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Hypomethylation of the aquatic invasive plant, *Ludwigia grandiflora* subsp. *hexapetala* mimics the adaptive transition into the terrestrial morphotype

Julien Genitoni[a,b], Danièle Vassaux[a], Alain Delaunay[b], Sylvie Citerne[c], Luis Portillo Lemus[a], Marie-Pierre Etienne[d], David Renault[e,f], Solenn Stoeckel[g], Dominique Barloy[a,*] and Stéphane Maury[b]

[a]ESE, Ecology and Ecosystem Health, Institut Agro, INRAE, Rennes 35042, France
[b]Laboratoire de Biologie des Ligneux et des Grandes Cultures (LBLGC), EA1207 USC1328 INRA, Université d'Orléans, Orléans 45067, France
[c]Institut Jean-Pierre Bourgin, INRAE, AgroParisTech, Université Paris-Saclay, Versailles 78000, France
[d]Institut Agro, CNRS, Université Rennes, IRMAR (Institut de Recherche Mathématique de Rennes) – UMR 6625, Rennes F-35000, France
[e]UMR CNRS 6553 EcoBio, University of Rennes 1, Rennes, France
[f]Institut Universitaire de France, 1 rue Descartes, Paris, France
[g]IGEPP, INRAE, Institut Agro, Université Rennes, Le Rheu 35653, France

Correspondence
*Corresponding author,
e-mail: dominique.barloy@agrocampus-ouest.fr

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Ongoing global changes affect ecosystems and open up new opportunities for biological invasion. The ability of invasive species to rapidly adapt to new environments represents a relevant model for studying short-term adaptation mechanisms. The aquatic invasive plant, *Ludwigia grandiflora* subsp. *hexapetala*, is classified as harmful in European rivers. In French wet meadows, this species has shown a rapid transition from aquatic to terrestrial environments with emergence of two distinct morphotypes in 5 years. To understand the heritable mechanisms involved in adjustment to such a new environment, we investigate both genetic and epigenetic as possible sources of flexibility involved in this fast terrestrial transition. We found a low overall genetic differentiation between the two morphotypes arguing against the possibility that terrestrial morphotype emerged from a new adaptive genetic capacity. Artificial hypomethylation was induced on both morphotypes to assess the epigenetic hypothesis. We analyzed global DNA methylation, morphological changes, phytohormones and metabolite profiles of both morphotype responses in both aquatic and terrestrial conditions in shoot and root tissues. Hypomethylation significantly affected morphological variables, phytohormone levels and the amount of some metabolites. The effects of hypomethylation depended on morphotypes, conditions and plant tissues, which highlighted differences among the morphotypes and their plasticity. Using a correlative integrative approach, we showed that hypomethylation of the aquatic morphotype mimicked the characteristics of the terrestrial morphotype. Our data suggest that DNA methylation rather than a new adaptive genetic capacity is playing a key role in *L. grandiflora* subsp. *hexapetala* plasticity during its rapid aquatic to terrestrial transition.

Abbreviations – ABA, abscissic acid; Am, aquatic morphotype; Ctrl, control; DMR, dry mass of roots; DMS, dry mass of shoots; FMR, fresh mass of roots; FMS, fresh mass of shoots; IAA, auxin; JA, jasmonic acid; JL, leucine jasmonyl acid; LI, length of internodes; LP, length of plant; nbB, number of nodes with buds; nbI, number of internodes; nbL, number of leaves; nbNR, number of nodes with roots; Rr, root ratio (FMR/DMR); SA, salicylic acid; Sr., shoot ratio (FMS/DMS); Tm, terrestrial morphotype; WC, water content ((FMS + FMR)/(DMS + DMR)); Zeb, zebularine.

Introduction

Global changes and human activity impact ecosystems and increase opportunities of geographic expansion of invasive species (van Kleunen et al. 2015, Early et al. 2016), thus increasing the cascading economic and ecological consequences (Theoharides and Dukes 2007). However, the capabilities of organisms to adapt rapidly to newly encountered environmental conditions are crucial for the establishment of a viable population, especially for plants that are sessile organisms. Moreover, invasion phenomenon can occasionally result in founder effects, i.e. a large decrease in the genetic diversity of the few establishing propagules. Phenotypic plasticity, which is the ability of one genotype to produce several phenotypes depending on the environment (Pigliucci 2005), is commonly cited as an important factor favoring the invasion success. Theoretically, organisms characterized by a greater plasticity should be able to thrive in a larger range of environmental conditions compared to their less-plastic relatives. Yet, the high level of phenotypic plasticity reported in some invasive species (Trussell and Smith 2000, Sexton et al. 2002, Daehler 2003, Davidson et al. 2011, Morris 2014) is still questioned (Chown et al. 2007, Godoy et al. 2011, Matzek 2012, Lamarque et al. 2013), and the underpinning mechanisms at the basis of this flexibility, including epigenetics, remain to be established.

Epigenetics can be defined as any non-genetic molecular modification of the genome that alters gene expression and that can be heritable (Meyer 2018). This definition broadly encompasses several levels of chromatin modifications, including DNA methylation, histone modifications and variants, and non-coding RNAs (Duncan et al. 2014, Allis and Jenuwein 2016). It has recently been suggested that epigenetics is a potential source of flexibility involved in the success of invasive species, allowing organisms to express advantageous phenotypes across a broader range of environments (Richards 2006, Richards et al. 2017, for a recent review see Marin et al. 2019).

DNA methylation is the most studied epigenetic marker; it consists of the addition of a methyl group to DNA cytosines. This addition is provided de novo and maintained by methyltransferase enzymes through three different sequence contexts in plants: CG, CHG and CHH (Meyer 2015). Over recent years, many studies have highlighted the role of DNA methylation in many biological and ecological mechanisms in plants, such as development (Guo et al. 2018, Li et al. 2018), responses to biotic and abiotic stressors (Tricker 2015, Crisp et al. 2016, Lämke and Bäurle 2017), cellular-stress response memory and priming (Mauch-Mani et al. 2017, Le Gac et al. 2018, 2019, Sow et al. 2018), phenotypic plasticity (Gourcilleau et al. 2010, Baulcombe and Dean 2014, Kooke et al. 2015, Conde et al. 2017, Lafon-Placette et al. 2018, Maury et al. 2019), and possibly adaptation (Bräutigam et al. 2013, Kawakatsu et al. 2016, Rey et al. 2016, Schmid et al. 2018, Gourcilleau et al. 2019). Kooke et al. (2015) used 99 Epigenetic Recombinant Inbred Lines (epiRILs) of *Arabidopsis thaliana*, consisting of crosses between the parents Col-0 and the hypomethylated mutant lines *ddm1* and *ddm2*, to compare the morphological responses of the plants exposed to saline conditions. These authors reported the existence of several distinct phenotypes among epiRILs and discovered that hypomethylation affects phenotypic plasticity during stress response.

Ludwigia grandiflora subsp. *hexapetala* (Nesom and Kartesz 2000), the water primrose native to South America, is an aquatic invasive plant in Europe (Hussner et al. 2016), which can colonize a wide range of habitats (Haury et al. 2014). In France, this plant invades aquatic habitats, such as static or slow-flowing waters, riverbanks, and has more recently been reported thriving in wet meadows (Dandelot et al. 2005, Lambert et al. 2010). As mentioned in Billet et al. (2018), *L. grandiflora* subsp. *hexapetala*, is an amphibious plant characterized by two distinct morphotypes: one being aquatic (Am), while the second one being terrestrial (Tm, wet meadows). In addition to raising ecological questions, the secondary colonization of wet meadows could lead to a loss of financial support for farmers because of depreciation in fodder value and abandonment of pasturing. In its original condition, aquatic plants are characterized by an elongated morphology with a low leaf number. Conversely, terrestrial plants present a bushy morphology with a greater root system and more leaves than the aquatic morphotype (Haury et al. 2014, Billet et al. 2018). In addition, Billet et al. (2018) identified an accumulation of metabolites in roots characterizing the plants thriving in the terrestrial condition, associated with the regulation of the following pathways: amino sugar and nucleotide sugar metabolism, starch and sucrose metabolism, as well as galactose, fructose and mannose metabolism. Two metabolic pathways for shoots in the aquatic condition were identified: the valine, leucine and isoleucine metabolism and degradation pathways.

Water primrose reproduces essentially by clonal propagation, and each population is thus expected to exhibit a low genetic diversity, as demonstrated in *Fallopia* spp. (Holm et al. 2017). According to Zhang et al. (2013), this kind of organisms make them good candidates for testing

the role of epigenetic mechanisms in invasive plants. Thus, the aim of this study is to explore the involvement of genetic and epigenetic components in the adaptation of *L. grandiflora* subsp. *hexapetala* to the terrestrial environment. We first examined the genetic differentiation of the aquatic and terrestrial morphotypes using single nucleotide polymorphisms (SNPs) and their epigenetic differentiation using global DNA methylation assessed by classical HPLC techniques (Lambé et al. 1997, Trap-Gentil et al. 2011, Teyssier et al. 2014, Alonso et al. 2016, Kurdyukov and Bullock 2016, Yong et al. 2016, Sow et al. 2018). Indeed, global DNA methylation (%mC) has been widely used in plants to obtain useful information on DNA methylation (Causevic et al. 2006, Gourcilleau et al. 2010, Trap-Gentil et al. 2011, Teyssier et al. 2014, Alonso et al. 2015, Alonso et al. 2016, Le Gac et al. 2018, Sow et al. 2018, Gourcilleau et al. 2019).

In a second part of the work, we explored whether DNA methylation is differently mobilized by terrestrial and aquatic morphotypes according to the environment, therefore possibly participating to the fast transition from aquatic to terrestrial life of the water primrose, as suggested for the amphibious plant, *Alternanthera philoxeroides* in response to change of water availability (Li et al. 2013). Here, we used a pharmacological approach using zebularine to perturb DNA methylation in aquatic and terrestrial morphotypes from the same populations of *L. grandiflora* subsp. *hexapetala* previously used by Billet et al. (2018). To characterize the hypomethylation effect of zebularine, a correlative approach and integrative analysis were conducted using morphological, physiological (metabolomics and phytohormones) and methylation percentage variables.

Materials and methods

Plant material and experimental design

Aquatic (Am) and terrestrial (Tm) morphotypes of *L. grandiflora* subsp. *hexapetala* grew as single dense continuous sympatric population in Mazerolles swamps (Nantes, France, N47 23.260, W1 28.206), separated at fine spatial scale in two subpopulations with different environments, respectively flooded the year round or ground-emerged a part of the year (as sympatric populations). Aquatic and terrestrial morphotypes were sampled from five collection points (50 × 50 cm frame), 10 m apart, giving a total of 10 collection points. After collection, plants were grown in a greenhouse in aquatic conditions for Am and Tm, as described in Billet et al. (2018).

For the zebularine experiment, a 10-cm portion from the stem apex was collected, without roots, buds and lateral stems. All plants were preconditioned for 2 weeks, as described in Billet et al. (2018), to promote root development. For morphological analyses, two biological replicates of nine plants were randomly grouped by three plants per container for aquatic (Am-a and Tm-a) and terrestrial (Am-t and Tm-t) conditions for both morphotypes, as described in Billet et al. (2018). The plants were then placed in a growth chamber (22°C, 16/8 h day/night). Similarly, three biological replicates of six plants were carried out for physiological (phytohormone and metabolite quantifications) and %mC analyses for each morphotype (Am and Tm), condition (-a and -t), and treatment (ctrl and zeb) combination, for a total of 288 plants. Zebularine (Sigma-Aldrich) treatment at 150 μM was applied in a liquid solution at the beginning of the experiment and renewed 7 days later. Plants without zebularine were grown as a control. Sampling for physiological and methylation analyses was conducted 14 days after the beginning of the experiment. De novo shoots and roots, taken 5 cm from the apex (including stems, leaves and buds) were sampled separately from six randomly collected plants per morphotype (Am or Tm), treatment (zebularine or control) and condition (aquatic or terrestrial). Samples for physiological analysis were pooled and immediately snap-frozen in liquid nitrogen, then lyophilized over 48 h using a Cosmos 20 K (Cryotec) and stored at −80°C.

Genetic differentiation

To assess the degree of genetic differentiation between the two sub-populations, we genotyped 15 individuals per group/morphotype at 38 SNP loci randomly sampled on the genome. Considering our sample size, we computed unbiased F_{ST} values at each locus as recommended by Hardy (2016) with the estimated average number of pairwise differences between two individuals sampled from the total population or within subpopulations (aquatic or terrestrial morphotypes).

Graph of genetic distances between phenotypes

To assess the homogeneity of the genetic background between terrestrial and aquatic individuals, we computed the allele sharing distances (ASD) between pairs of individuals within subpopulations and between subpopulations. ASD is a common metric well adapted to SNPs that measures the identity-by-state and relatedness between individuals (Stevens et al. 2011). ASD measures the probability of allele divergence between pairs of individuals across loci and is computed as the probability with which two SNP alleles, one drawn randomly from each of the two individuals, differ in state. Ranging from zero to one, low values of ASD mean that individuals are tightly related with identical genomes while high values of ASD mean less genetic relatedness and older

common ancestrality. For example, the ASD value between native Americans (Pima) from a same group is 0.6 ± 0.05 and 0.75 ± 0.05 between European coming from a same country (Mountain and Ramakrishnan 2005).

Genetic graphs are simple and common construction of more complex systems composed of nodes (here individuals) related by edges (here ASD values) to visualize the degree of ancestrality and admixture between individuals in population with different phenotypes and ecology (Rozenfeld et al. 2008, Gao and Martin 2009, Dyer 2015). Two adjacent individuals (nodes) linked by an edge are the most identical-by-state, thus the most genetically related in the considered sample. Genetic distance between pairs of distant individuals corresponds to the sum of all the edges to walk through between their nodes. The smaller the value between pairs of individuals, the more identical-by-state and closer related they are. If terrestrial phenotypes are supported by a hereditary genetic feature inherited from a single ancestral mutation and recombination event(s), we expect a clear separation (long edges, gap) between nodes, with higher magnitude of genetic distances between terrestrials and aquatics than within terrestrials.

Morphological analyses

Nine plants per morphotype, condition and treatment were analyzed. The following morphological, growth and fitness traits were measured:

Length of the plant (LP) in centimeters (cm), number of internodes (nbI) and number of leaves (nbL), were recorded in order to characterize plant morphology. The length of the internodes in centimeters was calculated as the ratio of LP/nbI. Number of nodes with roots (nbNR) and number of nodes with buds (nbB) variables are associated with the fitness of the plant. As mentioned in Billet et al. (2018) fitness of *L. grandiflora* subsp. *hexapetala* is considered as the ability to produce propagules from buds or nodes with roots. *L. grandiflora* subsp. *hexapetala* has the ability to form a new plant from small (>1 cm) plant fragments. Biomass of plants in grams (g) was determined by measuring the fresh mass of shoots (FMS) and roots (FMR). After oven drying the samples at 105°C for 48 h, we obtained their respective dry mass (DMS and DMR). To determine water content, the ratios of fresh/dry mass of shoots (Sr) and roots (Rr) were calculated.

DNA extraction and determination of global DNA methylation percentage by HPLC

Genomic DNA extraction was carried out using the CTAB protocol described in Lafon-Placette et al. (2013). Quantity and quality of gDNA were checked using the NanoDrop spectrometer and electrophoresis on agarose gel and ethidium bromide staining under UV light. Genomic DNA was hydrolyzed into nucleosides and analyzed by HPLC using a GeminiTM column (150 × 4.6 mm, 5 µm; Phenomenex). Finally, an isocratic mobile phase consisting of 0.5% methanol (v/v) and 5 mM acetic acid in water, as described by Zhu et al. (2013) and Le Gac et al. (2018) was used. Global DNA methylcytosine percentage (% mC) was calculated as follows:

$$\%mC = (mC/[C + mC]) \times 100$$

where C is the 2′-deoxycytidine content and mC is the 5-methyl-2′-deoxycytidine content. For each set of conditions, we analyzed three biological replicates for each morphotype, condition and treatment. Each sample was hydrolyzed twice and two HPLC runs were carried out by hydrolysis. Controls for this procedure included the use of co-migration with commercial standards (Sigma-Aldrich), confirmation by enzyme restriction analysis, and tests for RNA contamination were based on the HPLC detection of ribonucleosides which have already been detailed in Zhu et al. (2013).

Phytohormone quantification

Quantifications of abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA), jasmonyl Leucine acid (JLA) and auxins (IAA) were carried out in both shoots and roots. For each sample, 1 mg of dry powder was extracted with 0.8 ml of acetone/water/acetic acid (80/19/1 v:v:v). Stable labeled isotopes of phytohormones used as internal standards were prepared as described in Roux et al. (2014). An amount of 1 ng of each standard was added to the sample. The extract was vigorously shaken for 1 min, sonicated for 1 min at 25 Hz, shaken for 10 min at 10°C in a Thermomixer (Eppendorf®) and then centrifuged (8000 *g*, 10°C, 10 min). The supernatants were collected, and the pellets were re-extracted twice with 0.4 ml of the same extraction solution, then vigorously shaken (1 min) and sonicated (1 min; 25 Hz). After the centrifugations, the three supernatants were pooled and dried (final volume 1.6 ml).

Each dry extract was dissolved into 100 µl of acetonitrile/water (50/50 v/v), filtered, and analyzed using a Waters Acquity ultra performance liquid chromatography coupled to a Waters Xevo Triple quadrupole mass spectrometer TQS (UPLC-ESI-MS/MS). The compounds were separated on a reverse-phase column (Uptisphere C18 UP3HDO, 100 × 2.1 mm × 3 µm particle size; Interchim) using a flow rate of 0.4 ml min^{-1} and a binary gradient: (A) acetic acid 0.1% in water (v/v) and (B) acetonitrile with 0.1% acetic acid, the column temperature was 40°C. For ABA, SA, JA and indole-3-acetic acid we used the following binary gradient (time, % A):

(0 min, 98%), (3 min, 70%), (7.5 min, 50%), (8.5 min, 5%), (9.6 min, 0%), (13.2 min, 98%), (15.7 min, 98%). Mass spectrometry was conducted in electrospray and Multiple Reaction Monitoring scanning mode (MRM mode), in positive ion mode for the indole-3-acetic acid and in negative ion mode for the other hormones. Relevant instrumental parameters were set as follows: capillary 1.5 kV (negative mode), source block and desolvation gas temperatures 130 and 500°C, respectively. Nitrogen was used to assist the cone and desolvation (150 l h^{-1} and 800 l h^{-1}, respectively), argon was used as the collision gas at a flow of 0.18 ml min^{-1}.

Metabolomic fingerprint

Gas chromatography coupled with mass spectrometry (GC–MS) was used to scan for the metabolites that could be detected from the plant samples (amino acids, organic acids, polyamines, polyols, or sugars). For the preparation of the samples, the method described in Serra et al. (2013) was used and adapted as in Billet et al. (2018). Briefly, for each sample, an aliquot of 10 mg of lyophilized powder was homogenized in 600 µl of a solution of ice-cold methanol/chloroform (2:1, v/v). Then, a volume of 400 µl of ultra-pure water was added. Samples were homogenized and centrifuged for 10 min at 4000 *g* (4°C). The upper phase (128 µl) containing metabolites was transferred to new glass vials for roots and shoots, respectively. The derivatization of the samples was conducted with a CTC CombiPAL autosampler (CTC Analytics AG), as described in Serra et al. (2013). The GC–MS platform consisted of an Agilent 7890B gas chromatograph coupled to a 5977B mass spectrometer. The injector was held at 250°C, and the temperature of the oven ranged from 70 to 170°C at 5°C per min, 170 to 280°C at 7°C per min and 280 to 320°C at 15°C per min; at the end of the temperature ramps, the oven remained at 320°C for 4 min. A 30 m fused silica column (HP5 MS 30 m, I.D. 0.25 mm, thickness 0.25 µm, 5% Diphenyl/95% Dimethylpolysiloxan, Agilent Technologies) was used, with helium as the gas carrier at 1 ml min^{-1}. The temperatures of the transfer line and ion source were 280 and 230°C, respectively. The split mode (split ratio: 2:1) was used for the injection of 1 µl of each sample, and detection was realized by electronic impact (electron energy: 70 eV), in full scan mode. The detected peaks were annotated with MassHunter. Most detected metabolites were identified, and calibration curves were drawn for calculating the concentration of each metabolite.

Phenotypic plasticity

The phenotypic plasticity of the plants exposed to the different experimental conditions was calculated by using morphological and phytohormone variables and computing a 'relative distance plasticity index' RDPI, as suggested by Valladares et al. (2006). RDPI = $\sum$(dij → i'j'/[xi'j' + xij])/n), where n is the total number of distances, and j and j' are two individuals belonging to different treatments (i and I'). This index ranges from 0 (no plasticity) to 1 (maximal plasticity). The different RDPIs were calculated for morphological and phytohormone data by comparison of aquatic and terrestrial morphotype values in aquatic versus terrestrial conditions and treated and not treated with zebularine.

Statistical analysis

The effects of the zebularine treatment on the morphology and physiology of both water primrose morphotypes were assessed by running analyses of variance (ANOVAs) and principal component analyses (PCAs; FactomineR package).

Four-way ANOVAs were carried out with morphotype, treatment, condition and biological replicates as factors. Biological replicate and triple interaction effects are mainly not significant for all data. The effect of each factor is quantified by both type I and type II variance decomposition. As no difference between type I and type II have been observed, only ANOVA results of type I are presented in the text and tables of the article. Two and three-way interactions were then analyzed. The normality of residuals and the homoscedasticity were verified by Shapiro–Wilk's and Bartlett's tests, and normality was achieved, when required, by transforming morphological traits and the metabolite concentrations (Log10). Within the four ways analysis of variance models, the difference between groups have been examined through the comparisons of adjusted means thanks to the emmean package (Russell Lenth 2018) of the R software (Core Team 2018). The Tukey's test was used to conclude.

We also made two PCA incorporating morphological traits, phytohormones and a subset of six metabolites which are correlated with methylation percentage and some other metabolites significantly affected by hypomethylation according to the ANOVA. Then for the first PCA, we calculated the distances between the barycentres of the eight PCA groups and also made a hierarchical classification (HCPC) with FactomineR packages.

To link metabolites analysis to metabolic pathways, we performed pathway enrichment analyses in Metaboanalyst 3.0 (Xia et al. 2015). Fisher's exact test algorithm was performed for these pathway analyses with

Arabidopsis thaliana as the reference model. In this procedure, the number of hits between the metabolites in our dataset and all metabolites of a given pathway was calculated. Data presented in the figures are untransformed.

Results

Genetic differentiation between aquatic and terrestrial morphotypes of the invasive *L. grandiflora* subsp. *hexapetala* plant

Aquatic and terrestrial morphotypes showed low genetic differentiation with a median F_{ST} of 0.019. All the genotyped alleles were shared by the two subpopulations. Over the 38 genotyped SNPs, 27 loci showed no genetic differentiation between aquatic and terrestrial subpopulations (FST values under 0.05; Hartl and Clark 1997, Frankham et al. 2010). Only two SNPs showed high F_{ST} values (0.488 and 0.527) but those two values were due to skewed allele frequencies in a commonly shared allele pool rather than due to privative alleles or a narrow restricted ancestrality (Fig. 1). Genetic divergences (ASD) between pairs of individuals from different subpopulations were on average 0.328 (95% confidence interval: [0.315, 0.342]), on the order of values expected between sister plants sharing the same parents. ASD values within and between subpopulations were on the same order of magnitude, with no gap between subpopulations and even with intermingled individuals from different subpopulations (Fig. S1). The graph topology was nearly linear with regularly distributed individuals which was consistent with isolation-by-distance scenario (Templeton 2006). It showed some grapes of individuals diverging from only one to three alleles, which is expected under clonal reproduction (Rozenfeld et al. 2007). Altogether, the data suggested very recent common ancestors and even shared parents between individuals of different morphotypes.

Artificial hypomethylation of the aquatic and terrestrial morphotypes

A 14-day zebularine treatment was applied to induce DNA hypomethylation in Am and Tm morphotypes of the invasive *L. grandiflora* subsp. *hexapetala* plant grown in aquatic and terrestrial conditions. Only de novo-formed shoots during the treatment were used to assess the global DNA methylation percentage (%mC) using the HPLC method (Fig. 2; see Materials and Methods for procedure). The methylation percentages ranged from 25.9 to 29.8% for control plants and from 20.9 to 23.9% for plants treated with zebularine (Fig. 2). A four-way analysis of variance was carried out by comparison of morphotype (Am or Tm), treatment (zebularine or control), condition (aquatic or terrestrial) and biological replicates. The DNA methylation percentage was significantly affected by morphotypes, treatment and the interaction between condition and treatment (P = 8.82e-03, 4.88e-08 and 1.86e-2, respectively). ANOVA analysis revealed a significant decrease in %mC in zebularine-treated plants (P = 4.88e-08). Specifically, the zebularine treatment induced a methylation decrease ranging from 7.2 and 8.6% for Am and Tm grown in terrestrial conditions, respectively, and from 4.3 and 5.1% in aquatic conditions, respectively (Fig. 2). However,

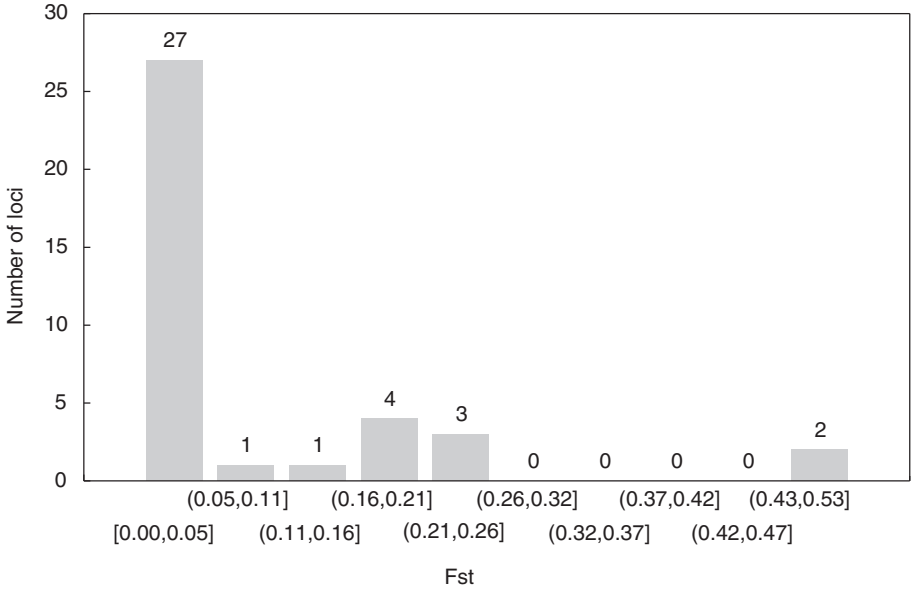

Fig. 1. Distribution of unbiased pairwise F_{ST} values per locus between aquatic and terrestrial subpopulations of *L. grandiflora* subsp. *hexapetala*. Overall median value: 0.019 [first quartile: 0.009; third quartile: 0.107].

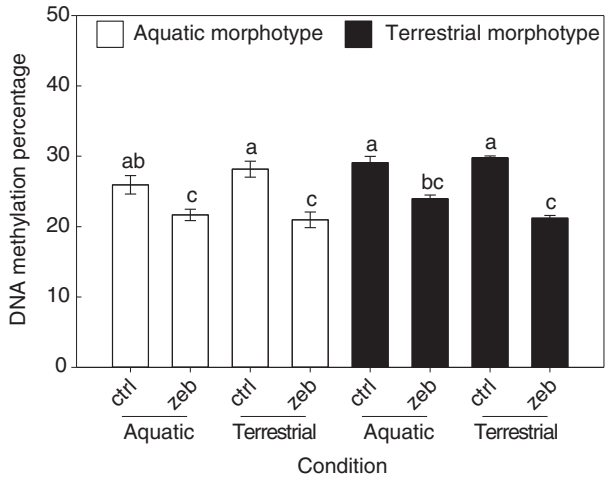

Fig. 2. Global DNA methylation percentages in de novo-formed shoot of aquatic and terrestrial morphotypes (white and black, respectively) in aquatic and terrestrial conditions with or without 14 days of zebularine treatment ('zeb' for 150 μM of zebularine and ctrl for control). Bars represent the mean value of three biological replicates with their standard errors. Thanks to a Tuckey HSD test based on the mean difference between groups, one or more letters are assigned to each group. Two groups sharing one letter have no significant difference.

we observed similar methylation levels between morphotypes independently of conditions for the two treatments.

The data showed that no significant variation in global DNA methylation was observed among morphotypes and conditions, but a significant hypomethylation was obtained after a 14-day zebularine treatment in both morphotypes and conditions.

Growth, morphology and phenotypic plasticity of hypomethylated aquatic and terrestrial morphotypes

After a 14-day treatment, Am and Tm grown in aquatic (-a) and terrestrial (-t) conditions were pictured (Fig. 3A), and morphology, biomass and water content were measured (Fig. 3B). No necrosis symptoms were observed for zebularine-treated plants (Fig. 3A). Analyses of variance were carried out and showed that morphology and growth were altered (Figs 3A,B and S2, Table S1). Hypomethylation had a significant impact on 6 of the 13 measured traits (Table S1). A significant impact was observed on hypomethylated plants, exemplified by the increased number of leaves in hypomethylated Am in aquatic conditions (Am-a; Fig. 3B). Effects of hypomethylation were also observed with the decrease in fresh mass of roots of the hypomethylated Tm in terrestrial conditions (Tm-t; Fig. 3B) Indeed, zebularine treatment impacted the two morphotypes differently when grown

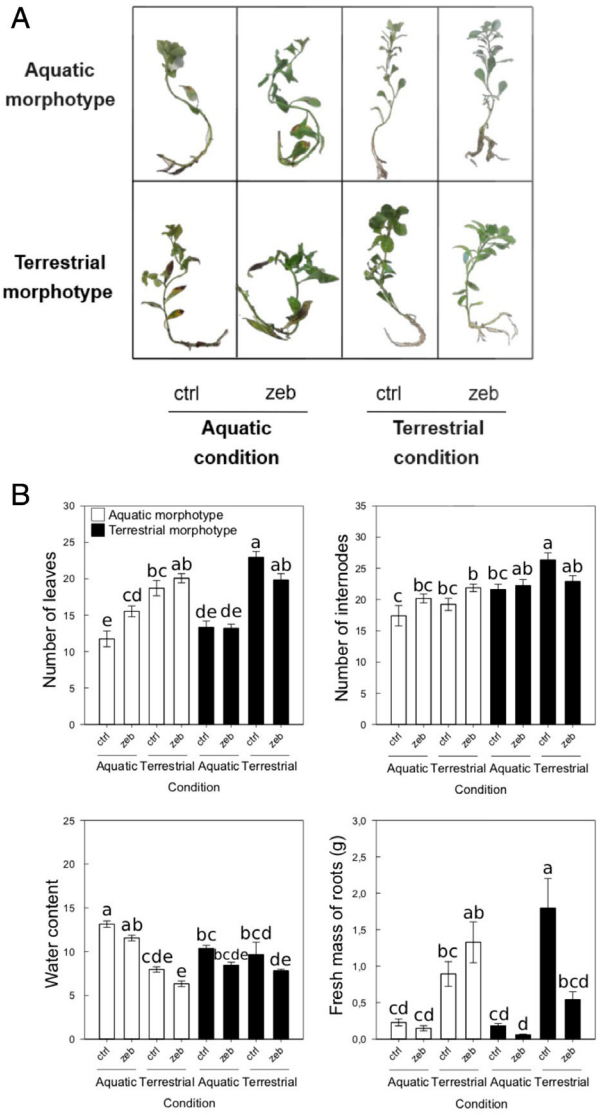

Fig. 3. Phenotype of hypomethylated aquatic and terrestrial morphotypes (white and black, respectively) of *Ludwigia grandiflora* grown in terrestrial or aquatic conditions. (A) Pictures of control and zebularine-treated plants in each condition. (B) Number of leaves, of internodes, water content and fress mass of roots. Bars represent the mean value of 10 biological replicates with their standard errors. Letters are assigned to groups thanks to a Tuckey HSD test. Two groups sharing one letter have no significant difference. Supplementary data for statistics are presented in Table S1.

in their original conditions (Am-a vs Tm-t), but also when conditions were shifted (Am-t vs. Tm-a; Figs 3B and S2, Table S1) with significant interaction between morphotype and treatment for some morphological traits. Hypomethylation also significantly decreased the water content (Fig. 3B and Table S1).

Another strong difference revealed by the analyses of variance was for the growth conditions (aquatic vs

terrestrial), independently of the zebularine treatment. Plants grown in aquatic conditions showed an elongated morphology, with a greater plant length and length of internodes, and greater water content and shoot ratio (Table S1, Fig. S2). On the contrary, plants having grown in terrestrial conditions were characterized by a greater number of leaves, number of internodes, biomass variables (fresh and dry masses of roots and shoots) and number of buds (Figs 3A,B and S2, Table S1).

To compare levels of phenotypic plasticity in response to DNA hypomethylation on the different variables, a relative distance plasticity index (RDPI; Valladares et al. 2006) ranging from 0 (no plasticity) to 1 (maximal plasticity) was calculated for each trait and morphotype across treatments (Tables 1 and S2). This index gives an unbiased estimation of the levels of phenotypic variation and allows the exploration of plasticity with strong statistical power to test for differences in plasticity between genotypes. Interactions between morphotype and treatment effects were detected for most variables (Table S2). However, the two morphotypes showed significantly distinct RDPI for a majority of morphological, biomass and water content variables (Tables 1 and S2). The terrestrial morphotype exhibited higher phenotypic plasticity for biomass variables as opposed to the aquatic morphotype, which showed higher RDPI (but weak RDPI values) for water content and morphological variables. In response to DNA hypomethylation, phenotypic plasticity of 8 out of 13 variables was affected in both morphotypes (Table 1). Furthermore, phenotypic plasticity of the terrestrial morphotype was negatively affected by DNA hypomethylation, while RDPI of aquatic morphotype was positively affected for biomass and water content variables.

Altogether, results showed that growth and morphology as well as phenotypic plasticity were affected in hypomethylated morphotypes in aquatic and terrestrial conditions.

Phytohormone balance in hypomethylated aquatic and terrestrial morphotypes

Quantification of phytohormones (SA, Auxin, LJA, JA and ABA) revealed that terrestrial condition is associated with the highest amounts of all phytohormones in comparison to aquatic condition, independently of morphotype (Figs 4 and S3, Table S3). Organ distribution between shoot and root showed that SA is more present in shoot, while LJA is measured in greater amounts in root. Auxin and JA did not show strong variation among organs.

Significant effects of hypomethylation on phytohormones were detected: for SA (lower in shoot and higher

Table 1. Phenotypic plasticity (RDPI values) of morphological traits in zebularine-treated or untreated aquatic and terrestrial morphotypes (Am and Tm) in both aquatic and terrestrial conditions (-a and -t). Letters are assigned to groups thanks to a Tuckey HSD test. Two groups sharing one letter have no significant difference. Highlighted in grey corresponds to a decrease in plasticity in response to hypomethylation and highlighted in bold corresponds to an increase. Supplementary data for statistics are presented in Table S2.

		RDPI value and Tukey means comparison						
	Traits	Am-ctrl		Am-zeb		Tm-ctrl		Tm-zeb
Morphological variables	Length	0.14	a	0.09	b	0.08	b	0.09
	Number of leaves	0.28	a	0.14	c	0.27	a	0.20
	Number of internodes	0.18	a	0.08	c	0.12	b	0.10
	Length of internodes	0.19	a	0.11	c	0.14	b	0.10
	Number of nodes with roots	0.23	ab	0.25	a	0.21	bc	0.19
	Number of buds	0.60	ab	0.68	a	0.60	ab	0.58
Biomass variables	Fresh mass of shoots	**0.18**	**c**	**0.25**	**ab**	0.29	a	0.23
	Fresh mass of roots	**0.57**	**b**	**0.73**	**a**	0.73	a	0.75
	Dry mass of shoots	**0.31**	**b**	**0.43**	**a**	0.40	a	0.26
	Dry mass of roots	**0.53**	**b**	**0.71**	**a**	0.64	a	0.67
Water content	Water content	**0.25**	**b**	**0.29**	**a**	0.15	c	0.07
	Shoots ratio	**0.27**	**b**	**0.31**	**a**	0.16	c	0.06
	Roots ratio	0.20	a	0.13	b	0.19	a	0.23
Phytohormones in shoot	Auxin	0.15	ab	0.28	a	0.11	b	0.29
	Salicylic acid	0.61	a	0.44	ab	0.55	a	0.26
	Abscissic acid	0.76	a	0.69	a	0.73	a	0.40
	Jasmonic acid	0.41	a	0.44	a	0.41	a	0.41
	Leucine jasmonyl acid	0.54	ab	0.42	b	0.86	a	0.35
Phytohormones in roots	Auxin	**0.15**	**b**	**0.40**	**a**	0.19	b	0.18
	Salicylic acid	0.14	a	0.14	a	0.11	a	0.12
	Jasmonic acid	0.52	a	0.42	a	0.58	a	0.47
	Leucine jasmonyl acid	0.69	a	0.57	a	0.66	a	0.74

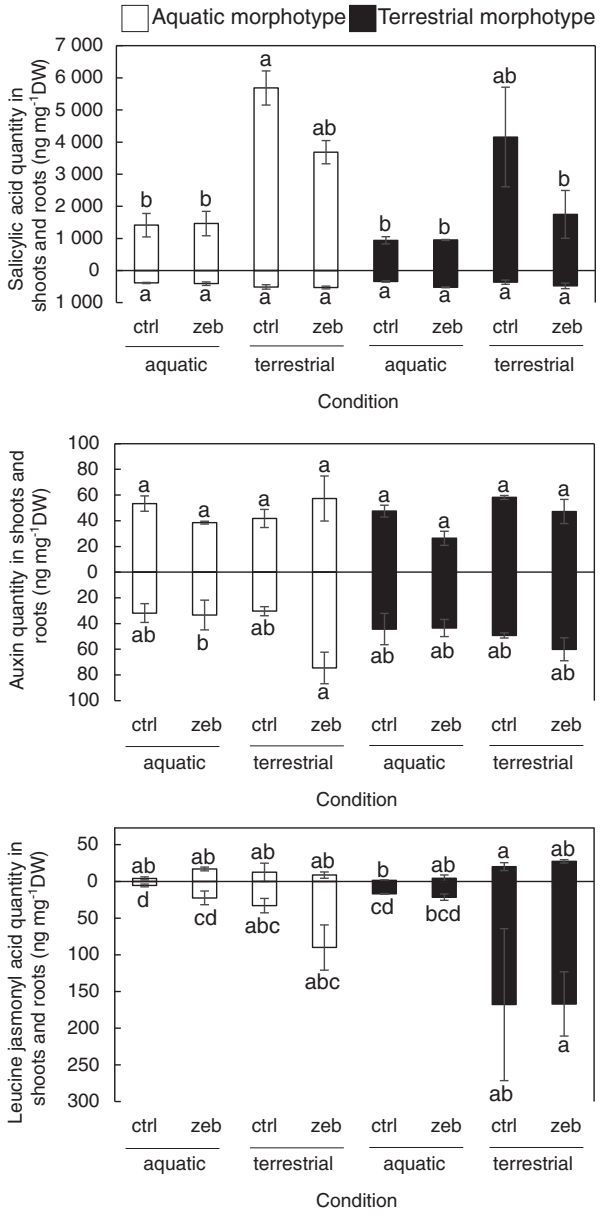

Fig. 4. Phytohormone quantities in roots and shoots of aquatic and terrestrial morphotypes (white and black) of *Ludwigia grandiflora* grown in terrestrial or aquatic conditions. Bars represent the mean value of three biological replicates with their standard errors. Letters are assigned to groups thanks to a Tuckey HSD test. Two groups sharing one letter have no significant difference. Shoots values are presented at the top and roots at the bottom. Supplementary data for statistics are presented in table S3.

in root) and LJA (higher in root; Table S3). Interaction between condition and treatment for SA was observed in shoots in favor of terrestrial condition and control. Analysis of RDPI for phytohormone contents showed a significant impact of hypomethylating treatment in shoots (Table S2) and lower values for the hypomethylated terrestrial morphotype (Table 1). To summarize, hypomethylation of morphotypes is associated to phytohormone balance changes in aquatic and terrestrial conditions. Phytohormone balance (contents and plasticity) is a sensitive physiological variable essentially affected by condition (-a vs -t) but also organ (shoots vs roots) and DNA hypomethylation (zebularine vs control).

Metabolomics profiling of hypomethylated aquatic and terrestrial morphotypes

The amount of several metabolites significantly differed in both shoots and roots depending on morphotypes, experimental conditions (aquatic, terrestrial) and treatments (zebularine-treated plants, control) (Table S4), revealing modifications of the metabolic activities. Condition (-a or -t) was the main factor discriminating the metabolic phenotypes of the plants ($P < 0.05$). Specifically, mainly in shoots, plants from aquatic conditions were characterized by a higher amounts of amino acids, including valine, leucine, serine, GABA and aspartic acid, in addition to higher concentrations of phosphoric acid, putrescine, glycerol-3-phosphate and quinic acid ($P < 0.05$; Table S4). In plants from terrestrial condition, larger quantities of sugars (fructose, glucose, galactose) were measured in both roots and shoots, and higher levels of polyols (sorbitol and inositol), glyceric acid, gluconolactone and malic acid in shoots ($P < 0.05$; Table S4).

Regarding treatment, the DNA hypomethylation altered the amounts of 10 and 8 metabolites in shoots and roots, respectively ($P < 0.05$; Figs 5 and S4). Six metabolites depicting hypomethylation-induced variability are presented in Fig. 5 (sugar maltose, glucose and fructose, and serine, fumaric and phosphoric acids), while variations of other metabolites are shown in Fig. S4. All metabolites affected by zebularine treatment were analyzed by Metaboanalyst, but no significant metabolomic pathway was identified, probably due to the low number of metabolites concerned here.

Our results show that hypomethylation affected amounts of these metabolites in a complex interaction between the morphotype, condition and organ.

Correlative approach and integrative analysis

The zebularine treatment discriminated plants from aquatic and terrestrial conditions (numbers of leaves and nodes with buds) as shown on the F1 axis of the PCA, while morphotypes were mainly separated on the second axis (Fig. 6A). Control vs zebularine treated-plants were relatively mixed along the axis. The global DNA methylation percentages were correlated with two

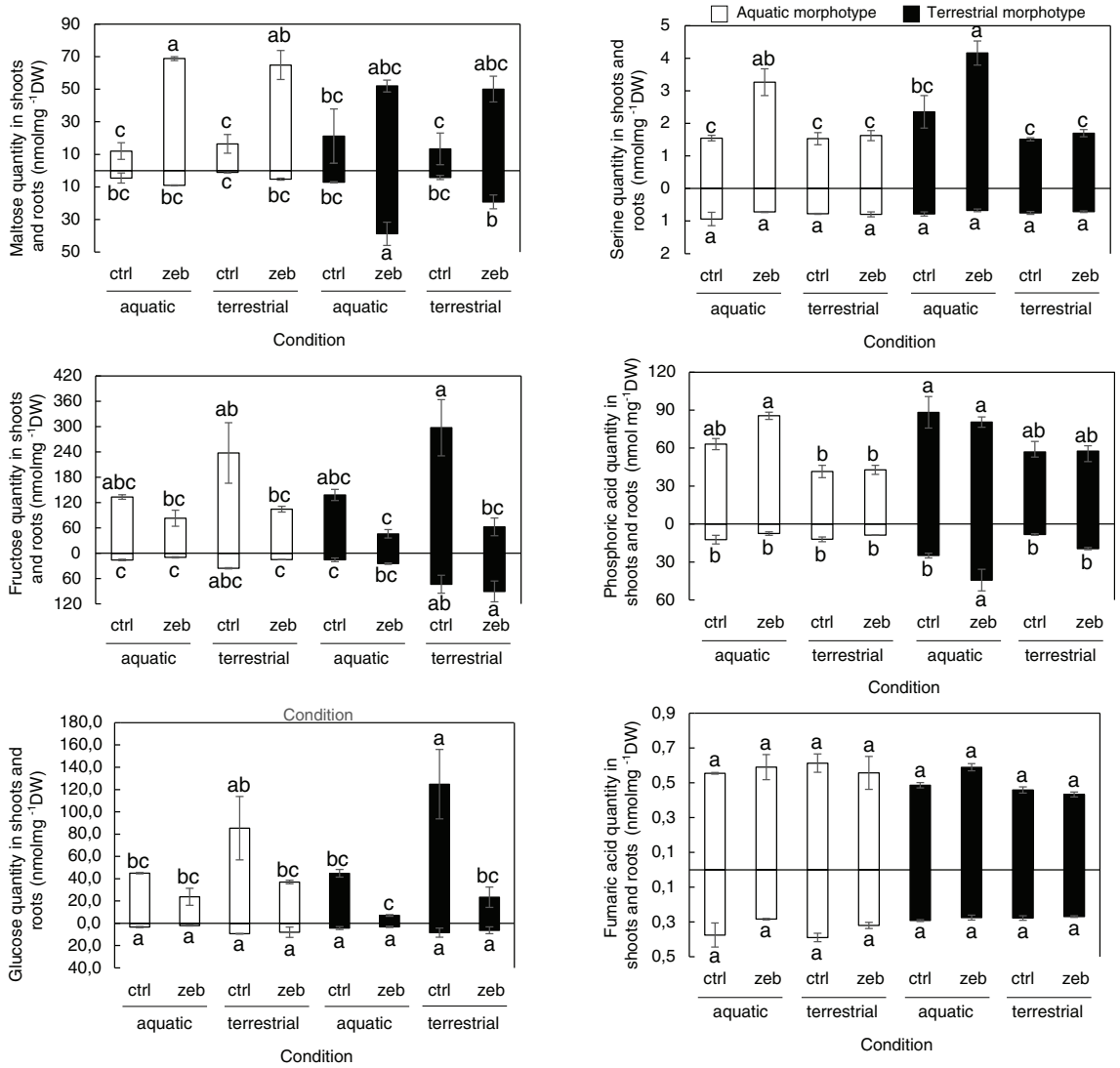

Fig. 5. Metabolite (maltose, serine, fructose, phosphoric acid, glucose, fumaric acid) quantities in roots and shoots of aquatic and terrestrial morphotypes (white and black) of *Ludwigia grandiflora* grown in terrestrial and aquatic conditions. Bars represent the mean value of three biological replicates with their standard errors. Shoots values are presented at the top and roots at the bottom. Letters are assigned to groups thanks to a Tuckey HSD test. Two groups sharing one letter have no significant difference. Supplementary data for statistics are presented in table S4.

variables of the PCA (Fig. S5): the roots ratio (Rr; r = 0.41 at $P < 0.05$,) and the SA in roots (r = −0.42 at $P < 0.05$). The main observation was that the hypomethylated aquatic morphotype in aquatic and terrestrial conditions (Am-a-zeb and Am-t-zeb; Fig. 6A) were getting closer to the control terrestrial morphotype in each condition (Tm-a-ctrl and Tm-t-ctrl, respectively).

A hierarchical classification analysis (HP) based on the PCA of morphological data highlighted six clusters mainly for aquatic or terrestrial conditions (Fig. S6) but was unable to clarify the effect of hypomethylation. Thus, calculation of the distances between barycenters and PCA groups were evaluated. The distances of the untreated aquatic morphotype in aquatic conditions (Am-a-ctrl) and the terrestrial morphotype control (Tm-a-ctrl; Am-a-ctrl/Tm-a-ctrl = 4.55) were greater than the distance between the hypomethylated aquatic morphotype (Am-a-zeb) and the terrestrial morphotype control (Tm-a-ctrl; Am-a-zeb/Tm-a-ctrl = 3.66), showing a hypomethylation-induced rapprochement of the morphotypes. This finding is also observed from the plants grown in terrestrial condition (Am-t-ctrl /Tm-t-ctrl = 6.21 vs. Am-t-zeb/Tm-t-ctrl = 5.94).

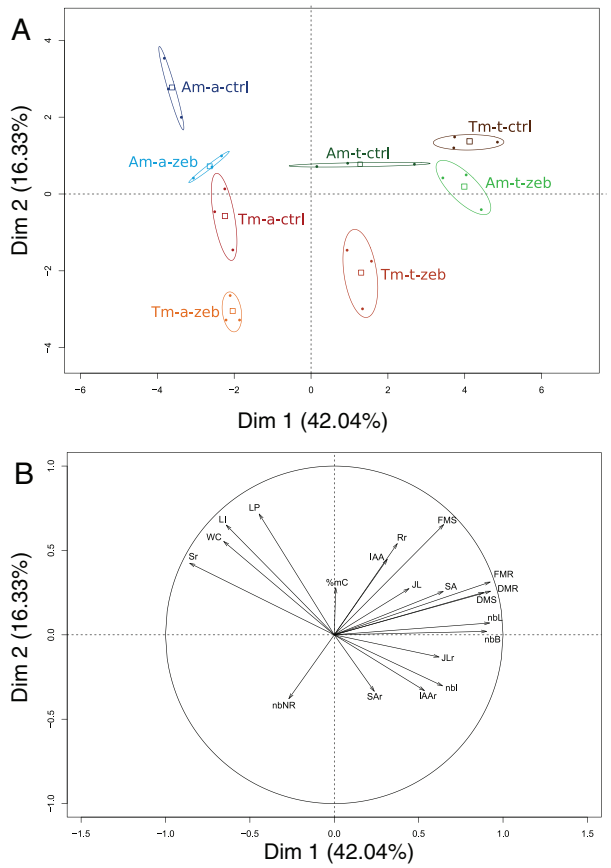

Fig. 6. Principal component analysis (PCA) representation allowing the characterization of the morphological and physiological variability of hypomethylated (zeb) or not (ctrl) aquatic and terrestrial morphotypes in aquatic (Am-a, Tm-a) and terrestrial (Am-t, Tm-t) conditions. (A) Individual factor map. Each data point represents one biological repeat. (B) Variables factor map. Morphological variables represented are number of leaves (nbL), length of internodes (LI), number of nodes (nbI), number of nodes with roots (nbNR), number of buds (nbB), fresh and dry mass of roots and shoots (FMS, FMR, DMS and DMR), shoots and roots ratio (FMS/DMS = Sr.; FMR/DMR = Rr), water content (WC). Phytohormones represented are auxin (IAA), salicylic acid (SA) and leucine jasmonyl acid (JL) in shoots and in roots (IAAr, SAr, JLr). Global DNA methylation percentages are indicated by %mC.

A second PCA with all data incorporating also metabolomic data was run (Fig. S7) and showed a closest proximity between Am-a-zeb and Tm-a-ctrl than between others. The effect of hypomethylation in aquatic conditions is explained principally by differences in water content (WC), shoot ratio (Sr) and some metabolites in shoots such as organic acid (phosphoric acid, aspartic acid), amino acid (phenylalanine) and putrescine (Fig. S7B). For these variables, based on the mean comparison using a Tuckey-test, no significant difference was observed after zebularine treatment between Tm-a-ctrl and Am-a-zeb, while significant differences between Tm-a-ctrl and Am-a-ctrl remained ($P < 0.05$ for WC, $P < 0.0001$ for Sr).

Altogether, our results suggest that hypomethylation of the aquatic morphotype induces a 'terrestrial morphotype-like' situation and mimics the transition to the terrestrial morphotype.

Discussion

Low genetic and global DNA methylation differentiations between aquatic and terrestrial subpopulations of the invasive *L. grandiflora* subsp. *hexapetala* plant

L. grandiflora is known to reproduce essentially by clonal propagation suggesting a low genetic diversity for each population (Richards et al. 2004, Holm et al. 2017) even if it is not a rule (VanWallendael et al. 2018). However, low genetic variation populations, when it can be assessed, make relevant candidates for testing the role of epigenetic mechanisms in invasive plants (Zhang et al. 2013). We first examined if aquatic and terrestrial morphotypes shared a recent common, local ancestry or descent from different two preadapted subpopulations by estimating their genetic differentiation using 38 SNPs. We also analyzed the genetic distances between all individuals to assess if terrestrial morphotypes originated from a recent single line, or even single clone, with a newly acquired adaptive genetic capacity. Median F_{ST} values observed (0.019) suggested a low genetic differentiation between aquatic and terrestrial morphotypes, well below the threshold value of 0.05 (Hartl and Clark 1997, Frankham et al. 2010), with a recently shared common ancestrality. This alleviates the possibility that fast-terrestrial transition of *L. grandiflora* subsp. *hexapetala* occurred from a new genetically based adaptive capacity. The distribution of F_{ST} values and the topology of genetic graph (Fig. S1) that we observed are commonly expected to be found by chance in spatially structured subpopulations with recent common ancestrality (Wright 1984, Hofer et al. 2009, Bierne et al. 2013). However, we found two SNPs with unusually high F_{ST} values. They can be expected under isolation by distance with strong demographical bottleneck in such spreading invasive populations out of equilibrium (Varvio et al. 1986, Rousset 2013), especially considering clonal propagation. But we cannot refute that they may arise by selection with genes implied in adaptation. Further investigations involving spatial genetic structure and genomic approaches should help disentangling between those hypotheses. However, even if such fast-terrestrial transition would have emerged from a newly acquired genetically based adaptive capacity hidden in a dominant pattern of isolation-by-distance, it would not exclude an

additive, complementary or even dominant epigenetic effect on their phenotypes (Lele et al. 2018, Banerjee et al. 2019).

We thus pushed forward to analyze if those two morphotypes occurring in one genetically homogeneous population may be caused by epigenetic and transcription variations, as commonly observed in invasive plants (Zhang et al. 2013). In plants, DNA methylation is involved in transposon silencing and gene expression, which can change the phenotype in response to environmental variations (Lande 2009, Meyer 2015, Kawakatsu et al. 2016, Bewick and Schmitz 2017, Seymour and Becker 2017). Although epigenomic methods such as whole genome bisulfite sequencing (WGBS) provide detailed information at the genomic scale, they require a complete reference genome that is still lacking in *L. grandiflora* subsp. *hexapetala*. In addition, this plant has been reported as being a decaploid genome leading to a complex genetic analysis (Dandelot et al. 2005). We measured the global DNA methylation (%mC) using the most approved HPLC technique (Zhu et al. 2013, Kurdyukov and Bullock 2016). Although this approach does not provide DNA methylation characterization at the detailed level of genes and genomic regions, it is a widely used and proven epigenetic indicator in plants accessible from the cell to the population level (Lambé et al. 1997, Causevic et al. 2006, Gourcilleau et al. 2010, Raj et al. 2011, Trap-Gentil et al. 2011, Latzel et al. 2013, Alonso et al. 2015, Garg et al. 2015, Alonso et al. 2016, Le Gac et al. 2018, Sow et al. 2018, Gourcilleau et al. 2019, Le Gac et al. 2019). Here, we found that global methylation levels in *L. grandiflora* subsp. *hexapetala* (estimated genome size around 700 Mb, personal communication D. Barloy) was about ~28% of mC which is five times higher than in *Arabidopsis thaliana* (5%; 135 Mb) and twice as high as in *Antirrhinum majus* (15%; 400-500 Mb), which is consistent with the relation among global DNA methylation (%mC) and the genome size reported in angiosperms (Alonso et al. 2015). However, in our study, we could not detect any significant variation in %mC between morphotypes and conditions, as reported recently in isogenic lines of *Antirrhinum majus* in different shade avoidance conditions (Gourcilleau et al. 2019). Thus, given the absence of differences in global DNA methylation between morphotypes and conditions, we hypothesize that the phenotypic differences between morphotypes reflect differences in DNA methylation at specific loci. The gold standard epigenomics method is Whole-Genome Bisulfite Sequencing (WGBS), which can provide detailed information on the status of cytosines over the entire genome (Yong et al. 2016) but requires a complete reference genome and represent a financial cost. Further investigations into methylation polymorphism in our system will have actually to use approaches that do not require a reference genome e.g., as methods as epigenome bisulfite sequencing (epiGBS), bisulfite converted restriction site associated DNA sequencing (bsRADseq), Epi RADseq and methylated DNA immunoprecipitation (meDIP) (Trucchi et al. 2016, van Gurp et al. 2016). However, in this instance our objective was to perform an initial epigenetic characterization with a widely used epigenetic indicator (%mC), and to assess the effect of zebularine treatment.

Zebularine induced hypomethylation on the *L. grandiflora* subsp. *hexapetala* morphotypes in terrestrial and aquatic conditions

There are several non-exclusive methods for the study of DNA methylation (Kurdyukov and Bullock 2016, Yong et al. 2016) notably in model organisms such as *A. thaliana*, for which a large number of genetic resources are available. This includes notably manufacturing mutants for enzymes that maintain DNA methylation or disrupting DNA via RNAi. However, pharmacological investigations can also be conducted by disrupting de novo methylation with a hypomethylating agent, such as zebularine and 5-azacytidine. This strategy is cost effective and, importantly, does not require genomic resources, and has been successfully implemented not only in model plants such as *Arabidopsis thaliana* (Baubec et al. 2009, Amoah et al. 2012) but also in many others (Burn et al. 1993, Brock and Davidson 1994, Finnegan et al. 1998, Fieldes et al. 2005, Causevic et al. 2006, Trap-Gentil et al. 2011). Yet, it has never been used in invasive plants. Hypomethylant chemical agents such as zebularine and 5-azacytidine are used in cancer research (Cheng et al. 2004). In addition, there is a renewed use of the hypomethylant chemical agent for the study of DNA methylation and its role in organism responses to the environment (Baubec et al. 2009, Bossdorf et al. 2010, Richards et al. 2010, Hudson et al. 2011, Zhong et al. 2013, Latzel et al. 2016, Osorio-Montalvo et al. 2018, Puy et al. 2018). Zebularine is an inhibitor of methyltransferase, which causes a hypomethylation of de novo tissues. Our study was carried out using concentrations and durations in accordance with the cited literature. We assessed the %mC using the HPLC method in de novo-formed shoot apexes, as this is where new tissues start their differentiated growth and development on contact with zebularine (Aranda et al. 2017). We found that the 14-day zebularine treatment induces a significant decrease in %mC in de novo-formed shoots for all situations (morphotypes × conditions), and ranging from 4 to

9% as it was previously reported in *Arabidopsis thaliana* (Baubec et al. 2009).

Altogether, zebularine is an effective way of reducing the percentage of methylation in plants' de novo-formed tissues. This allows us to generate hypomethylated *L. grandiflora* morphotypes in both conditions for the first time to assess the impact of DNA hypomethylation on the fast adaptation of invasive plants.

Induced-hypomethylation affects morphotypes and their plasticity proposing that DNA methylation plays a role in the fast transition from aquatic to terrestrial morphotypes

In this study, we first reported comparison among the two untreated morphotypes with a lower number of nodes and an elongated morphology, smaller number of leaves and stronger water content in the aquatic morphotype as compared with the terrestrial one. On the contrary, a greater number of leaves and mass of roots significantly characterized the terrestrial morphotype. In parallel, higher quantities of sugars in the terrestrial conditions and amino acids in the aquatic conditions were found. All these findings confirm the conclusions from Billet et al. (2018). Here, we carried out a new analysis with phytohormone quantification and found that phytohormones mainly varied between conditions (plasticity) rather than between morphotypes. Thus, ABA, JA and SA were present in greater amounts in terrestrial conditions in good agreement with their role in drought responses (Kohli et al. 2013). Then, hypomethylated morphotypes were compared showing that DNA hypomethylation and growth conditions (-a or -t) positively or negatively affect several morphological, biomass and water content traits, depending on morphotypes. Opposite effects could be observed between the two morphotypes in their original conditions. Hypomethylated morphotypes in both terrestrial and aquatic conditions were shown to exhibit significant variations compared to controls at developmental, hormonal and metabolic levels. Developmental effects induced by variations in DNA methylation have already been largely reported in plants: for example, Baubec et al. (2009) showed a reduction in growth after zebularine treatment in *Arabidopsis thaliana*. Alonso et al. (2017) observed a decrease in leaf numbers after treatment with 5-azacytidine, and Tatra et al. (2000) reported stem elongation in *Stellaria longipes* in response to shade by using 5-azacytidine. Our observations are in line with these studies, except that we found a positive effect of hypomethylation on development, such as an increase in leaf and internode numbers.

Furthermore, the measured traits can be associated with the fitness of the plant. Hussner (2009) has shown that any fragment of water primrose can regenerate an entire plant, thus contributing to the species survival. As a result, the number of buds, as well as the size of the plants are important variables in the development of *L. grandiflora* subsp. *hexapetala*. Biomass is recognized as an important criterion that can be linked to the fitness of the plant (Younginger et al. 2017). As *Ludwigia* from aquatic and terrestrial conditions exhibit different morphologies, these differences likely contribute to its survival and invasive success.

Another main aspect of our analysis was to combine developmental analysis with measurements of hormone and metabolite levels. Phytohormones contribute not only to plant development but also to stress responses of the individuals. In addition, there is growing evidence of the existence of a crosstalk between hormonal balance and epigenetic regulation (Yamamuro et al. 2016, Zhang et al. 2016, Campos-Rivero et al. 2017, Maury et al. 2019). Here, we showed that hormonal balances and their plasticity were affected by hypomethylation. In addition, we detected a significant negative correlation between %mC and SA amount in roots. This is in accordance with Zhang et al. (2016), who reported that the chromatin remodeler DDM1, involved in DNA methylation patterns, promotes hybrid vigor by regulating SA. In addition, connections between DNA methylation and hormone pathways in the shoot apex have recently been highlighted (Lafon-Placette et al. 2018, Le Gac et al. 2018, Maury et al. 2019). Few studies also proposed the existence of relationships between phytohormones, metabolites and epigenetic mechanisms (Latzel et al. 2012, Kooke et al. 2018, Schvartzman et al. 2018). Specifically, Kooke et al. (2018) found some correlations between several *Arabidopsis thaliana* EpiRILs (Epigenetic Recombinant Inbred Lines) and some secondary metabolites. Shen et al. (2016) summarized the interaction between metabolic/redox signaling and epigenetics, in terms of plant adaptation to environmental conditions, suggesting a 'bi-directional interplay' of epigenetics and metabolism. Here, we found several metabolites (amino acids, sugars and organic acids) affected by hypomethylation, and six of them could be correlated to the %mC levels. However, the complex interplay among epigenetics and metabolism needs more insight before being clearly interpreted.

Our fine characterization of the hypomethylation effects on the two invasive morphotypes in terrestrial and aquatic conditions at developmental, hormonal and metabolic levels allows us to carry out an integrative approach using PCA. As we hypothesized, DNA hypomethylation impacted aquatic and terrestrial morphotypes of *L. grandiflora* subsp. *hexapetala* differently in response to the two conditions. The main outcome of this

integrative analysis was that inducing hypomethylation on the aquatic morphotype gives a terrestrial morphotype-like phenotype. Altogether, our data showed that hypomethylation of the invasive aquatic morphotype mimics the terrestrial fast transition suggesting an implication of the epigenome in the appearance of this new terrestrial invasive morphotype. However, different scenarios can be discussed here. The implication of DNA methylation could be causal through the control of gene expression or transposable element activation as well as a downstream marker of changes resulting from genetic changes (Banerjee et al. 2019, Marin et al. 2019). In addition, our data showed a weak genetic population structuration, but we cannot exclude that the terrestrial morphotype has been selected from the aquatic population with some genetic diversity (for example transposable elements) and become successful in terrestrial conditions. It is also possible that interactions between genetics and epigenetics at specific loci could be involved. Indeed, plant phenotype can reflect past environments of parents or even grandparents due to transgenerational effects as proposed in the clonal plant *Trifolium repens* (Rendina González et al. 2018). There is potential for environment-induced, heritable DNA methylation to exist in addition to other parental effects (Rendina González et al. 2018)

A future challenge will be to disentangle genetic and epigenetic effects notably for invasive plants (Banerjee et al. 2019, Marin et al. 2019). One solution concerning clonal invasive plants such as *L. grandiflora* subsp. *hexapetala* could be to focus on a single clone vegetatively propagated for each morphotype and to develop an epi/-genomic strategy in parallel to the creation of genomic resources (genome and transcriptome). Banerjee et al. (2019) present an experimental framework for the characterization of the three-way relationship between environmental cue, phenotypic plasticity and epigenetic changes. They recommend also 'combining trait and molecular data and including comparative analysis of fitness functions between native and introduced ranges of a species and explore adaptive differentiation in invaders, while accounting for non-adaptive evolutionary changes'. It is also important to evaluate the combined roles of epigenetics and transposable elements on a genomic scale, which could be key sources of flexibility for invasive species during the colonization of new environment (Gao et al. 2010, Richards et al. 2012, Xie et al. 2015, Marin et al. 2019).

Author contributions

J.G., S.M. and D.B. designed and conducted the research. J.G., D.V. and D.B. performed zebularin treatment and measured morphological traits. A.D. and S.M. carried out DNA methylation analysis, S.C. quantified phytohormones, D.R. with J.G. realized metabolomic analysis and S.S. with L.P. performed SNP and genetic differentiation analyses. M.P.E. and J.G. done the statistical analyses. J.G., S.M. and D.B. analyzed the data, conceived the idea and the initial structure of the manuscript. All authors have read and approved the final manuscript.

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Data availability statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Morphological biomass and water content variables in aquatic and terrestrial morphotypes.

Table S2. Phenotypic plasticity (RDPI) of morphological traits in aquatic and terrestrial morphotypes.

Table S3. Phytohormones quantities in roots and shoots in aquatic and terrestrial morphotypes.

Table S4. Metabololites amounts in shoots and roots in aquatic and terrestrial morphotypes.

Fig. S1. Minimum spanning graph of the genetic relatedness.

Fig. S2. Morphological variables measured in aquatic and terrestrial morphotypes.

Fig. S3. Phytohormones measured in aquatic and terrestrial morphotypes.

Fig. S4. Metabolites measured in aquatic and terrestrial morphotypes (Am and Tm).

Fig. S5. Correlation matrix between morphological, phytohormone and metabolites variables and global DNA methylation percentages.

Fig. S6. Cluster dendrogram of morphological and phytohormones variables.

Fig. S7. Principal Component analysis (PCA) of global methylation percentages, morphological traits, phytohormones and metabolites in aquatic and terrestrial morphotypes.

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