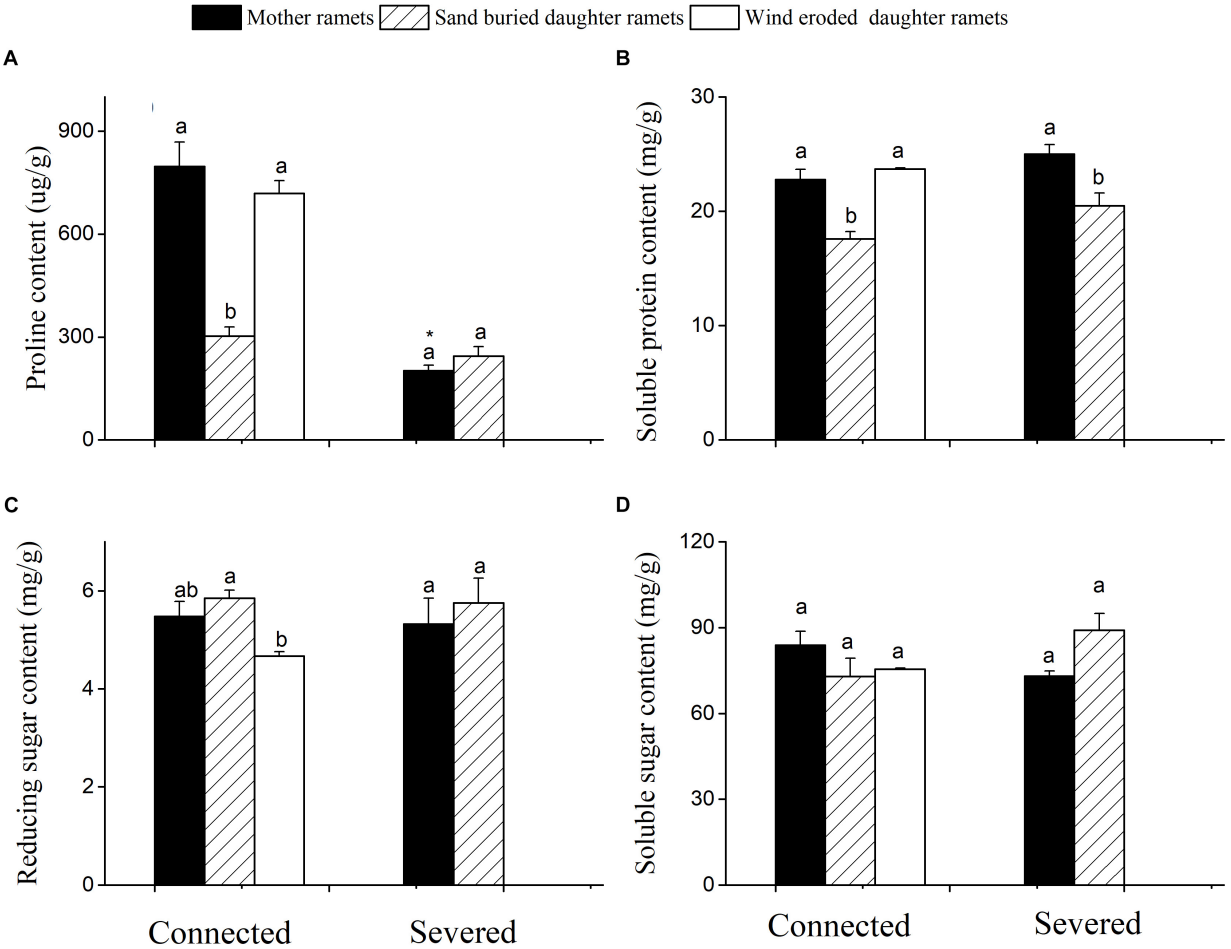

FIGURE 4 | Changes in the biochemical characteristics of *C. mongolicum* mother and daughter ramets when severed or connected under wind eroded and sand burial environments. **(A)** Proline content; **(B)** Soluble protein content; **(C)** Reducing sugar content; **(D)** Soluble sugar content [Different lowercase letters denote significant differences ($p < 0.05$) among ramets before or after being severed, * denotes significant difference ($p < 0.05$) between the same type of ramets before and after being severed].

bud site on the roots (rhizomes) (**Table 2**), and provide a vital connection with the mother plant and the ramets, thereby significantly contributing to colonization and dispersal. However, sand movement causes heterogeneous habitats for horizontal rhizomes and hence variance in their survival and function. We found that horizontal rhizomes can extend several meters from the mother ramet, and that longer roots occur at wind-eroded sites than at sand-buried sites because natural wind erosion effectively exposes horizontal rhizomes. On the other hand, we also found that when exposed to the air bud bank density and overall survival rates were much lower at wind-eroded sites than at sand burial sites, where such exposure does not occur (**Table 2**). This difference in survival likely happens because wind erosion denudes roots, thereby modifying the bud bank size (i.e., the number of buds) and the overwinter survival rate. Because survival and bud production were both higher at sand burial sites, we concluded that moderate sand burial environments promote clonal reproduction to a greater degree than exposure from wind erosion (**Table 2**). Our results are aligned with the findings of previous studies that indicated that moderate sand burial maintains a moist environment around clonal fragments and protects them from drying out (Maun, 1996; Luo and Zhao, 2015a).

Adaptive Strategies to Mobile Sand Dune Environments

Though sand movement significantly affects the clonal regeneration, *C. mongolicum* takes some strategies to adapt to mobile sand dune environments. First, the extensive root architecture reflects the plant's adaptive ability to make best use of unevenly distributed soil resources (Fitter and Stickland, 1991). In our study the root length (vertical principal root) to shoot length ratio found in mature natural populations was lower than 1.0. While each mother ramet of *C. mongolicum* can often include several horizontal rhizomes, and each can extend beyond several meters from the mother ramets, indicating that *C. mongolicum* allocates few resources to principal vertical root tissues, with increasing resource allocation to

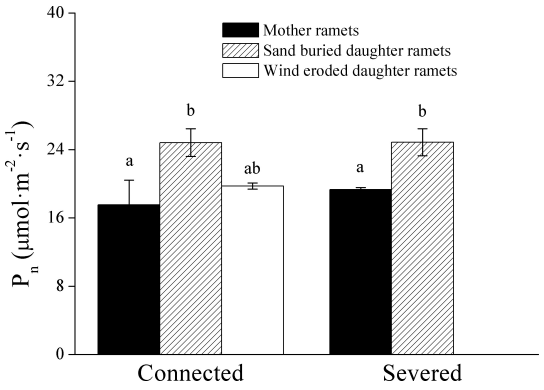

FIGURE 5 | The effect on net photosynthetic rate (P_n) of severing the connection between *C. mongolicum* mother (M) and daughter (R) ramets when severed or connected under eroded (Rw) or sand burial (Rs) environments. [Different lowercase letters denote significant differences ($p < 0.05$) among clonal fragments before or after being severed.

horizontal roots. This was previously documented for two other rhizomatous dune species, *Ammophila breviligulata* (Maun, 1984) and *Sporobolus virginicus* (Balestri and Lardicci, 2013). The spread of horizontal rhizomes substituting for vertical roots may be an important adaptive strategy used by clonal plants to colonize harsh mobile sand dune habitats (Pitelka and Ashmun, 1985; de Kroon and Hutchings, 1995). This adaptive strategy may also increase plant survival after heavy burial or wind erosion, and/or may permit *C. mongolicum* to forage for water in less affected parts of the dune. This foraging strategy was identified in the clonal plant *Hedysarum laeve* (Li et al., 2015) and a similar strategy may be used by the woodland strawberry, *Fragaria vesca* (Waters and Watson, 2015).

Second, we found a proliferation of ramets on rhizomes that had been buried by sand in the early growing season from 0 ∼ 25 cm depth. However, most ramets initiated from these buds died within 1 month of emergence. The high mortality rate of juvenile ramets occurred in the early growing season in both sand environments, with only 10% remaining at the end of the growing season (**Table 2**). Thus, *C. mongolicum* demonstrated a high ramet turnover in its early life stage, in both mortality and initiation, which could be attributed to the continual windy and drying conditions in the Minqin region. This result is consistent within plants of the same genus (*C. arborescens*) under severe sand burial depths (Luo and Zhao, 2015a). In a moderate sand burial environment, survival depends mainly on plant density as water and nutrient in the Minqin dune environment are quite poor, thus the competition between ramets is severe, and fast ramet turn over may assist in avoiding localized water and nutrient depletion (Dong and Alaten, 1999; Li et al., 2015). This could also be described as an adaptive strategy used by *C. mongolicum* to cope with the highly variable sand environment in this region.

In summary, *C. mongolicum* rhizomes can extend considerable distances and have a high capacity for ramet regeneration, however, this capacity is highly dependent on sand burial state. A habitat with alternating wind erosion and sand burial, i.e., moderate sand burial environment is ideal for clonal reproduction and colonization. In a harsh desert sand environment, we also find that *C. mongolicum* exhibit an exploratory foraging strategy, similar to those found in other clonal plants.

Effects of Physiological Integration on Clonal Growth in Different Sand Environments

Despite the adaptive strategies of *C. mongolicum*, wind erosion remains a major stress factor in the Minqin region. Results from this study demonstrate that wind erosion led to a reduction in leaf attributes on both mother and daughter ramets. Even so, ramets at wind eroded sites that were unfavorable for growth still survived if they remained connected to the parental plant via the rhizome. Physical connections between ramets transports resources within clonal plants, this integration significantly ensured young ramet survival and permitted continuing development in the harsh wind-blown environment. These findings are consistent with those of Hartnett and Bazzaz (1983); Salzman and Parker (1985), and Roiloa and Hutchings (2012), who demonstrated that resource transport occurs from ramets under favorable conditions to developing ramets in unfavorable sites.

In contrast, sand buried daughter ramets had improved assimilating shoot elongation. This appeared very important for daughter clonal fragment of *C. mongolicum* compared to mother ramets, which increased the number of assimilating shoots per cluster (branchlets), presumably to enhance photosynthesis. Even when rhizomes were severed, daughter ramets maintained growth status as with the mother ramets. The reason for this was explained by Yu et al. (2004); Dong et al. (2010, 2011); and Balestri and Lardicci (2013), who noted that the internodes of clonal plants contain storage materials that can be remobilized by ramets when necessary. Thus, under moderate sand burial conditions, *C. mongolicum* rhizomes and their nodes could also mobilize water and nutrients to the ramets after being disconnected from the mother ramet. Moreover, daughter ramets survived by carrying out photosynthesis, and were able to absorb enough photosynthate for normal growth. Physiological integration through rhizome connection increased the colonization capacity and ramet survival of *C. mongolicum* ramets occupying wind eroded environments.

Sand-buried ramets produced more chlorophyll a, chlorophyll b and total chlorophyll contents than wind eroded ramets when connected. As stated previously these ramets also have a higher photosynthetic capacity. When connected, if daughter ramets suffered from wind erosion, mother ramets showed the same trend as they mobilize resources to reduce plant cell membrane damage by increasing proline and soluble protein content (Luo et al., 2014; Ye et al., 2016). Our results demonstrated that at wind-eroded sites mother and (stressed) daughter ramets had higher proline and soluble protein content than sand buried (i.e., unstressed) ramets did. In parallel with this finding, the photosynthetic rate (P_n) and chlorophyll fluorescence parameters (F_m, F_o, Φ_{PSII}, qP, and ETR) showed similar trends, much greater

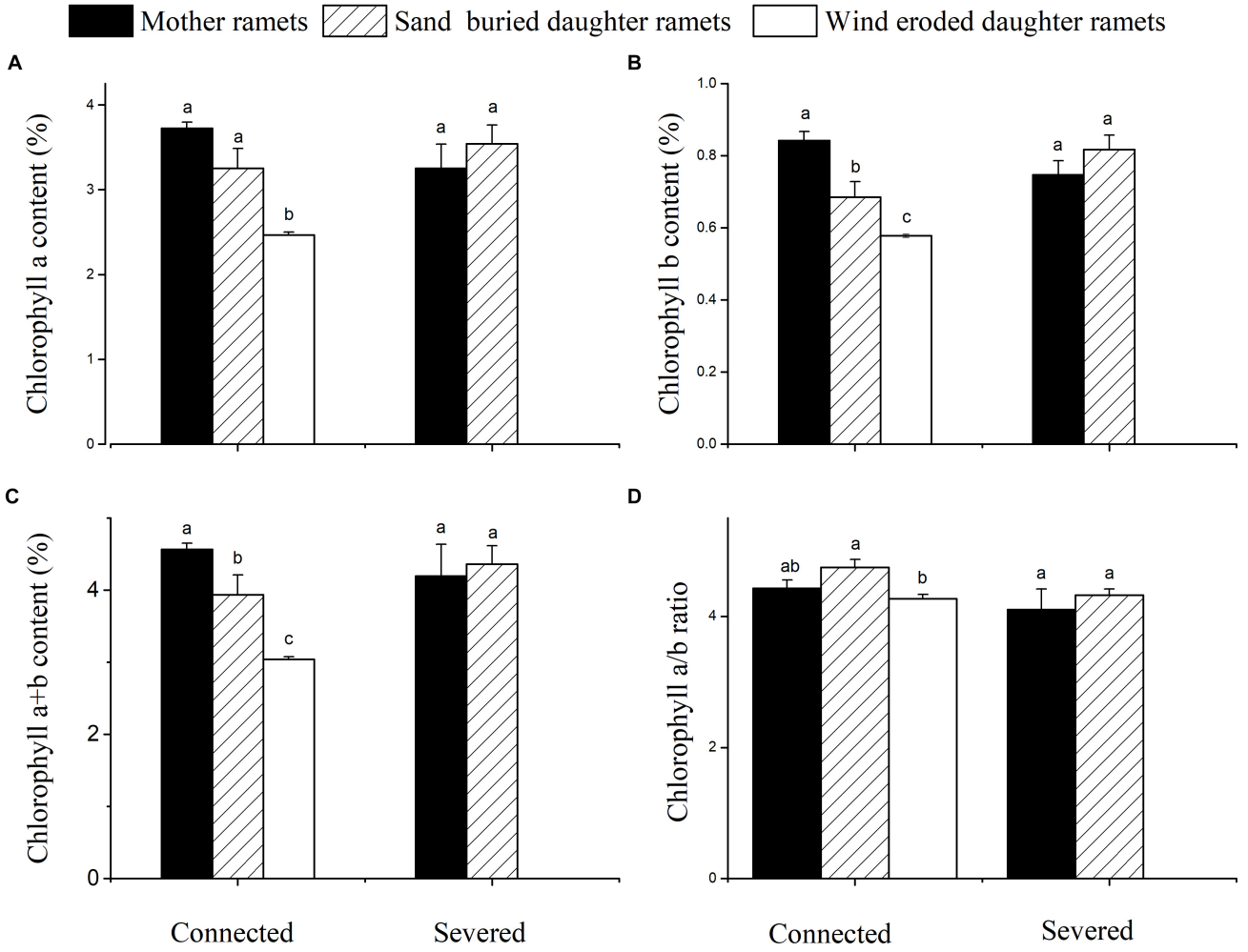

FIGURE 6 | Chlorophyll content changes in mother and daughter ramets when connected and severed in *C. mongolicum* growing in wind eroded and sand accumulated environments. **(A)** chlorophyll a; **(B)** chlorophyll b; **(C)** chlorophyll a + b; **(D)** chlorophyll a/b. [Different lowercase letters denote significant differences ($p < 0.05$) among clonal fragments before or after being severed.

TABLE 4 | Chlorophyll fluorescence parameters in assimilating shoots of different types of ramets under connected and severed conditions.

		Mother ramet	Sand buried ramet	Wind eroded ramet
F_m	Connected	1.42 ± 0.05bA	2.16 ± 0.27aA	1.27 ± 0.17b
	Severed	2.04 ± 0.22aB	1.92 ± 0.22aA	–
F_o	Connected	0.34 ± 0.01bA	0.47 ± 0.06aA	0.29 ± 0.04b
	Severed	0.46 ± 0.04aB	0.44 ± 0.05aA	–
F_v/F_m	Connected	0.76 ± 0.01aA	0.78 ± 0.01aA	0.78 ± 0.01a
	Severed	0.77 ± 0.01aA	0.77 ± 0.01aA	–
ΦPSII	Connected	0.59 ± 0.01aA	0.50 ± 0.07abA	0.43 ± 0.03b
	Severed	0.56 ± 0.01aA	0.54 ± 0.01aA	–
qP	Connected	0.86 ± 0.01aA	0.74 ± 0.08abA	0.65 ± 0.04b
	Severed	0.82 ± 0.02aA	0.81 ± 0.01aA	–
ETR	Connected	25.15 ± 0.29aA	21.26 ± 2.94abA	18.16 ± 1.85b
	Severed	24.17 ± 0.43aA	23.07 ± 0.42aA	–

Different lowercase letters denote significant difference ($p < 0.05$) among clonal fragments before or after being severed; different uppercase letters denote significant differences ($p < 0.05$) between the same type of ramets before and after being severed.

values were recorded from sand buried ramets than from wind eroded ramets under connected conditions. Once daughter ramets were disconnected, P_n values were similar in mother and sand-buried daughter ramets, showing that sand buried ramets had more stable photosynthetic systems. Ashraf and Harris (2013) proved that moderately sand buried conditions promoted the clonal growth of *C. mongolicum*. However, once daughter ramets were disconnected, proline content, F_o and F_m in mother ramets significantly declined to levels found in buried ramets. Taken together, these results demonstrated that mother ramets in favorable conditions could experience stress when connected daughter ramets come under stress. Mother ramets then coordinate a physiological response to the stress. Moreover, this response does not occur for daughter ramets that occupy favorable sites.

CONCLUSION

The study focused on the effect of sand movement on population regeneration capacity in *C. mongolicum*. This shrub is uniquely adapted to shifting sand environments in deserts. However, despite its best efforts to mobilize plant internal resources, normally buried plant parts when exposed to the air-causes high mortality. Wind erosion and sand burial were both found to affect physiological, biochemical, and morphological characteristics of both parent and clonal ramets. The study concluded that this plant has a number of strategies to mobilize and coordinate resources to maintain colonization though its clonal rhizomes. In the variable conditions of arid sandy deserts, daughter ramets benefit from clonal integration with the parent ramets, especially under wind eroded and unfavorable environments, which should be carefully considered in shrub and sand dune management of sand fixation plantations of *C. mongolicum*. However, to fully understand the transmission capacity of water, nutrients and energy between *C. mongolicum* clonal ramets, and apply it to plantation management, further research under sand burial and wind erosion conditions is essential.

AUTHOR CONTRIBUTIONS

BF conceived and conducted the field study, analyzed the data, and wrote the manuscript. CZ supervised the manuscript. XZ analyzed part of the data. KS supervised and reviewed the manuscript. All authors contributed critically to the drafts and provided approval of the final version of the manuscript for publication.

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Transgenerational Effects and Epigenetic Memory in the Clonal Plant *Trifolium repens*

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Transgenerational effects (TGE) can modify phenotypes of offspring generations playing thus a potentially important role in ecology and evolution of many plant species. These effects have been studied mostly across generations of sexually reproducing species. A substantial proportion of plant species are however reproducing asexually, for instance via clonal growth. TGE are thought to be enabled by heritable epigenetic modification of DNA, although unambiguous evidence is still scarce. On the clonal herb white clover (*Trifolium repens*), we tested the generality of clonal TGE across five genotypes and five parental environments including soil contamination and above-ground competition. Moreover, by genome wide-methylation variation analysis we explored the role of drought, one of the parental environments that triggered the strongest TGE. We tested the induction of epigenetic changes in offspring generations using several intensities and durations of drought stress. We found that TGE of different environments were highly genotype specific and all tested environments triggered TGE at least in some genotypes. In addition, parental drought stresses triggered epigenetic change in *T. repens* and most of the induced epigenetic change was maintained across several clonal offspring generations. We conclude that TGE are common and genotype specific in clonal plant *T. repens* and potentially under epigenetic control.

Keywords: clonal reproduction, *T. repens*, plant memory, abiotic stress, transgenerational effects

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INTRODUCTION

Adaptive phenotypic plasticity allows plants to adjust their morphology to actual environmental conditions in order to maintain or increase their relative fitness. However, plant phenotype can also reflect past environments of parents or even grandparents (e.g., Miao et al., 1991; Latzel et al., 2014; Lampei et al., 2017) due to transgenerational effects (TGE). TGE had been studied mostly across sexual generations and only rarely among clonal generations (e.g., Raj et al., 2011; Verhoeven and van Gurp, 2012; Rendina González et al., 2016, 2017). Nonetheless, clonal reproduction is very common reproductive strategy in many plant communities and is often the main reproductive strategy for most plant species. For example, up to 70% of central European meadow species reproduce clonally (Klimeš et al., 1997). Clonal plants usually also exhibit complex and sophisticated behavior such as active foraging for resources (Bell, 1984; Waters and Watson, 2015) or division of labor (Alpert and Stuefer, 1997) where individual ramets might be adjusted to different functions like

soil resources acquisition vs. photosynthesis. In this regard, each new ramet can be considered as a new generation potentially independent from the main mother plant carrying on environmental information (Latzel et al., 2016). It has been recently shown that the behavior of clonal plants can be governed not only by actual environmental condition but also by their past experiences, i.e., by TGE (Louapre et al., 2012; Rendina González et al., 2016, 2017). It is evident that TGE in clonal plants should get more attention in order to improve our understanding of their role in ecology and evolution.

TGE can be a simple consequence of carry over effects when chemicals and/or pathogens are passed from parents to offspring (Roach and Wulff, 1987; Rossiter, 1996; Huxman et al., 2001). Although, more often, TGE are thought to be targeted by a pre-programming of offspring phenotypes likely via *epigenetic mechanisms* (Bruce et al., 2007; Ginsburg and Jablonka, 2009; Ding et al., 2012; Thellier and Lüttge, 2013; Müller-Xing et al., 2014). Epigenetic mechanisms comprise histone modification, methylation of cytosine residues of DNA and small RNA molecules regulating gene expression, which are intimately interconnected (Wagner, 2003; Vanyushin, 2006). DNA methylation is shown to be environmentally inducible and, in some cases, heritable (Boyko et al., 2010; Angers et al., 2010; Hauser et al., 2011; Wibowo et al., 2016; Lämke and Bäurle, 2017). Nonetheless, most of the environmentally induced epigenetic changes are maintained within generations and rarely passed to sexually derived offspring due to meiosis that resets most of the environmentally induced epigenetic variation (Feng and Jacobsen, 2011; Paszkowski and Grossniklaus, 2011; Heard and Martienssen, 2014; Tricker, 2015). Clonal plants, on the other hand, can reproduce asexually and thus bypass meiosis. Therefore, it has been proposed that environmentally induced epigenetic change can be better maintained in clonal than sexual generations. Heritable environmentally induced epigenetic change can consequently enable a rapid adaptation to changing environments and its implications in short-term microevolution of clonal plants (Latzel and Klimešová, 2010; Verhoeven and Preite, 2014; Dodd and Douhovnikoff, 2016).

Indeed, mounting evidence showed that epigenetic differentiation of clonal plant populations can be at least partly caused by environmental induction. One of the first evidences provided Verhoeven et al. (2010) who showed that environmental stress in parental generation can trigger changes in DNA methylation that can be passed to next apomictic (clonal) generation of dandelions (*Taraxacum officinale*) with high fidelity. The environmental induction of DNA methylation changes was genotype-specific and represents, at least partly, a stress-induced increase of seemingly untargeted DNA methylation variation (Preite et al., 2018). In another study, they pointed out that the epigenetic differentiation (DNA methylation variation) of natural populations of apomictic dandelions can be environmentally determined (Preite et al., 2015). Furthermore, Richards et al. (2012) showed in Japanese knotweed and Gao et al. (2010) in alligator weed that genetically uniform populations of clonal plants can be epigenetically structured, and that this structure is likely due to environmental conditions. Raj et al. (2011) observed in clonal offspring of poplar trees that drought stress response was associated with origin of a genotype and was likely mediated by epigenetic variation. Finally, Robertson et al. (2017) identified specific epigenetic variation in clonal *Spartina alternifolia* populations related to water pollution. Although these pioneering studies provided first evidence that epigenetic change can be triggered by environment, they did not detect direct phenotypic effects of epigenetic variation (e.g., Verhoeven et al., 2010) and were not able to distinguish between epigenetic variation originated from environmental induction or selection of certain epigenotypes (e.g., Richards et al., 2012; Spens and Douhovnikoff, 2016; Robertson et al., 2017).

Here we provide results of two experiments focusing on clonal TGE and epigenetic changes in *Trifolium repens* induced by various environments. In the first experiment, we tested the effect of five parental environments – control, drought, contaminated soil (salt and copper), and shading, on the induction of clonal transgenerational phenotypic effects in the common clonal herb *T. repens*. Since TGE can be genotype specific (Groot et al., 2017; Lampei et al., 2017; Münzbergová and Hadincová, 2017), we tested the generality of environmentally induced TGE of the five environments across five genotypes. Since drought stress triggered the strongest phenotypic TGE in one of the tested genotypes, we focused in the second experiment on drought stress for this single genotype only. By methylation-sensitive amplification polymorphism (MSAP), we tested the role of different intensities and durations of drought periods on induction of epigenetic change in clonal offspring generations. We tested the following hypotheses: (i) environmental stress experienced by the parental clone triggers clonal TGE observable at the phenotype level of clonal offspring ramets, (ii) clonal TGE are genotype specific, (iii) different intensity and duration of drought stress in the parental generation induces changes in DNA methylation that is passed to clonal generations.

MATERIALS AND METHODS

First Study – Transgenerational Effects

Five genotypes of *Trifolium repens* L. were randomly collected from grasslands in surrounding of Průhonice town, Central Bohemia, Czech Republic in 2013. All genotypes thus experienced same climatic conditions and very similar (a)biotic interactions. Despite this, the genotypes differed in their growth where two of them produced more biomass than other three genotypes (see also below). Their propagation took place in a greenhouse with controlled temperature and light regime set up at 17 h light and 7 h dark. Plants were cultivated in 30 × 40 × 8 cm trays filled with commercial Agro lawn soil substrate (mixture of compost, peat and sand, same substrate was used in all steps of the study) for 4 months to even out possible environmental effects and to pre-cultivate plant material.

Study Design

Ten cuttings consisting of eight internodes of each genotype were individually transplanted to 30 × 40 × 5 cm trays filled with soil substrate and were let to propagate. After 1 month plants were proportionally and randomly assigned to the

following environments for 2 months: control – no manipulation, drought – limited watering only when leaves were wilting (eight cycles of drought stress during the run of experiment), copper contaminated soil – regular application (three times in a week, 24 applications in total) of 60 ml of 16 mM solution of copper (II) sulfate pentahydrate ($CuSO_4 \times 5H_2O$) with estimated final concentration of 500 mg copper in 1 kg soil, salt contaminated soil – regular application of 100 ml of 4.3 mM solution of salt (NaCl) with estimated final concentration 1.5 g salt in 1 kg soil, and shading by growing plants under green plastic sheet that reduced light intensity at 50%. After 2 months of cultivation in all environments, 10 cuttings (maternal ramets) consisting from 4 internodes and apical end labeled by a plastic ring were created from each treatment and genotype combination and individually transplanted to 20 × 30 × 4 cm trays (one cutting per tray) filled with soil substrate without any further manipulation. All ramets were without root system with only emerging root tip usually at the fourth internode. All trays were randomly distributed in the greenhouse, and their positions were changed four times during the cultivation period. This randomized design resulted in 250 plants (5 genotypes × 5 treatments × 10 plants) in total. After 50 days from transplantation all plants were harvested. Every harvested plant was cut at the position of the plastic ring (position of the apical end at the time of transplantation), and only the parts that had developed after transplantation were considered. Every plant was divided into the maternal stolon (the main axis of growth of the transplanted maternal ramet, see also **Figure 1**) and into lateral branches, here considered as the collection of daughters (offspring) ramets due to the monopodial growth of *T. repens* (i.e., maternal ramets is elongating in the main axis and producing offspring ramets via the lateral axillary buds). Maternal stolon as well as all offspring ramets were dried at 80°C for 24 h and weighed.

Second Study – Drought Induced DNA Methylation Variation in Clonal Offspring

Morphological data as well as experimental design of the study has been already published (Rendina González et al., 2016) and, therefore, we provide here only a reduced description of the study. We used the same growing setup as in previous study. Thirty cuttings of pre-cultivated genotype of *T. repens* were individually planted to a tray (30 × 40 × 8 cm) filled with commercial Agro lawn soil substrate (mixture of compost, peat and sand). After 30 days, plants were randomly assigned (six trays per treatment) to each of the following five treatments: (1) Control; (2) Long-Intense drought; (3) Short-Intense drought; (4) Long-Medium drought; and (5) Short-medium drought. Plants assigned to control treatment were watered regularly to maintain the soil permanently moist. For the intense drought treatments, plants were watered with approximately 200 ml of water only when most leaves were wilting. Such stress occurred within 4 to 7 days. The Long-Intense drought treatment was applied for 4 months (December 2014 to March 2015), whereas the Short-Intense drought treatment was subjected to the control treatment of the first two of these months (December 2014 to January 2015) and to the water stress for the next 2 months (February to March 2015). The medium drought plants experienced half of

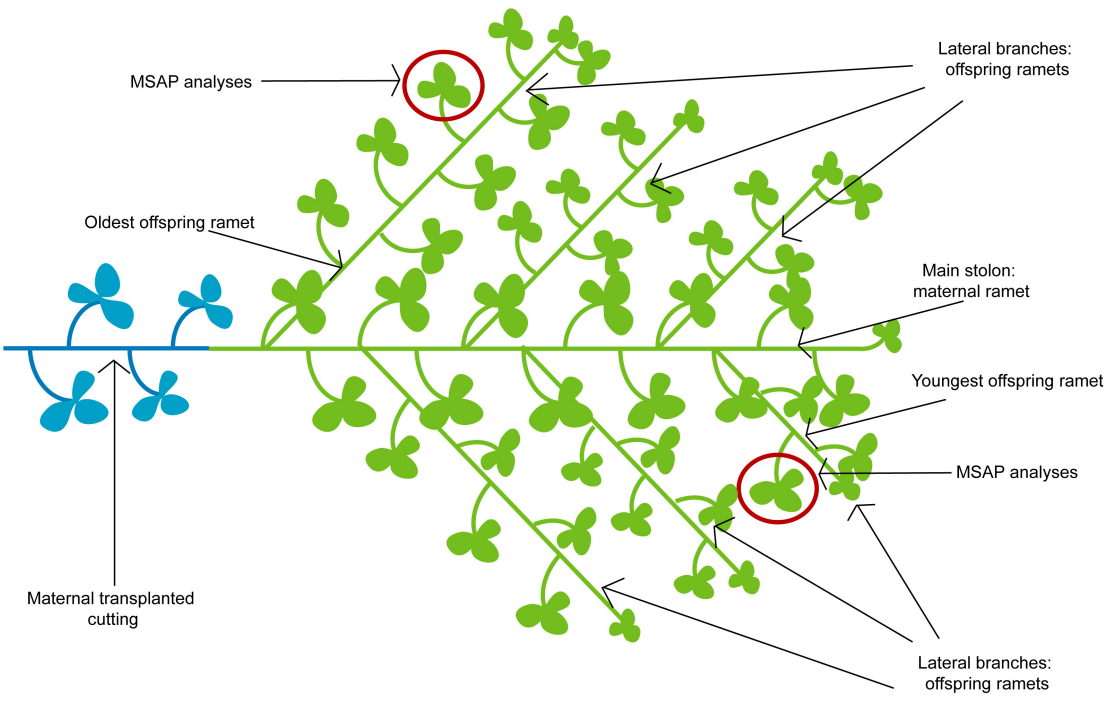

FIGURE 1 | Idealized scheme of *T. repens* plant developed after transplantation of maternal cutting to a control environment. Highlighted are leaves collected for MSAP analyses in the second experiment and the cohorts of offspring ramets (i.e., the oldest and youngest offspring ramets).

the drought cycles experienced by plants in the intense drought treatments, which was achieved by applying water stress to them during alternating periods of water stress experienced by plants in the intense drought treatment. The Short-Medium drought and Long-Medium drought corresponded to those already described for the intense drought treatments (for more detailed information see Rendina González et al., 2016). In April 2015, all drought treatments were terminated, and plants were cultivated for another 14 days in control conditions with a saturated water regime. After the period, five standardized apical cuttings from each parental plant from all treatments were created. These cuttings consisted of four nodes and the apical end and were planted individually into 18 × 10 × 6 cm trays filled with standard potting soil, i.e., one cutting per tray. Fifteen replicated plants from each parental treatment, 75 plants altogether, were randomized and grown for 2 months in control treatment conditions. After 2 months above-ground biomass was harvested (results published in Rendina González et al., 2016). Leaf samples for MSAP analyses were collected from five randomly selected plants from all treatments. Collected were fully developed leaf of the youngest and oldest offspring ramet that had developed after transplantation to the control environment (**Figure 1**). Generally, around five lateral branches, corresponding to five consequent generations, developed in our experiments. Together were collected 50 leaf samples (5 treatments × 5 replicated plants × 2 leaves). However, in total, only 41 samples from the offspring ramets were used for molecular analyses due to insufficient size for DNA extraction of 6 leaf samples from the Short-Medium drought treatment and 3 not properly amplified samples (2 from Short-Intense and 1 from Long-Intense treatments).

MSAP Analysis

Total genomic DNA was extracted from 8 mg of dry leaf material with NucleoSpin® Plant II kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany), and the quality was examined by electrophoresis in agarose gel 1% (w/v). Purity and quantification were measured spectrophotometrically with NanoDrop2000 (Thermo Scientific). DNA digestion was performed with 100 ng of DNA, 8 units of *HpaII* (New England) as frequent cutter and 8 units of *EcoRI* (New England) as rare cutter. The digested ends were ligated with 0.5 μl of *HpaII* adapter [50 μM] and 0.5 μl of *EcoRI* adapter [10 μM] (**Table 1**) and 4 units of T4 DNA ligase (New England). All samples were then diluted 6.66× fold.

HpaII recognizes CCGG sequences but cuts only if the cytosines are unmethylated or if the external cytosine is hemimethylated. Cleavage is blocked if the cytosine is fully methylated. Thus, in clonal plants, assuming the absence of genetic variation, MSAP loci can be interpreted as a direct variation in the methylation status of the restriction site (Verhoeven et al., 2010). MSAP protocol was adapted from Aina et al. (2004). A pre-amplification step was carried out with *HpaII* primer (5′GACTGCGTACCAATTC) and *EcoRI* with one selective nucleotide (5′GACTGCGTACCAATTC+A). PCR mix contained: 2 μl of diluted DNA; 2 μl Buffer 10× (TS); 1 μl $ClMg^{+2}$, 0.8 μl dNTPs [5 μM], 0.4 μl. H-M primer [10 μM], 0.4 μl EcoR1 primer [10 μM]; 1 unit of *Taq* DNA Polymerase (ThermoScientific) in a final volume of 20 μl. The amplification conditions were: 94°C for 2 min; 10 cycles at 94°C 30 s, touchdown of −1°C 65°C for 30 s, 72°C for 2 min, 25 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 2 min and a final elongation step at 60°C for 10 min. The samples were then examined by electrophoresis in agarose gel 1% (w/v).

A second step of selective amplification was conducted with three fluorescently labeled primer pairs chosen from a preliminary selection test of 12 primers and 5 μl of the pre-amplificated DNA 10×, 2.5 μl Buffer 10× (TS); 1.5 μl $ClMg^{+2}$, 1 μl dNTPs [5 μM], 0.5 μl BSA (TS), 0.5 μl HpaII selective primer [10 μM], 0.5 μl EcoR1 selective primer [10 μM]; 1 unit of *Taq* DNA Polymerase (ThermoScientific) in a final volume of 20 μl. The selective PCR conditions were as following: 94°C for 4 min 30 cycles at 94°C for 45 s, 60°C for 30 s, 72°C for 30 s and a final elongation step at 72°C for 5 min. The primer combinations used were EcoRI-AAG/HpaII-CAC, EcoRI-ATT/HpaII-TTA, EcoRI-ACA/HpaII-TCAA.

Following selective amplification, 1 μl of each the PCR products were mixed with a solution of 10 μl of Hi-Di formamide and 0.2 μl of molecular weight marker LIZ500 and denatured at 95°C for 5 min followed by quick cooling on ice.

TABLE 1 | Results of GLM testing the effect of parental treatments and genotype on the biomass of maternal ramets of *T. repens* after transplantation to the control environment.

	Df	SS	*F*	*P*
Genotype (G)	4	1.714	29.808	**<0.0001**
Treatment (T)	4	0.889	15.463	**<0.0001**
G × T	16	0.331	1.437	0.128

Significant P values are in bold face.

Fragment Analysis

The amplified fragments were separated by capillary electrophoresis in the ABI 3130 Genetic Analyzer (Applied Biosystems) and measured with the GeneScan™-500 LIZ® Size Standard (Applied Biosystems, Warrington, United Kingdom). Presence (1) and absence (0) of fragments were scored from the obtained electropherograms using GeneMarker 2.2.0 software (SoftGenetics® LLC) to construct an epigenetic binary matrix. Fragments from approximately 100–500 bp were scored based on the presence of at least one peak height over 50 relative fluorescence unit and visually compared between all samples relative to each primer combination. Loci present in the negative controls and the ones which contained more than three miss-matches between technical replicates were removed from the analysis (26 in total). 10% of the samples were replicated to estimate the error rate together with the negative controls (Bonin et al., 2004). The error rate for all primer combinations was 6.7%.

Statistical Analyses

First Study – Transgenerational Effects

The effects of maternal treatments and genotype on the dry biomass of maternal stolon and offspring ramets were tested using general linear model (GLM) with the two-way full factorial

design. Since maternal stolon biomass had weak but positive effect on offspring biomass (correlation biomass of maternal ramet with offspring biomass R^2 = 0.065), maternal stolon biomass was included as a covariate to the statistical model when offspring biomass was analyzed.

To meet the assumptions of homoscedasticity and normality, all measured variables were log transformed prior to analyses. All statistical analyses were performed using JMP statistical software (JMP 10, SAS Inst.).

Second Study – DNA Methylation Variation

For the analysis of the binary matrix, the "msap" package for R was used (Pérez-Figueroa, 2013). Population differentiation was tested using analyses of molecular variance (AMOVA) that estimates Phi-st as fixation index (an analog of Fst for molecular data, Excoffier et al., 1992) by means of the package "pegas" with 10,000 permutations and includes the package "ade4" for the principal coordinates analysis (Pérez-Figueroa, 2013). In total, all offspring ramets that had developed after transplantation of the maternal ramets to the control environment were included in the analysis (n = 41).

RESULTS

First Study – Transgenerational Effects

Maternal Stolon

Maternal treatments altered growth of maternal stolon after its transplantation to the control environment (**Figure 2** and **Table 1**). However, the *post hoc* test revealed that the significant difference was mainly due to copper treatment (**Figure 2**).

TABLE 2 | Results of GLM testing the effects of maternal treatments and genotypes on the biomass of offspring ramets of *T. repens* developed after transplantation of maternal ramets to the control environment.

	Df	SS	F	P
Maternal stolon	1	3.034	41.206	**<0.0001**
Genotype (G)	4	8.252	28.0186	**<0.0001**
Treatment (T)	4	2.352	7.9847	**<0.0001**
G × T	16	3.321	2.819	**0.0004**

Significant P values are in bold face.

Offspring Ramets

Maternal treatments affected biomass of offspring ramets, and this effect showed to be genotype specific (**Table 2** and **Figures 3**, **4**). The offspring biomass was highest for the offspring of mothers that experienced copper contamination, the lowest biomass for the offspring of mothers that experienced drought stress (**Table 2** and **Figures 2**, **3**). Nonetheless these effects were strongly genotype dependent (**Table 2** and **Figure 4**). Copper treatment increased ramet biomass in two genotypes (C and F) but had no effect on offspring biomass of other genotypes. Shading had no effect on offspring biomass in all but one genotype. Salt contamination either decreased or increased offspring biomass depending on the genotype (**Figure 4**).

MSAP Analysis

AMOVA analysis of methylation profiles of leaf samples collected from the offspring ramets of all treatments that had developed after maternal ramets' transplantation to the control environment showed a significant but low differentiation between treatment groups [Phi_ST = 0.07628 (P = 2e−04) see **Figure 5**]. Based on the epigenetic distances calculated from the binary matrix

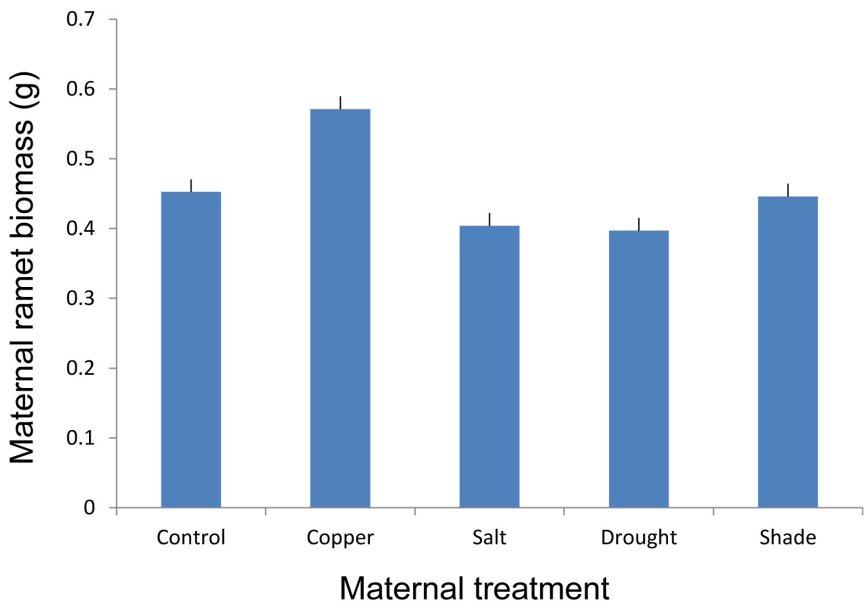

FIGURE 2 | The effect of maternal treatments on the above-ground biomass of maternal ramets of *T. repens* that had developed after transplantation to the control environment. Means and SE are shown.

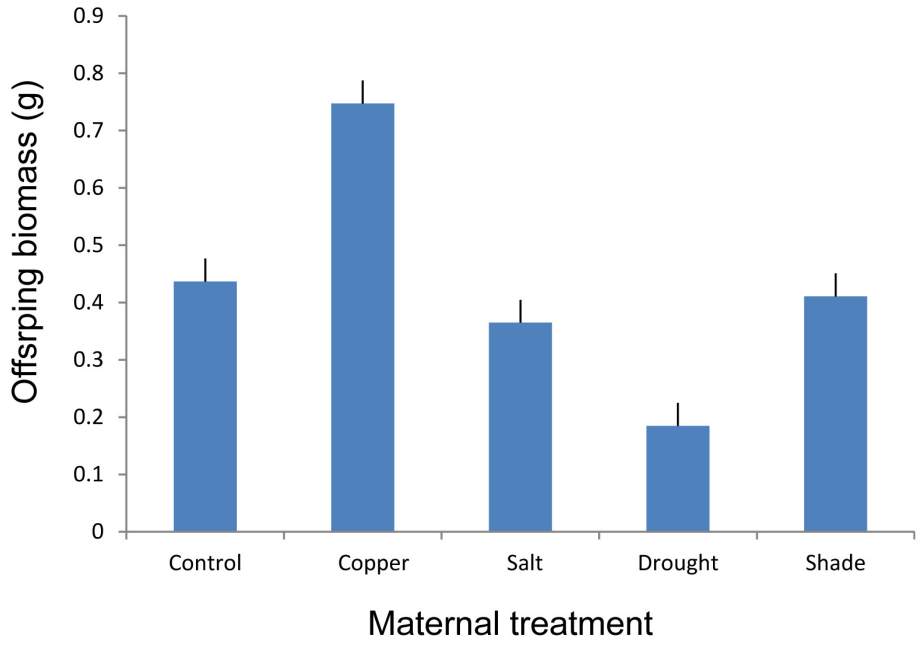

FIGURE 3 | The effect of maternal treatments on the above-ground biomass of offspring ramets of *T. repens* that had developed after transplantation to the control environment. Means and SE are shown.

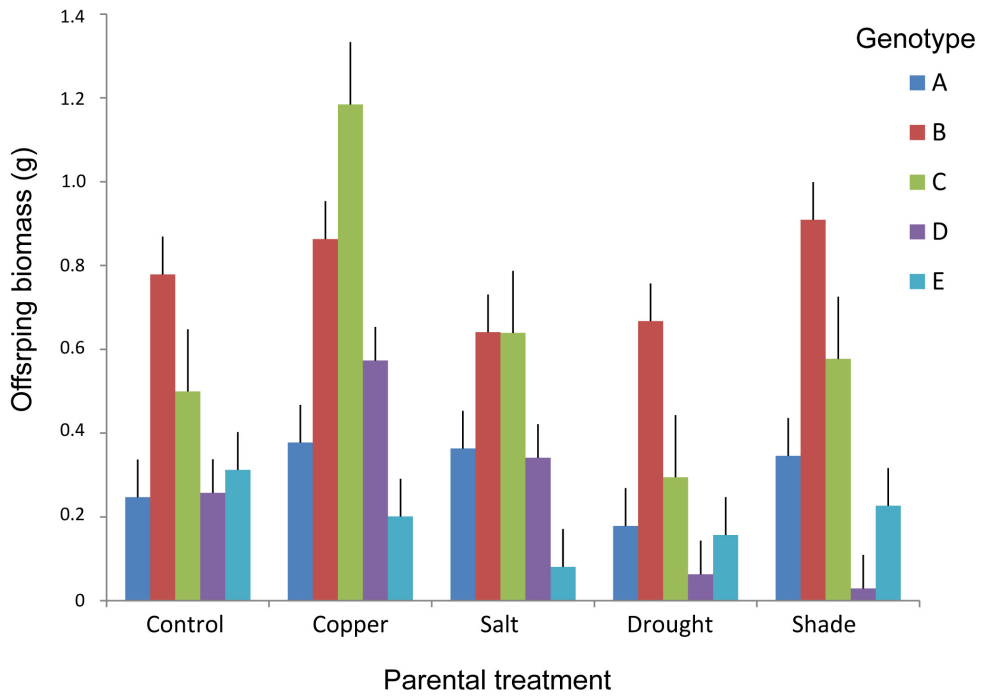

FIGURE 4 | Above-ground biomass of offspring ramets of *T. repens* in relation to maternal treatments and genotypes. Means and SE are shown.

for the enzyme *HpaII*, two distinct groups formed in the PCoA with 21.8% of the variance explained in both axis (**Figure 5**). On the top the Short-medium differentiated from the rest of the treatments with the control treatment appearing in the middle of the plot, whereas the other three treatments (Short-intense, Long-intense, and Long medium) clustered together alongside the control treatment. Also, comparisons of individual treatments with controls show that epigenetic status of all

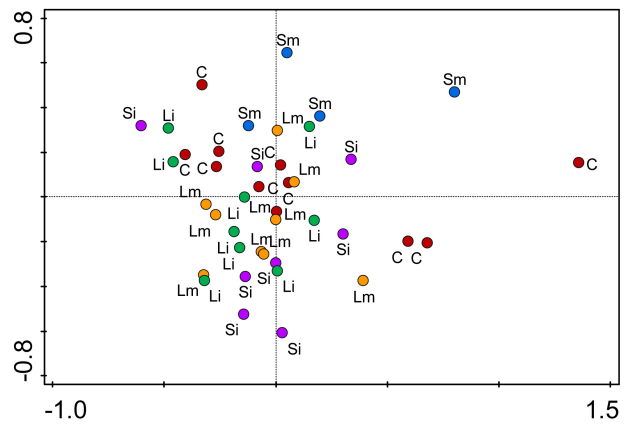

FIGURE 5 | Principal Coordinate Analysis (PCoA) of epigenetic differentiation on offspring ramets of *T. repens* between treatments. C, control (red); Si, Short-Intense drought (purple); Sm, Short-medium drought (blue); Li, Long-Intense drought (green); Lm, Long-medium drought (yellow). The two coordinates explain 21% of the variance. Individuals are shown as points.

treatments but short-medium drought differed from controls (**Figure 6**).

DISCUSSION

Transgenerational effects are thought to play an important role in the adaption and evolution of clonal plants in predictable fluctuating environments (Latzel and Klimešová, 2010; Verhoeven and Preite, 2014; Tricker, 2015; Bilichak and Kovalchuk, 2016). Our results indicate TGE positively or negatively altered clonal offspring biomass in *T. repens* depending on the type of maternal stress (drought, soil contaminations, and shading). However, the direction and strength of these TGEs were genotype specific. Finally, by testing the variation of genome-wide DNA methylation with MSAP, the second study shows that drought stress induces DNA methylation changes that are inherited in clonal offspring plants. Thus, DNA methylation has the potential to mediate at least some of the observed phenotypic TGE; however, the causal role of DNA methylation in the TGE remains to be demonstrated.

First Study – Transgenerational Effects Due to Various Parental Stress Types

Copper contaminated soil in the maternal generation triggered a positive TGE on offspring biomass, whereas drought stress in maternal generation triggered a negative TGE on offspring biomass. Copper is an essential metal for plant growth and development since it is involved in a wide range of physiological processes, although it can be toxic at high concentrations (Sommer, 1931; Yruela, 2005). The threshold of copper, for which it becomes toxic to the plant is species dependent (Adrees et al., 2015). We observed a significant increase in the biomass production of maternal stolons and offspring ramets suggesting that the concentration applied in our study was probably below the toxic level for *T. repens*. Indeed, there are cases of copper tolerant plants that involve mechanisms to overcome the toxic effect of heavy metals. For example, the excess of copper can be sequestered into metabolically inactive parts like vacuole, apoplast, and epidermal cell walls (Adrees et al., 2015). This might be also the potential mechanism enabling phenotypic TGE due to the copper residual that can be transmitted from maternal plant to the clonal offspring, although TGE due to copper were found only in three out of five genotypes analyzed. Interestingly, despite that TGE were genotype specific, the copper treatment had similar effect on maternal ramets of different genotypes after their transplantation to control environment.

Repeated drought cycles in the maternal generation triggered the strongest TGE with a significant negative effect both on the maternal and offspring biomass. The most apparent mechanism enabling observed phenotypic TGE can be small size of maternal ramets due to their reduced growth in the dry maternal environment. Small size of maternal ramets can be translated into small size of offspring ramets due to reduced resources provided by maternal ramets. However, we controlled for this effect by including the size of maternal ramets as a covariate in statistical analyses. Even after accounting for the effect of maternal ramets' size the results remained strongly significant suggesting that other mechanisms than the size of maternal ramets were also enabling TGE. These results are in line with our previous study (Rendina González et al., 2017) where we showed that despite drought-induced TGE significantly reduced clonal offspring biomass in optimal conditions, TGE were adaptive in the actual presence of drought, i.e., offspring ramets of mothers from dry environment performed better than offspring ramets of control mothers in the presence of drought. Another previous study (Rendina González et al., 2016) also suggested that TGE due to maternal drought were partly controlled by heritable DNA methylation change given that phenotypic TGE were not detected in plants that were treated with 5-azacytidne, a demethylating agent that removes epigenetic marks on DNA. Moreover, our second study (see below) also suggest that DNA methylation change can be at least partly responsible for observed TGE due to drought in maternal generation.

Because water availability is limited in salt contaminated soils, it is expected that salt stress should have similar physiological effects on plants as drought (Bartels and Sunkar, 2005; Uddin et al., 2016). Indeed, similarly to TGE due to drought, we found negative effect of TGE induced by salt stress on offspring biomass, although the effect was weaker than TGE triggered by drought. TGE due to salt can be adaptive as demonstrated Suter and Widmer (2013). They discovered an acquired salt tolerance in the offspring phenotype of stressed *Arabidopsis thaliana* plants, and this effect was strongest when both parental lines were stressed. The authors suggest that the observed TGE dependency on plant genotype can be explained by the interaction between the genetic background and the inheritance of environmentally induced epigenetic patterns (Suter and Widmer, 2013).

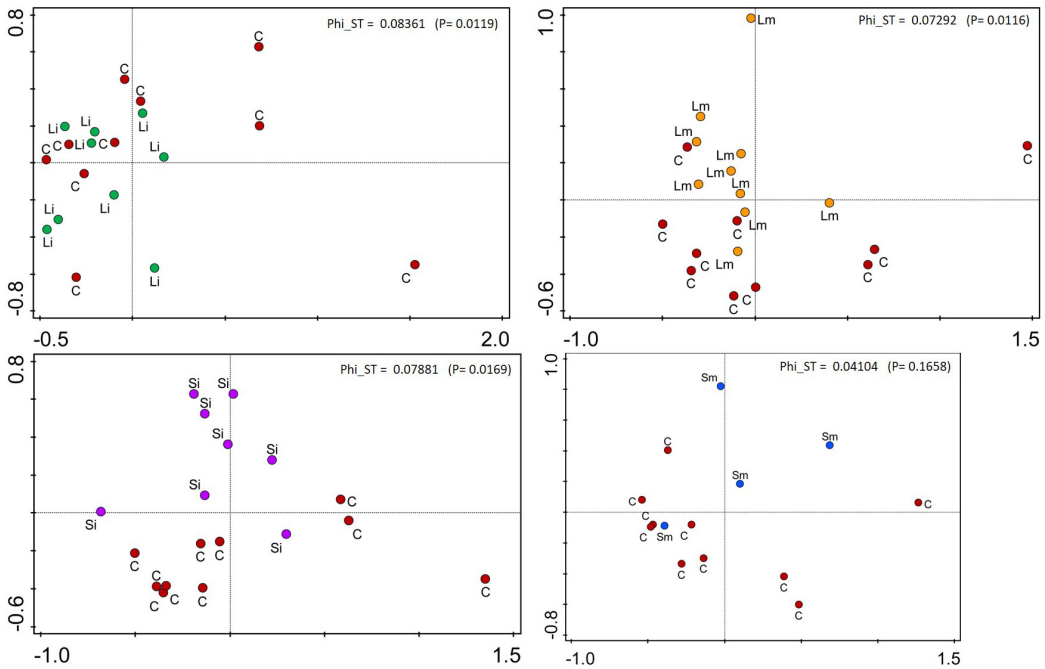

FIGURE 6 | Representation of the PCoA on offspring ramets of *T. repens* for individual treatments vs. Controls. C, Control; Si, Short-Intense drought; Li, Long-Intense drought, Long-medium drought; Sm, Short-medium drought. Fixation index Phi_ST and *p*-value are shown for each plot.

Of all stress treatments analyzed, shade did trigger TGE in one genotype of *T. repens* only. Previous studies have shown the role of light in fitness and memory of plants (Galloway and Etterson, 2007; Müller-Xing et al., 2014). For example, a study on an annual herb *Campanulastrum americanum* showed that different maternal light environments differently determine offspring germination rate and fitness (Galloway and Etterson, 2007). Since *T. repens* grows mostly in open biotopes such as grasslands or disturbed biotopes, it is likely that shade is not crucial stressor for the species and TGE due to shade were not evolutionary relevant.

Also, other studies have reported similar phenotypic responses of clonal offspring to drought or other biotic and abiotic stresses in clonal plants and model species (e.g., Verhoeven et al., 2010; Raj et al., 2011; Verhoeven and Preite, 2014; Herman and Sultan, 2016) and suggest that epigenetic mechanisms are likely involved (Wang et al., 2010; Zheng et al., 2013; Tang et al., 2014; Guarino et al., 2015; Douhovnikoff and Dodd, 2015; Ahn et al., 2017; Yaish, 2017).

Observed genotype specificity of most of TGE in our first study are in line with majority of other studies (Raj et al., 2011; Suter and Widmer, 2013; Latzel et al., 2014; Groot et al., 2017). Such specificity of TGE can have multiple origins. Each genotype can differ in its response to environmental stimuli, which can be translated into variation in TGE. In addition, genotypes likely differ in their selection history (although we collected the genotypes from very similar conditions) and thus also TGE could evolved differently in different genotypes. In this regard, the response of individual genotypes to various stresses can considerably differ highlighting thus the importance of considering the degree of genetic variation that is involved in phenotypic plasticity and its correlation with epigenetic variation and its inheritance. Various known molecular mechanisms are interconnected to give rise to the observed phenotype, and it is still not fully understood to what extent environmentally induced DNA methylation is independent from genetic control (Angers et al., 2010; Richards et al., 2012; Eichten et al., 2014). The interaction of hormones, stress responsive genes, small RNA involved in the RNA-directed DNA methylation pathway, and histone modifications are important players in the epigenetic landscape and its stability (Grant-Downton and Dickinson, 2006; Lauria and Rossi, 2011; Crisp et al., 2016). One mechanism playing part in epigenetic regulation is related to the activation and/or silencing of transposable elements. Some of these elements are thought to work dynamically when a genotype is challenged with stressful environments and are under strict epigenetic control (McClintock, 1984; Fedoroff, 2012). Thus, the mobility of transposable elements and changes in gene expression due to environmental cues might be heritable through DNA methylation that still retains a degree of reversibility (soft inheritance) in case of environmental fluctuations, which enables a stress "memory." This soft inheritance might account even more in organisms reproducing clonally, avoiding the complex genetic shuffling that occurs during a meiotic event (Jablonka and Lamb, 2008; Latzel et al., 2016). Nevertheless, it remains an open question which molecular mechanisms are involved within each genotype and its differential reaction under same environmental conditions.

Second Study – Methylation Profiles of Clonal Offspring

We found that the methylation profiles of offspring of all drough treatments (Long-intense, Long-medium, and Short-Intense) except the Short-medium drought stress, differentiated from controls (**Figure 6**). Morphological data on the same plants (Rendina González et al., 2016) also showed significant morphological differences between offspring of drought stressed mothers and controls. The role of DNA methylation in TGE was also indirectly supported by experimental demethylation of part of the plants in Rendina González et al. (2016), where the observed TGE disappeared after demethylation with 5-azacytidine under the same drought treatments. Although MSAP data do not provide insight in functional loci it shows DNA methylation differences. Our observations thus seem to be consistent with the idea that epigenetic variation was at least partly involved in observed TGE. However, there are other potential factors that can contribute to TGE like somatic transfer of hormones involved in response to drought (e.g., Roach and Wulff, 1987).

Our study adds to the mounting evidence that heritable epigenetic variation in plants can play a responsive role in the presence of stressful environments (Hauser et al., 2011). For instance, Herman and Sultan (2016) observed drought-triggered TGE in *Polygonum persicaria* that were removed after demethylation treatment with zebularine, thus indicating that DNA methylation was likely involved in the expression of the offspring phenotypes. On the other hand, Preite et al. (2018) observed a build-up of DNA methylation variation after three generations in two lineages of the apomictic dandelion concluding that these changes were inherited in a genotype and context-specific manner. Another study reported heritable DNA hypomethylation and enhance tolerance to heavy metal stress in the unstressed offspring of rice (Ou et al., 2012). Nevertheless, in order to gain a better insight into the heritability and stability of environmentally induced epigenetic modifications, it will be necessary to employ advanced methodological techniques into further studies questioning the role of environmental stresses in adaptation and evolution of epigenetic mechanisms in clonal plants.

CONCLUSION

Overall, our results show that TGE are genotype specific in *T. repens* (and probably in other clonal plants too) and that there is potential for environment-induced, heritable DNA methylation changes to mediate TGEs. However, such DNA methylation based TGE probably exist in addition to other parental effects, such as carry over effects, e.g., copper residuals inherited somatically. This opens the question how behavior and/or ecology of clonal plants can be determined by their previous experiences (e.g., Latzel et al., 2016). Since clonal plants can exhibit very sophisticated behavior like foraging for resources, division of labor, or resources and information exchange among ramets, TGE can have strong potential to modify clonal plant behavior and thus their ecology and evolution. Nonetheless, to get more accurate overview of the role of TGE in ecology and evolution of clonal plants it would be necessary to test the adaptiveness of TGE (e.g., Rendina González et al., 2017) and importantly, the stability of TGE across several clonal generations and their overall generality across clonal species.

AUTHOR CONTRIBUTIONS

VL designed the experiments. VL and AG performed the experiments and statistical analyses. AG performed the molecular analysis. VP and KV aided in interpreting the results and worked on the manuscript. AG and VL wrote the manuscript with input of all authors.

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Context-Dependent Developmental Effects of Parental Shade Versus Sun Are Mediated by DNA Methylation

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Parental environment influences progeny development in numerous plant and animal systems. Such inherited environmental effects may alter offspring phenotypes in a consistent way, for instance when resource-deprived parents produce low quality offspring due to reduced maternal provisioning. However, because development of individual organisms is guided by both inherited and immediate environmental cues, parental conditions may have different effects depending on progeny environment. Such context-dependent transgenerational plasticity suggests a mechanism of environmental inheritance that can precisely interact with immediate response pathways, such as epigenetic modification. We show that parental light environment (shade versus sun) resulted in context-dependent effects on seedling development in a common annual plant, and that these effects were mediated by DNA methylation. We grew replicate parents of five highly inbred *Polygonum persicaria* genotypes in glasshouse shade versus sun and, in a fully factorial design, measured ecologically important traits of their isogenic seedling offspring in both environments. Compared to the offspring of sun-grown parents, the offspring of shade-grown parents produced leaves with greater mean and specific leaf area, and had higher total leaf area and biomass. These shade-adaptive effects of parental shade were pronounced and highly significant for seedlings growing in shade, but slight and generally non-significant for seedlings growing in sun. Based on both regression and covariate analysis, inherited effects of parental shade were not mediated by changes to seed provisioning. To test for a role of DNA methylation, we exposed replicate offspring of isogenic shaded and fully insolated parents to either the demethylating agent zebularine or to control conditions during germination, then raised them in simulated growth chamber shade. Partial demethylation of progeny DNA had no phenotypic effect on offspring of shaded parents, but caused offspring of sun-grown parents to develop as if their parents had been shaded, with larger leaves and greater total canopy area and biomass. These results contribute to the increasing body of evidence that DNA methylation can mediate transgenerational environmental effects, and show that such effects may contribute to nuanced developmental interactions between parental and immediate environments.

Keywords: ecological epigenetics, DNA methylation, non-genetic inheritance, maternal effects, phenotypic plasticity, transgenerational plasticity, shade tolerance, seed provisioning

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INTRODUCTION

A fundamental question in understanding phenotypic variation is how organisms integrate environmental cues with inherited biological information to guide development. This information includes more than genes alone, because organisms also inherit environmentally induced developmental factors from their parents, such as altered provisioning of resources to the embryo and epigenetic modifications of genetic material (reviewed by Roach and Wulff, 1987; Herman and Sultan, 2011; Bonduriansky et al., 2012; English et al., 2015). A great deal remains to be determined about both the nature of these inherited developmental effects and their transmission mechanisms.

Initial studies showed that, depending on the plant species, environmentally stressed maternal individuals may either increase or decrease the quantity of nutritive tissues allocated to developing seeds (Haig and Westoby, 1988; Schmitt et al., 1992; Sultan, 1996; Donohue and Schmitt, 1998; Fenner and Thompson, 2005). Such alterations in the amount of provisioning are expected to result in consistently expressed effects on development. For instance, increased provisioning may cause a "silver spoon" effect, endowing progeny with overall growth benefits regardless of their environmental conditions (Grafen, 1988; Uller et al., 2013). In other cases, however, the effects of parental environment on offspring phenotype differ depending on the conditions that offspring themselves encounter (e.g., Miller et al., 2012; Salinas and Munch, 2012). Such context-dependent effects suggest a more targeted form of inherited information, such as epigenetic modifications to specific DNA sites or cytoplasmically transmitted signaling molecules, that can alter gene expression pathways (Jablonka and Raz, 2009; Danchin et al., 2011; Feil and Fraga, 2012; e.g., Scoville et al., 2011) and hence modulate the phenotypic responses of progeny to their own environments (Gapp et al., 2014).

As noted, studies of parental environmental effects on progeny phenotypes have focused largely on the amount of maternal provisioning, which can be easily estimated in most plants by weighing individual seed units or early germinants (Wulff and Bazzaz, 1992; Sultan, 1996; Zas et al., 2013). While changes to cytoplasmic factors are more difficult to test, methods for studying certain epigenetic modifications – in particular DNA methylation – are now well established (Bossdorf et al., 2008; Verhoeven et al., 2016; Richards et al., 2017). In both plants and animals, the addition or removal of methyl groups from cytosine nucleotides at specific loci may be induced by environmental conditions and the altered DNA subsequently transmitted to offspring (e.g., Verhoeven et al., 2010; Dowen et al., 2012; Pastor et al., 2013; Yu et al., 2013; Zheng et al., 2013; Skinner, 2014). Because such DNA methylation state changes can alter patterns of gene activity (reviewed by Law and Jacobsen, 2010; He et al., 2011; Jones, 2012; Schubeler, 2015), they may result in substantial phenotypic consequences (e.g., Zhang et al., 2013; Cortijo et al., 2014; Akkerman et al., 2016; Herman and Sultan, 2016). The role of DNA methylation in mediating inherited environmental effects can be tested by using chemical methyltransferase inhibitors such as 5-azacytidine (Jones, 1985) or zebularine (Cheng et al., 2003) to experimentally reduce methylation (e.g., Bossdorf et al., 2010; Boyko et al., 2010; Herrera et al., 2012; Verhoeven and van Gurp, 2012; Alvarado et al., 2015; Akkerman et al., 2016; Herman and Sultan, 2016). Zebularine causes transient, genome-wide demethylation at levels that can be dosage-regulated (Baubec et al., 2009). It is thus preferable to 5-azacytidine, which has broadly toxic effects and can be biased to specific loci (Cheng et al., 2003; Ghoshal and Bai, 2007; Hagemann et al., 2011).

We investigated inherited developmental effects of shade, a key environmental variable. Because understory shade versus sun is an ecologically critical aspect of plant habitats (Valladares et al., 2016 and references therein), developmental responses of individuals to these alternative environments are an exceptionally well-studied aspect of plasticity both within and across generations (Schlichting and Smith, 2002; Schmitt et al., 2003; Valladares and Niinemets, 2008; Sultan, 2010; Fitter and Hay, 2012; Marin et al., 2018). Plant plasticity to understory shade is distinct from the well-studied adaptive "shade avoidance" syndrome, which is a suite of phenotypic adjustments in response to neighbor shade characterized by reduced branching, slower leaf development, and greater stem and petiole elongation (Dudley and Schmitt, 1996; Smith and Whitelam, 1997). Unlike the shade cast by a neighbor's shoot, understory shade cannot be easily evaded via plastic avoidance responses such as extending petioles to reposition leaves. Instead, plants generally respond to understory shade by altering phenotypes in ways that maximize light interception under reduced photon flux density, for instance by allocating proportionally more biomass to leaf tissue and producing broader, thinner leaves (Sultan and Bazzaz, 1993; Evans and Poorter, 2001; Navas and Garnier, 2002; Niinemets et al., 2003; Herr-Turoff and Zedler, 2007; Valladares and Niinemets, 2008; Matesanz et al., 2012; Marin et al., 2018).

In addition to these immediate phenotypic adjustments, individual plants may also respond to shaded versus open conditions by modifying their offspring in ways that affect seedling development (e.g., Schmitt et al., 1992; Sultan, 1996; Galloway and Etterson, 2007). As with most cases of inherited environmental effects or *transgenerational plasticity* (Herman and Sultan, 2011; Salinas et al., 2013; Akkerman et al., 2016; Bell and Stein, 2017), the transmission mechanisms for effects of parental shade versus sun remain unclear (McIntyre and Strauss, 2014). Shade habitats are often characterized by specialist taxa with constitutively large seeds, which provide their seedlings with sufficient initial energy reserves to quickly produce a large shoot that affords tolerance of understory conditions (Leishman and Westoby, 1994; Fenner and Thompson, 2005; Leck et al., 2008; Muller-Landau, 2010). If transgenerational effects of shade were based on a similar provisioning mechanism, then, in taxa that inhabit diverse light conditions, shaded parent individuals would be predicted to plastically increase the amount of seed nutritive tissue. Such provisioning effects would likely be consistently expressed, enhancing growth of seedling offspring regardless of their environmental conditions (Haig and Westoby, 1988). However, in several studies, the effects of parental light environment on such functional progeny traits as leaf size and specific area were found to be expressed differently depending on offspring conditions (Galloway and Etterson, 2009;

McIntyre and Strauss, 2014), pointing to inherited developmental modifications that more precisely alter progeny development. To date, however, tests have not been conducted in any plant system to determine whether DNA methylation or other epigenetic modifications play a role in mediating the inherited effects of parental shade versus sun.

Here we present the results of a glasshouse experiment testing for inherited effects of parental shade versus sun on progeny developing in alternative (sun and shade) conditions, together with experimental data on the roles of provisioning and DNA methylation in mediating these effects. Our experimental material consisted of naturally evolved (field-based) genotypes of the well-studied plasticity model system *Polygonum persicaria*, a colonizing annual of diverse temperate habitats. Because this species occurs in open, moderately shaded, and patchy light environments (Sultan et al., 1998), variation in parental light conditions may represent an important source of phenotypic variation among and within natural populations. We addressed the following questions: (i) How does parental shade versus sun influence offspring development with respect to ecologically important leaf traits and total seedling growth? (ii) Does parental light environment differently affect seedling development occurring in shade versus in sun? and (iii) Do seed provisioning and/or DNA methylation play a role in mediating inherited effects of shade versus sun on progeny phenotypes?

MATERIALS AND METHODS

Study System

Polygonum persicaria is a common herbaceous species introduced from Eurasia to North America by European settlers (Mitchell and Dean, 1978; Staniforth and Cavers, 1979). Experimental genotypes were sampled from three ecologically distinct introduced-range populations: an open, moist pasture (full sun; MHF population, Northfield, MA, United States), a shaded horse paddock (moderate canopy shade; TP population, Dover, MA, United States), and an organic farm (full sun with neighbor shade; NAT population, Natick, MA, United States, site details in Sultan et al., 1998). Field-collected achenes (1-seeded propagules) were inbred under uniform favorable glasshouse conditions for four generations to produce highly inbred (selfed full-sib) genetic lines (hereafter "genotypes"). Because *P. persicaria* has a mixed breeding system with a high degree of natural self-fertilization (Mulligan and Findlay, 1970), such intensively inbred lines can be generated for field-collected genotypes without inbreeding depression (Herman and Sultan, 2016). This allows for a fully factorial design in which replicate plants of each inbred genotype are grown in contrasting parental environments, to produce genetically uniform offspring that differ only in parental environment (Sultan, 1996; Herman et al., 2012; Herman and Sultan, 2016).

Parental Generation

Fifth-generation inbred achenes of five genotypes (2 MHF, 2 TP, and 1 NAT; see above) were stratified in distilled water at 4°C for 7 weeks, sown into flats of moist vermiculite, and randomly positioned on a glasshouse bench (6/1/12). At the first true leaf stage (4–6 days after emergence; 6/13/12), seedlings of each genotype were individually transplanted into 1 L clay pots filled with a 1:1:1 mix of sterilized topsoil:horticultural sand:fritted clay (Turface^{TM}, Profile Products, Buffalo Grove, IL, United States) pre-moistened with 250 mL water. Five days after transplant, two replicate seedlings of each genotype were randomly assigned to one of two parental glasshouse treatments. In the Parental Sun treatment, plants received 100% of incident light (c. 1300 μmol m^{-2} s^{-1} midday photosynthetically active radiation or PAR; Baker, unpublished data), with a Red:Far Red spectral ratio of c. 1.0 (as measured with an SKR R:FR meter; Skye Instruments, Llandrindod Wells, United Kingdom). The Parental Shade treatment consisted of a metal frame covered by 80% neutral-density shade cloth (PAK Unlimited Inc., Cornelia, GA United States) overlaid with strips of green plastic filter (#138, Lee Filters, Burbank, CA United States), providing plants with c. 260 μmol m^{-2} s^{-1} midday PAR and a R:FR ratio of c. 0.7, which agrees with measured R: FR ratios under the mixed canopy shade under which annual *Polygonums* occur (Griffith and Sultan, 2005). To simulate natural understory, equidistant holes 3.5 cm in diameter were cut in the shade cloth so that each Parental Shade plant received a daily 15 min sunfleck (Matesanz et al., 2014). Parental plants in both treatments were kept at field capacity moisture and grown for 9 weeks, with bench positions re-randomized weekly. Self-fertilized, full-sib achenes produced by the 10 experimental parents (5 genotypes × 2 parental treatments) were harvested, air dried, and stored at 4°C.

Offspring Development

Fifty – eighty achenes from each experimental parent were stratified and germinated as described in the section "Parental Generation." Individual seedlings were transplanted at the first true leaf stage (5/29/15 – 6/1/15) into 200 mL clay pots of 1:1:1 topsoil:sand:fritted clay mix (see section "Parental Generation"). Ten replicate offspring of each experimental parent were randomly assigned to Offspring Sun and Offspring Shade treatments (identical to Parental Sun and Parental Shade treatments; details above), for a total experimental sample of N = 200 seedlings (5 genotypes × 2 parental treatments × 2 offspring treatments × 10 replicate seedlings per offspring treatment). Seedlings received 75% sun and were well-watered for 1 day to ensure recovery from transplant shock before they were randomly positioned within treatments and kept at field capacity moisture throughout the experiment.

For each seedling, **stem elongation** (cm from base to apex) was measured after 6, 12, and 19 days in treatment and **leaf number** was counted after 8, 14, and 19 days in treatment. Individual offspring were harvested on day 20 (6/18/15–6/21/15). For each seedling, the two most recent fully expanded leaves were scanned on a LI-3100 leaf area meter (LICOR Inc., Lincoln, NE, United States), oven-dried (at 100°C for 1 h and then at 65°C for ≥48 h), and weighed to estimate specific leaf area (**SLA**: cm^2 leaf surface area per g leaf tissue) and **mean single-leaf area** (cm^2). Remaining leaves were separated from stems, and these tissues were oven-dried (at 100°C for 1 h and then at 65°C for ≥48 h)

and weighed. **Total leaf area** for each seedling was estimated by multiplying its **SLA** by its total leaf biomass (including the mass of the two leaves sampled for SLA). Root systems were manually washed, dried at 65°C for ≥48 h, and weighed. **Total biomass (g)** was calculated as [total leaf biomass + stem biomass + root biomass], and **% biomass allocation** to each tissue was calculated as [total leaf, stem, or root biomass/total biomass × 100%]. The final sample lacked 14 seedlings due to insufficient germination or abnormal development; in addition 1 seedling was missing data for root mass, 1 seedling was missing data for total leaf area, and 3 outliers were excluded from the analysis (likely due to treatment error): final sample sizes were $N = 185$ for number of true leaves and stem elongation, and $N = 184$ for all other traits.

Demethylation Experiment

Twenty-four – forty-eight achenes from each experimental parent (genotype × parental treatment combination, see section "Study System") were individually weighed on a Cahn C-33 microbalance (Cahn Instruments, Cerritos, CA, United States) and stratified in distilled water at 4°C for 5 weeks. The quantity of **seed provisioning** (mg) for each seedling was estimated as initial air-dried achene mass minus air-dried pericarp mass (retrieved after germination).

Chemical demethylation was imposed during germination. Achenes were sown in Petri plates (9/14/16) on solidified 0.8% agar containing either 0 or 45 μM zebularine (hereafter Control and Demethylation germination treatments, respectively). This dose of zebularine had no adverse developmental effects on *P. persicaria* seedlings in a prior study (Herman and Sultan, 2016), and is similar to a dosage used by Baubec et al. (2009) that reduced global 5-methyldeoxycytidine levels by 15–18% in *Medicago truncatula* and *Arabidopsis thaliana*. Petri plates were positioned randomly on a glasshouse bench and re-randomized daily. Each seedling was transplanted 6 days after germinating so that all plants in the Demethylation germination treatment received the same dose of zebularine.

Eight replicate Control and Demethylation seedling offspring of each experimental parent were transplanted (9/23/16–10/4/16) into individual 200 mL clay pots as described in the section "Parental Generation" and placed in a randomized complete block design in an E-7 dual Conviron growth chamber (Controlled Environments Ltd., Winnipeg, MB, Canada) at a 25°C:18°C, 14:10 h day:night cycle. To simulate a uniform understory treatment, the growth chamber was modified with a metal internal frame covered by 30% neutral-density shade cloth (PAK Unlimited Inc., Cornelia, GA United States) lined with green plastic filter (see section "Parental Generation") with regularly spaced 1 cm circulation holes; experimental seedlings received c. 220 μmol m^{-2} s^{-1} PAR (Baker, unpublished data). Seedlings were kept at field capacity moisture and re-randomized weekly within blocks. The total experimental sample was $N = 160$ seedlings (5 genotypes × 2 parental treatments × 2 germination treatments × 8 replicate offspring per germination treatment).

Seedlings were grown for 25 days before being harvested (10/18/16–10/29/16). At harvest, an overhead photograph was taken of the entire canopy of each seedling and digitized (EasyLeafAreaV2 software; Easlon and Bloom, 2014) to estimate **canopy area**, a functional trait that accounts for leaf overlap. As described in the section "Offspring Development," a subsample of two leaves was used to estimate **mean single leaf area** before plant tissues were oven dried and weighed to calculate **total biomass**. Eight seedlings were removed from the final sample due to experimental error or abnormal growth, resulting in a final sample size of $N = 152$.

Data Analysis

All statistical analyses were performed with JMP Pro 13 (SAS Institute, Cary, NC, United States) and graphing was performed with R version 3.3.3 (R Core Team, 2017).

Offspring Development

Analysis of Variance (ANOVA) with type III sums of squares was used to analyze the (fixed) effects on each offspring trait of *parental treatment* (PT, Parental Shade vs. Parental Sun), *offspring treatment* (OT, Offspring Shade vs. Offspring Sun), *genotype*, and all two-way and three-way interactions (see Herman et al., 2012 for a similar analysis). *Genotype* was treated as a fixed effect because the genotypes in this study do not represent a random sample of the species' genetic diversity; rather, the sample was drawn from specific populations in order to encompass the full range of *P. persicaria* light habitats (Sultan et al., 1998, see Herman et al., 2012 for a previous analysis of this genotype sample). To resolve the specific phenotypic effects of parental treatment and genotype within each offspring treatment, separate ANOVAs were performed analyzing the effects of *parental treatment, genotype*, and their interaction on seedling phenotype in each offspring treatment. In the full analysis, **total biomass** was Box-Cox transformed to meet ANOVA assumptions, but transformation was not required for the **total biomass** ANOVA within each offspring treatment, or for any other trait. For each trait, the mean percent change due to Parental Shade compared with Parental Sun (pooled across genotypes) was calculated in each offspring treatment using the equation: 100% × (trait $\text{mean}_{\text{parentalshade}}$ − trait $\text{mean}_{\text{parentalsun}}$)/trait $\text{mean}_{\text{parentalsun}}$.

MANOVA was used to test the effects of *parental treatment* (Parental Shade vs. Parental Sun), *offspring treatment* (Offspring Shade vs. Offspring Sun), *genotype*, and all two-way and three-way interactions on **% biomass allocation** to roots, leaves, and stems. To investigate the significant PT × OT interaction effects, separate ANOVA were performed in each offspring treatment analyzing the effects of *parental treatment, genotype*, and their interaction on **% stem**, **% leaf**, and **% root allocation**. Multivariate repeated-measures ANOVA (Scheiner and Gurevitch, 2001) was used to analyze main and interaction effects of PT, OT, and genotype on **stem elongation** and **leaf number** over time. Following a significant sphericity chi-square test, multivariate Wilks' Lambda was used to assess significance (Cole and Grizzle, 1966).

Demethylation Experiment

ANOVA was used to analyze the (fixed) effects on seedling traits of *parental treatment* (PT, Parental Shade vs. Parental Sun), *germination treatment* (GT, Control vs. Demethylation), *genotype*, all two-way and three-way interactions, and spatial

block. To resolve the distinct effects of Demethylation on offspring of shade and of sun parents, separate ANOVA were performed testing the effects of *germination treatment, genotype*, and their interaction on seedling phenotype in each parental treatment group. For each trait, the mean percent change (pooled across genotypes) due to Demethylation vs. Control germination treatments was calculated in each parental treatment group using the equation: 100 × (trait $\text{mean}_{\text{Demethylation}}$−trait $\text{mean}_{\text{Control}}$)/trait $\text{mean}_{\text{Control}}$. Student's *t*-test was used to test the effect of *parental treatment* on **seed provisioning**. For the full model, **seed provisioning** was also tested as a covariate for **total biomass** but was excluded from the final ANOVA due to non-significance ($p = 0.1673$). The effect of **seed provisioning** on **total biomass** was also tested by regression, both for the full sample and within each Parental Treatment × Germination Treatment group.

RESULTS

Parental Shade Had Strong, Treatment-Specific Effects on Offspring Traits

All seedlings grown in Offspring Shade had higher SLA, but lower total biomass, total leaf area, and mean single-leaf area compared to seedlings grown in Offspring Sun (**Figure 1** and **Table 1**, *offspring treatment* $p < 0.0001$ for all four traits). On average, seedling offspring of Parental Shade plants had greater mean values for these four growth traits than offspring of Parental Sun plants (**Figure 1** and **Table 1**, effect of *parental treatment*, $p \leq 0.0152$ for all traits). For all four traits, these average effects of Parental Shade versus Parental Sun were greater in magnitude than those of genotype (cf. *F*-values, **Table 1**). However, for total biomass, mean single-leaf area, and SLA, the effect of Parental Shade varied significantly depending on the offspring growth treatment (**Table 1**, *PT* × *OT* interaction effects $p \leq 0.0452$). In the Offspring Sun treatment, Parental Shade resulted in small, non-significant increases in all four traits compared to Parental Sun (**Figures 1A–D**). For seedlings growing in shade, effects of Parental Shade compared with Parental Sun were dramatic: in the Offspring Shade treatment, progeny of Shade parents produced 44% more total biomass, 60% greater total leaf area, 51% greater mean single-leaf area, and 13% higher SLA than progeny of Sun parents (**Figure 1**; effect of *parental treatment* from ANOVA within Offspring Shade treatment $p \leq 0.0001$, 0.0001, 0.0001, and 0.0188, respectively). These parent-environment effects on total biomass, mean single-leaf area, and total leaf area were greater than the largest *genotype* effects (across treatments) for these traits (*parental treatment* and *genotype* effect *F*-values within Offspring Shade, respectively = 29.9 vs. 5.9 for total biomass; 21.0 vs. 5.8 for single leaf area; 5.7 vs. 1.9 for SLA; and 25.8 vs. 5.6 for total leaf area). For instance, the largest *genotype* effect on total leaf area was a 36% difference between MHF1 and TP2, compared to the 60% greater total leaf area conferred by Parental Shade on average, across genotypes. The *genotype* × *parental treatment* interaction effect was significant for total leaf area and mean single-leaf area (**Table 1**, $p = 0.0292$ and 0.0536, respectively), and the *genotype* × *offspring treatment* interaction effect was significant or marginally significant for all traits (**Table 1**, $0.0283 \leq p \leq 0.0879$). The three-way interaction (OT × PT × Gen) was non-significant for all four growth traits.

With respect to tissue allocation, all seedlings grown in Offspring Shade allocated more biomass to leaf and stem tissues, and less biomass to root tissues, than seedlings in Offspring Sun (**Figure 2**; effect of *offspring treatment* based on MANOVA Wilks' Lambda $p < 0.0001$). The *parental treatment* effect on biomass allocation varied with *offspring treatment* (PT × OT interaction effect, Wilks' Lambda $p \leq 0.0055$): Parental Shade resulted in increased biomass allocation to leaf tissue and lower allocation to stem tissue for progeny growing in Offspring Shade, but did not change leaf allocation, and *increased* stem allocation, for progeny growing in sun (based on ANOVA for each trait within treatments, **Figures 2A,B**). Effects of *parental treatment* on root allocation within each offspring treatment were non-significant (**Figure 2C**). As a result of these progeny treatment-specific effects, the main effect of *parental treatment* on proportional biomass allocation was non-significant (effect of *parental treatment*, Wilks' Lambda $p = 0.8647$).

Progeny of Parental Shade plants produced more leaves than progeny of Parental Sun plants in both Offspring Sun and Offspring Shade treatments, an effect that increased over time (**Figure 3A**; effect of *parental treatment* × *time*, multivariate repeated-measures ANOVA Wilks' Lambda $p = 0.0136$), especially for progeny growing in shade (3-way interaction of *parental treatment* × *offspring treatment* × *time,* Wilks' Lambda $p = 0.0218$; **Figure 3A**). The positive but less pronounced effect of Parental Shade on stem elongation also increased over time in both seedling environments (**Figure 3B**, effect of *parental treatment* × *time* based on multivariate repeated-measures ANOVA, Wilks' Lambda $p = 0.0003$).

Partial DNA Demethylation Caused Progeny of Sun Plants to Develop Similarly to Shade Progeny

As expected, the effects of Parental Shade on control-germinated seedlings grown in growth chamber shade in the Demethylation experiment were consistent with parental effects on seedling development in the Offspring Shade glasshouse treatment (described above), where transgenerational effects of parental environment were most strongly expressed: control progeny of Parental Shade plants produced greater total biomass, canopy area, and mean single-leaf area than offspring of Sun parents. The phenotypic impact of Parental Shade versus Sun was substantially altered by partial demethylation with zebularine (**Figures 4A–C**); for all three traits, the demethylation treatment had different effects on Sun and Shade progeny (**Table 2**, *PT* × *GT* interaction effects; these contrasting effects explain the lack of significant PT and GT main effects). For seedling progeny of Shade parents, demethylation slightly (and non-significantly) reduced biomass, canopy area, and leaf size (5–9% mean trait reductions; **Figures 4A–C**). However, demethylation significantly and substantially altered phenotypic expression

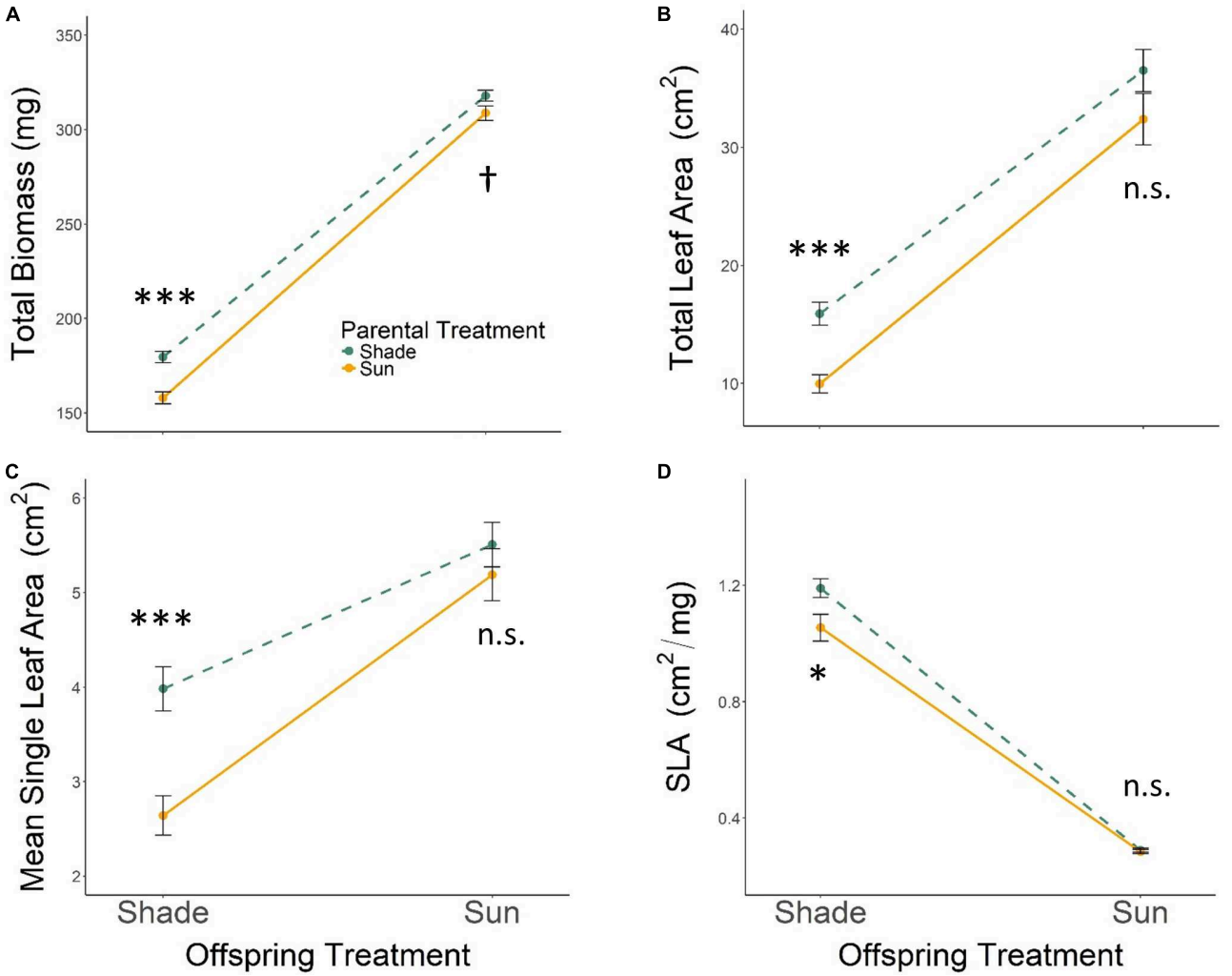

FIGURE 1 | Means ± SE shown (N = 184) for **(A)** total biomass, **(B)** total leaf area, **(C)** mean single leaf area, and **(D)** SLA for offspring of Parental Shade (dashed green line) and Parental Sun (solid yellow line) plants that were grown either in Offspring Shade or Offspring Sun treatments. Results of significant tests for the effect of parental shade versus sun in each offspring treatment are shown, based on separate within-treatment ANOVAs ($^{\dagger}p < 0.10$, $^{*}p < 0.05$, $^{***}p < 0.001$, n.s. $p > 0.1$, see section "Materials and Methods" for details).

TABLE 1 | Results of significance tests for effects of shade versus sun *parental treatment* (PT), shade versus sun *offspring treatment* (OT), and *genotype* (Gen) on seedling traits based on a three-way ANOVA (N = 184; details in Materials and Methods).

Source of variation	**Total biomass (mg)** R^2_{adj} **= 0.92**		**Total leaf area (cm²)** R^2_{adj} **= 0.58**		**Mean single leaf area (cm²)** R^2_{adj} **= 0.42**		**SLA (g/cm²)** R^2_{adj} **= 0.83**	
	F	***p*-value**	***F***	***p*-value**	***F***	***p*-value**	***F***	***p*-value**
Parental treatment	24.0282	**<0.0001*** **	11.1616	**0.0010****	13.3534	**0.0003*****	6.0195	**0.0152***
Offspring treatment	2177.4669	**<0.0001*** **	222.6994	**<0.0001*** **	87.7163	**<0.0001*** **	867.184	**<0.0001*** **
Genotype	3.705	**0.0065****	3.3402	**0.0117***	6.3299	**<0.0001*** **	1.9913	0.0982†
PT × OT	4.0714	**0.0452***	0.3937	0.5312	4.9554	**0.0274***	5.4526	**0.0208***
Gen × PT	1.5589	0.1877	2.7662	**0.0292***	2.3824	0.0536†	0.67	0.6137
Gen × OT	2.5469	**0.0414***	3.5605	**0.0082****	2.7876	**0.0283***	2.0638	0.0879†

*Significant p-values are shown in bold (†p <0.10, *p < 0.05, **p < 0.01, ***p < 0.001). The three-way interaction (offspring treatment × parental treatment × genotype) was non-significant for all traits but was included in the model.*

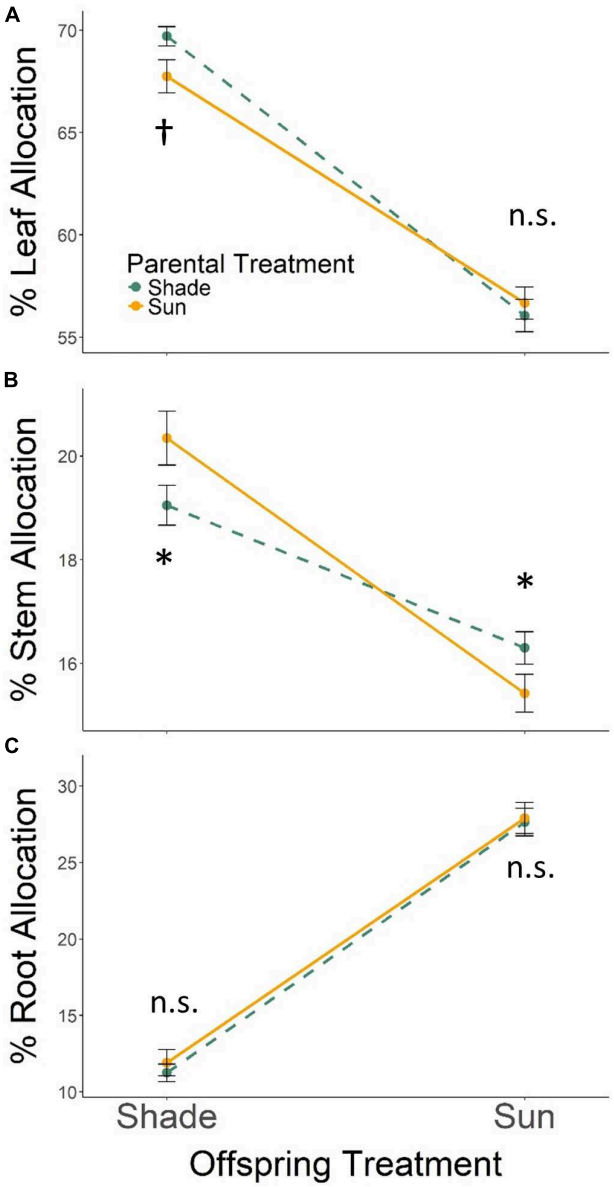

FIGURE 2 | Means ± SE shown (N = 184) for **(A)** % leaf, **(B)** % stem, and **(C)** % root biomass allocation for offspring of Parental Shade (dashed green line) and Parental Sun (solid yellow line) plants that were grown either in Offspring Shade or Offspring Sun treatments. Results of significant tests for the effect of parental shade versus sun in each offspring treatment are shown, based on separate within-treatment ANOVAs ($^{\dagger}p < 0.10$, $^{*}p < 0.05$, n.s. $p > 0.1$, see section "Materials and Methods" for details).

in progeny of Sun parents, resulting in seedlings with 25% greater total biomass, 22% increased canopy area, and 13% larger leaves than Control-germinated sun-plant progeny (effect of Control vs. Demethylation *germination treatment* within Parental Sun treatment, $p = 0.0042$ for total biomass, $p = 0.0448$ for canopy area, and $p = 0.0091$ for mean single-leaf area, based on separate ANOVA within each parental treatment). As a result, demethylated progeny of Sun parents (**Figures 4A–C**, red triangles in Parental Sun Treatment) developed very similarly to Control progeny of Shade parents (**Figures 4A–C**, black squares in Parental Shade Treatment). Although genotypes differed significantly on average for all traits (main effect of *genotype*, **Table 2**), 2- and 3-way interaction effects of *genotype* with *parental treatment* and *germination treatment* were non-significant.

Seed Provisioning Did Not Mediate the Growth Effects of Parental Shade vs. Parental Sun

Achenes produced by Shade parent plants had 12% lower seed provisioning on average than achenes of Sun parents (Student's t-test for effect of Parental Shade vs. Parental Sun $p = 0.0002$; $N = 152$). Despite this lower seed provisioning, Parental Shade offspring produced greater total biomass and larger leaves than Parental Sun offspring (see previous section). Based on a regression analysis, there was no significant (positive or negative) relationship between seed provisioning and seedling total biomass (**Figure 5**, $R^2_{adj} = 0.0154$, $p = 0.0687$, $N = 152$). Linear regressions calculated separately for each of the 4 *parental treatments* × *germination treatment* seedling groups were also non-significant ($R^2_{adj} \leq 0.07$ in all cases, $p > 0.05$ in all cases) and explained c. 7% of the variation or less within each group.

DISCUSSION

Parental Shade Resulted in Specific Alterations to Offspring Phenotypes That Were Functionally Appropriate for Growth in Shade

In isogenic seedlings differing only in parental environment, parental shade versus sun caused specific developmental modifications to offspring: increased allocation to leaf tissues, more rapid shoot development (stem elongation and leaf production), and larger, thinner leaves, resulting in greater total leaf area and seedling biomass. Earlier work on *P. persicaria* also showed specific, but somewhat different, developmental effects of parental shade immediately after germination: after 96 h of growth in a common controlled environment, seedling offspring of shaded parents had produced similar biomass but 30% shorter roots than offspring of full-sun parents, indicating increased proportional allocation to shoot growth during initial development (Sultan, 1996). These data add developmental insights to transgenerational field studies showing that parental light conditions may influence seedling growth and survival in herbaceous species (Galloway and Etterson, 2007; McIntyre and Strauss, 2014).

Increases to light acquisition traits such as leaf biomass allocation, leaf size, and SLA are well known immediate plastic responses to understory shade (Bradshaw, 1965; Bazzaz, 1996; Fitter and Hay, 2012; Sultan, 2015). These allocational, morphological, and structural adjustments are well known to offset the negative growth effects of reduced photon

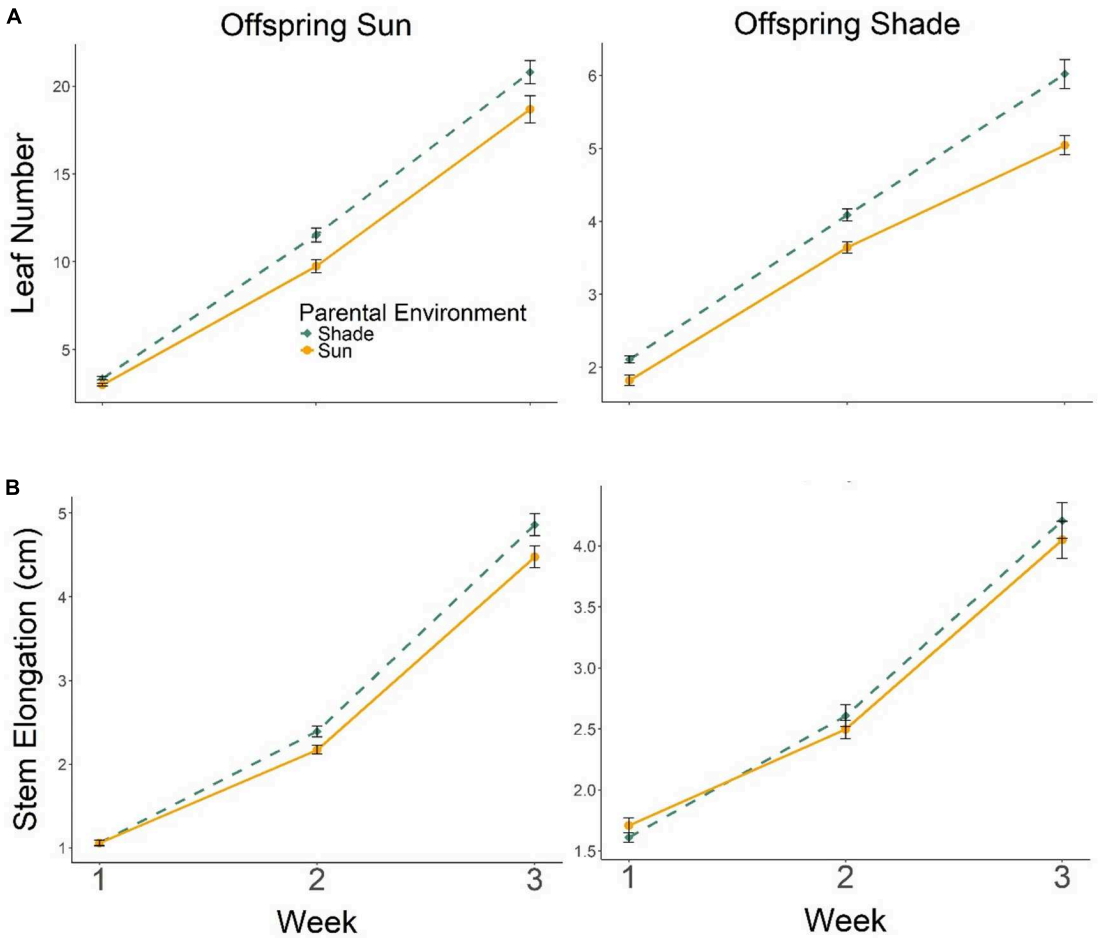

FIGURE 3 | Means ± SE shown (*N* = 185) for **(A)** leaf production and **(B)** stem elongation over 3 weeks growth for offspring of Parental Shade (dashed green line) and Parental Sun (solid yellow line) plants that were grown either in Offspring Shade or Offspring Sun treatments.

flux density by increasing photosynthetic surface area (Sultan and Bazzaz, 1993; Evans and Poorter, 2001; Navas and Garnier, 2002; Niinemets et al., 2003; Herr-Turoff and Zedler, 2007; Marin et al., 2018; additional references in Valladares and Niinemets, 2008). To our knowledge, the data we report here show for the first time that similar adjustments to these key functional traits can also occur as a result of *inherited* effects of shade experienced by parent plants. In a growing number of plant and animal studies, parent individuals in stressful conditions have been found to produce offspring with specific phenotypic alterations that provide functional adaptation if progeny encounter those same stresses (*adaptive transgenerational plasticity;* e.g., predation, Agrawal et al., 1999, light-limited field microsite, Galloway and Etterson, 2007, drought stress, Sultan et al., 2009, simulated leaf herbivory, Scoville et al., 2011, elevated water temperature, Salinas and Munch, 2012, high dissolved CO_2 concentration, Miller et al., 2012; additional examples and references in Mousseau and Fox, 1998; Herman and Sultan, 2011; Salinas et al., 2013). A subsequent experimental study of these *P. persicaria* genotypes confirmed that, for progeny that were grown to maturity in either extreme understory or neighbor shade, inherited effects of parental shade were associated with significantly higher lifetime fitness (total reproductive output) compared with parental sun (Baker et al., unpublished).

Developmental Effects of Parental Shade Versus Sun Varied Depending on Offspring Environment

Although these developmental modifications were qualitatively similar across sun and shade offspring treatments, their degree of expression varied significantly: inherited effects of parental shade versus sun on trait expression were far more pronounced in seedling offspring that were themselves growing in shade. Such context-dependent expression of parental environment effects has been documented in a number of plant and animal taxa (e.g., Schmitt et al., 1992; Galloway, 1995; Hereford and Moriuchi, 2005; Miller et al., 2012; Leverett et al., 2016). In Sheepshead minnow fish (*Cyprinodon variegatus*), for example, the effects of parental temperature treatment were expressed differently depending on the temperature experienced by juvenile

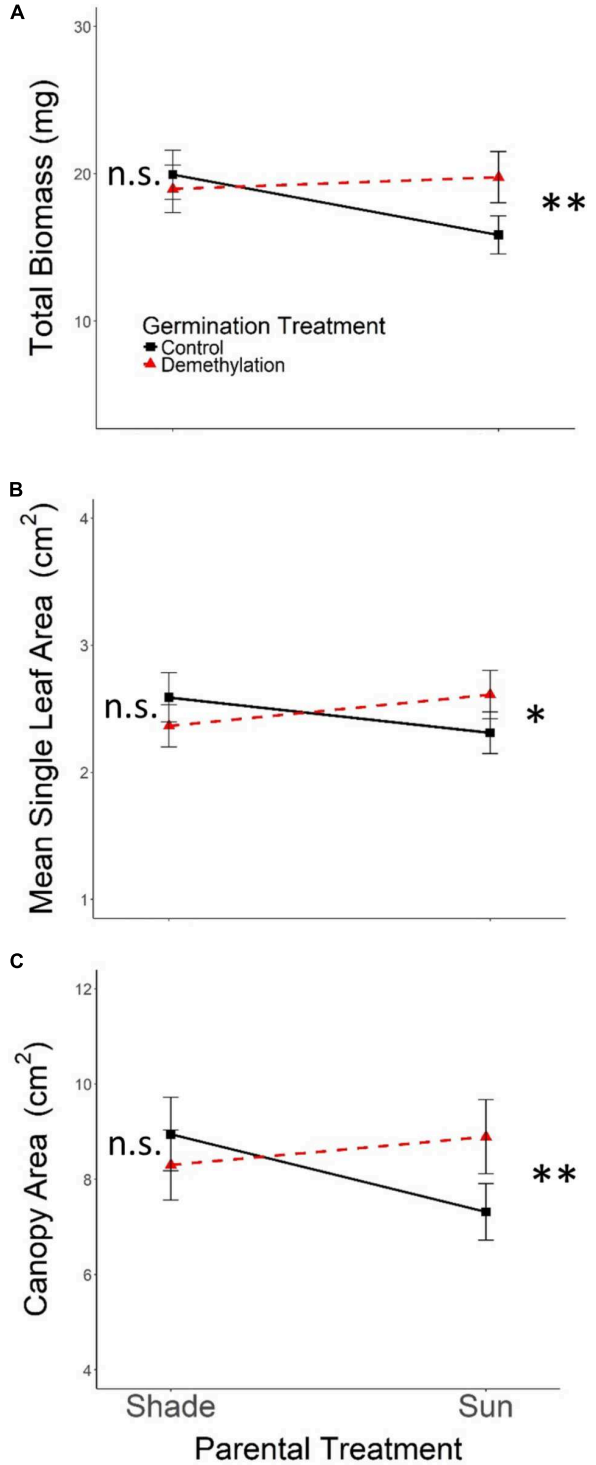

FIGURE 4 | Means ± SE shown (N = 152) for **(A)** total biomass, **(B)** mean single leaf area, and **(C)** canopy area for offspring of Parental Shade and Parental Sun plants that were exposed to either 0 μM (Control, solid black lines) or 45 μM zebularine (Demethylation, dashed red lines) during germination. Results of significant tests for the effect of Control versus Demethylation in each parental treatment group are shown, based on separate within-treatment ANOVAs (*$p < 0.05$, **$p < 0.01$, n.s. $p > 0.1$, see section "Materials and Methods" for details).

offspring (Salinas and Munch, 2012). In plants, such context-specific expression of inherited environmental influences are widespread; the effects of parental drought (Sultan et al., 2009; González et al., 2017), shade (Galloway and Etterson, 2009; McIntyre and Strauss, 2014), nutrient availability (Latzel et al., 2010, 2014), CO_2 concentration (Lau et al., 2008), salinity (Van Zandt and Mopper, 2004; Vu et al., 2015; Groot et al., 2016; Moriuchi et al., 2016), and temperature (Whittle et al., 2009; Zhang et al., 2012) have all been shown to be expressed differently in alternative offspring environments.

Context-dependent parental effects are captured statistically by significant *parent environment* × *offspring environment* interaction terms as sources of phenotypic variation. Such complex patterns of expression arise from the various ways that within- and trans-generational environmental influences are integrated by developing organisms (Leimar and McNamara, 2015; Sultan, 2015; Auge et al., 2017). In some cases, favorable immediate conditions in offspring environments may mask or overcome negative transgenerational effects of parental stress. For instance, parental nutrient stress in *Plantago lanceolata* resulted in delayed flowering for progeny in nutrient-poor soil, but this negative developmental effect was not observed when progeny were grown in nutrient-rich soil (Latzel et al., 2014). Conversely, resource-limited progeny environments can mask *positive* parental effects on growth: for instance, parental sun resulted in higher fitness than parental shade when *Claytonia perfoliata* offspring were grown in full-sun, but not when progeny developed in shade, where reproductive output was low regardless of parental light conditions (McIntyre and Strauss, 2014).

In the present case, the more pronounced expression of parental shade effects in offspring that were developing in shade indicates an adaptively integrated response to a particular combination of like inherited factors and immediate cues. Similarly, drought-stressed *P. persicaria* parents produced offspring with an enhanced root extension rate that was further increased when these progeny developed in dry rather than moist soil (Sultan et al., 2009). Investigating the possible selective evolution of this kind of integrated response system is a considerable challenge that researchers are just beginning to approach (Herman et al., 2014; Leimar and McNamara, 2015; McNamara et al., 2016; Sultan, 2016). Such investigations require further information about environmental correlation patterns across generations (Marshall and Uller, 2007; Uller, 2008; Herman et al., 2014), and about other potential sources of variation in the distribution and impact of transgenerational effects, such as differential expression among the progeny of a given parent. For instance, species with complex shoot or inflorescence architectures may evolve position-dependent parental effects on offspring phenotypes. In the closely related annual *P. hydropiper*, which produces achenes at both the axial base and the tip of its flowering spikes, parental shade resulted in shade-adaptive seedling development (faster leaf production and stem extension as well as greater total biomass) in terminal achenes but not in those produced in axillary positions (Lundgren and Sultan, 2005). Such position-dependent expression of parental effects may either provide bet-hedging for

TABLE 2 | Results of significance tests for effects of shade versus sun *parental treatment* (PT), control versus demethylation *germination treatment* (GT), and *genotype* on seedling traits based on a three-way ANOVA (N = 152; details in Materials and Methods).

Source of variation	Total biomass (mg) R^2_{adj} = 0.74		Mean single leaf area (cm^2) R^2_{adj} = 0.76		Canopy area (cm^2) R^2_{adj} = 0.76	
	F	*p*-value	*F*	*p*-value	*F*	*p*-value
Parental treatment	3.1868	0.0767†	0.00865	0.9261	1.4398	0.2325
Germination treatment	5.0495	**0.0264***	1.31143	0.2543	3.2114	0.0756†
PT × GT	3.9503	**0.0490***	3.19571	0.0763†	4.8668	**0.0292***
Genotype	53.439	**<0.0001*****	83.934	**<0.0001*****	81.1713	**<0.0001*****

Significant p-values are shown in bold ($^{\dagger}p < 0.10$, $^{**}p < 0.05$, $^{***}p < 0.001$). All interactions with genotype were non-significant (but were included in the model).

uncertain progeny conditions, or allow for alternative phenotypes when progeny are likely to have different dispersal distances from the maternal plant and hence different probabilities of encountering similar microsites (see Donohue and Schmitt, 1998).

Inherited Developmental Effects of Parental Shade Versus Sun Were Not Mediated by Seed Provisioning

Depending on the species, parent plants in light-limited environments may either increase (Jenner, 1979; Peet and Kramer, 1980) or decrease (Schmitt et al., 1992) the mass of individual seeds, a direct proxy for the amount of endosperm or other nutritive tissues provided to offspring that is often strongly and positively correlated with seedling growth (Roach and Wulff, 1987; Haig and Westoby, 1988, i.e., "silver spoon" *sensu* Grafen, 1988; Uller et al., 2013). In the present study, the progeny of shaded *P. persicaria* parents had slightly lower seed mass on average (after removing the outer pericarp), yet this reduced provisioning was not associated with lower seedling biomass as would be expected in a simple "silver spoon" model for transgenerational effects.

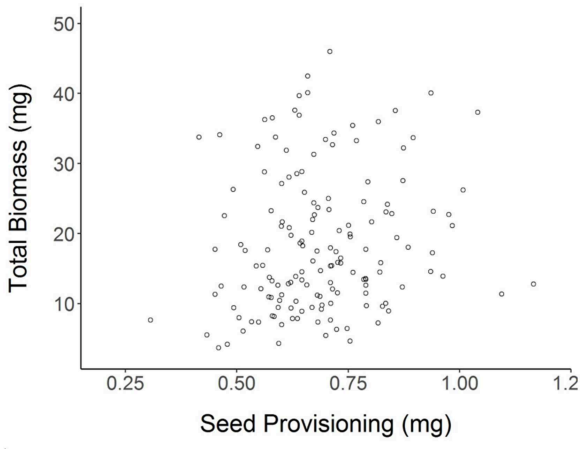

FIGURE 5 | Total biomass of each (day 20) seedling as a function of its initial seed provisioning (R^2_{adj} = 0.0154 n.s., N = 152; details in section "Materials and Methods").

Instead, contrary to expectation, the offspring of shaded parents produced *greater* total biomass on average than offspring of full-sun parents, and significantly so for offspring growing in shade. Seed provisioning explained only a very small proportion of variation in seedling biomass, and we found no significant relationship between provisioning and biomass either overall, or within each parent environment-offspring treatment group. Similarly, in an earlier study of *P. persicaria,* isogenic parent plants that were drought-stressed rather than amply watered produced progeny with very different seedling phenotypes, yet seed provisioning (which was similar for both sets of progeny) had no significant effect on variation in either developmental traits or biomass (Herman and Sultan, 2016).

Changes to seed size induced by stressful parental conditions (e.g., Stanton, 1984; Marshall, 1986) have generally been considered the primary mechanism of transgenerational effects on seedling development (Roach and Wulff, 1987; Donohue and Schmitt, 1998; Fenner and Thompson, 2005; while they are not seedling traits *per se*, effects on dormancy and germination have also been intensively studied. However, these result largely from direct changes to maternal [seed coat and fruit] tissues; Penfield and MacGregor, 2017). Results for *Polygonum* suggest that this view be re-examined, since quantity of seed provisions alone may be a less robust predictor of offspring phenotypes than previously believed. To confirm this predictive relationship and infer causation, genetically uniform mother plants must be grown in contrasting conditions and their seeds weighed individually, so that the effect of any resulting seed mass differences on growth traits can be tested using covariate analysis (e.g., Agrawal, 2002; Hereford and Moriuchi, 2005; Herman et al., 2012). When researchers have taken this rigorous approach, results have not always confirmed a major role for provisioning in mediating inherited environmental effects. Using this approach to test transgenerational effects of parental nutrient conditions, for instance, seed provisioning was found to account for most (Stratton, 1989), some (Wulff, 1986; Schmid and Dolt, 1994; Hereford and Moriuchi, 2005; Zas et al., 2013), or none (Wulff and Bazzaz, 1992) of the resulting variation in progeny phenotypes for herbaceous taxa. A second well-studied case is elevated parental CO_2 concentration, which is well known to result in both increased seed size and progeny growth modifications (Jablonski et al., 2002). A rigorous study by Lau et al. (2008) found that, although maternal

CO_2 concentration strongly affected offspring traits in three different species, there was no evidence that these effects were mediated by seed mass. As in studies of both parental shade versus sun and parental drought versus moist soil in *Polygonum*, the lack of provisioning effects in these cases, despite substantial changes to progeny development, points to an alternative mechanism for mediating inherited effects of parental environment.

The quantity of seed provisioning is only one of several possible factors whereby parental environment may influence progeny phenotypes. Indeed, recent studies of transgenerational effects have revealed a surprisingly diverse set of biological inheritance mechanisms (Day and Bonduriansky, 2011; English et al., 2015). For instance, along with changes to the *quantity* of seed provisioning, parental stresses may induce modifications to the *quality* or composition of seed constituents, including changes in protein content (Parrish and Bazzaz, 1985; Donohue, 2009), hormone concentration (Jha et al., 2010), and stored seed transcripts (Vu et al., 2015). Such changes to inherited signaling molecules may result in specific alterations of progeny development and environmental response pathways, providing a plausible mechanism for adaptively integrated transgenerational effects. Although we found no evidence that changes in seed mass mediate the effects of parental shade versus sun on *Polygonum* offspring, additional studies are needed to determine whether changes to seed constituents involved in regulatory pathways might play a role in this system. Note that changes in the quantity and compositional quality of seed provisioning need not be mutually exclusive; progeny development may be influenced by several types of environmentally induced heritable factors acting cumulatively or interactively (Herman and Sultan, 2011).

DNA Methylation Changes Play a Role in Mediating the Parental Effects of Shade Versus Sun

Transgenerational effects on progeny may also be mediated by environmentally induced, heritable epigenetic modifications such as changes to methylation state, histone modifications, or non-coding RNAs (Jablonka and Raz, 2009; Sultan, 2015). Because these modifications affect gene activity and hence developmental pathways, they are plausible mediators of context-dependent expression of parental effects. Although other modes of epigenetic transmission may be involved as well (Bonduriansky and Day, 2009; Akkerman et al., 2016), DNA methylation is increasingly viewed as a likely transmission mechanism for transgenerational effects of parental conditions (Kappeler and Meaney, 2010; Herman et al., 2014; Colicchio et al., 2015). In plants, changes in DNA methylation states are known to mediate the effects of several types of environmental stress on progeny phenotypes, e.g., salinity (Boyko et al., 2010), nitrogen deficiency (Kou et al., 2011), drought (Alsdurf et al., 2015; Herman and Sultan, 2016), and herbivory (Akkerman et al., 2016) (additional examples in Bossdorf et al., 2008; Bonduriansky and Day, 2009; Verhoeven et al., 2010, 2016; Herman and Sultan, 2011; Holeski et al., 2012; Richards et al., 2017).

Our experimental demethylation test confirmed that DNA methylation states are involved in mediating transgenerational effects of parental shade versus sun in *Polygonum*. However, the direction of the mediating state change was unexpected. In the few other available studies, chemical demethylation removed the adaptive effects of parental stresses on progeny development, including salt stress in *Arabidopsis thaliana* (Boyko et al., 2010), drought in *P. persicaria* (Herman and Sultan, 2016), and simulated herbivory in *Mimulus guttatus* (Akkerman et al., 2016). In these cases, parental stress apparently leads to stress-adapted progeny via induced *addition* of methyl groups, such that knocking down methylation levels removes the adaptive effect. In this case, by contrast, shade-adaptive progeny phenotypes evidently result from a *removal* of methyl groups that is induced by parental shade: chemically demethylated progeny of sun-grown parents developed the same shade-adaptive features as the progeny of shaded parents, but when progeny of shaded *Polygonum* parents were demethylated, their development was unaltered. To our knowledge, these are the first experimental data showing that adaptive developmental effects of parental stress on progeny can be affected by demethylation rather than addition of methyl groups. These results for parental shade, together with those of Herman and Sultan (2016) for parental drought, show that, even within a given system – here, the same genotypes within a species – adaptive developmental effects of parental stresses on progeny may be established by either methylation or demethylation [i.e., since methylation generally reduces transcriptional activity (Jones, 2012) by either down- or up-regulating relevant components of response pathways].

While these results confirm a role for DNA methylation change in the inheritance of parental shade effects, further molecular work is needed to determine precisely how these effects are transmitted to progeny. Unlike in mammals, where DNA methylation is mostly reset during embryogenesis, methylation states are meiotically stable in plants (Kakutani et al., 1999; Becker et al., 2011; Schmitz et al., 2011). Accordingly, it is possible that shade-induced methylation state changes at loci involved in plastic shade responses may be maintained through meiosis and directly transmitted to offspring. Alternatively, DNA methylation patterns may be reconstructed during embryogenesis (Bouyer et al., 2017) or in developing progeny (Vu et al., 2015) by inherited regulatory molecules (such as hormones, proteins, or non-coding RNAs) that can direct DNA methylation and demethylation (Bonduriansky and Day, 2009; Mahfouz, 2010; Boyko and Kovalchuk, 2011; Zhang and Zhu, 2011; Holeski et al., 2012; Duncan et al., 2014; Matzke et al., 2015). It is also not known whether shade-induced methylation state changes are targeted to specific loci. In this study, genome-wide partial demethylation by zebularine mimicked the parental effects of understory shade on progeny phenotypes, suggesting that parental shade effects may be mediated by similarly non-specific demethylation. Such genome-wide demethylation may result from the loss of methylation marks across cell division (Duncan et al., 2014), for instance due to a shortage of available methyl groups or to reduced activity of DNA methyltransferases (Zhang and Zhu, 2012), perhaps initiated by a metabolic feedback.

Although data are not available with respect to shade, other environmental conditions are known to alter these epigenetic regulators (e.g., in *Arabidopsis*, Dowen et al., 2012; reviewed by Meyer, 2015). Alternatively, shade may induce targeted methylation changes, if certain DNA loci are more sensitive than others to changed levels of methyltransferases or signaling molecules. Methylation changes may also interact with changes in the amount or quality of seed provisions (Herman and Sultan, 2011). Assessing the precise roles and relative impact of these inheritance mechanisms is a substantial experimental challenge (Donohue, 2009).

Although it is well established that both biotic and abiotic stresses may induce DNA methylation changes at specific loci (Kovar et al., 1997; Chinnusamy and Zhu, 2009; Dowen et al., 2012) and that these changes may be inherited by descendent generations (Verhoeven et al., 2010; Kou et al., 2011; Zheng et al., 2013), few if any published cases document that these inherited epigenetic changes actually result in tolerance to the inducing stress (Meyer, 2015). Conversely, some studies convincingly link specific epigenetic state changes to adaptive effects, but without demonstrating their stress-induction or heritable transmission (e.g., Xie et al., 2015). Resolving the entire causal pathway, from stress induction, to precise epigenetic changes and their transmission, to phenotypic effects and functional consequences, is a demanding task indeed. More broadly, understanding the mechanisms, dynamics, and adaptive importance of transgenerational effects in plant populations will require not only improved genomic tools for epigenetic studies in non-model species (Richards et al., 2017), but collaborative investigations that draw on molecular, developmental, and ecological expertise.

DATA AVAILABILITY STATEMENT

The datasets generated and analyzed for this study can be found in the Figshare repository: https://figshare.com/articles/Baker_2018_parental_shade_DNA_methylation_xlsx/6884945.

AUTHOR CONTRIBUTIONS

BB, LB, and SS designed the glasshouse experiments, which was conducted by BB and LB. BB and SS designed the demethylation experiments and BB conducted it and carried out the statistical analyses. BB and SS jointly interpreted results and wrote the manuscript.

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