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Summary

DMSP (dimethylsulfoniopropionate) is an ecologically important sulfur metabolite commonly produced by marine algae and by some higher plant lineages, including the polyploid salt marsh genus *Spartina* (Poaceae). The molecular mechanisms and genes involved in the DMSP biosynthesis pathways are still unknown. In this study, we performed comparative analyses of DMSP amounts and molecular phylogenetic analyses to decipher the origin of DMSP in *Spartina* that represents one of the major source of terrestrial DMSP in coastal marshes. DMSP content was explored in 14 *Spartina* species using ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy and Ultra Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS). Putative genes encoding the four enzymatic steps of the DMSP biosynthesis pathway in *Spartina* were examined and their evolutionary dynamics were studied. We found that the hexaploid lineage containing *S. alterniflora*, *S. foliosa* and *S. maritima* and their derived hybrids and allopolyploids are all able to produce DMSP, in contrast to species in the tetraploid clade. Thus, examination of DMSP synthesis in a phylogenetic context implicated a single origin of this physiological innovation, which occurred in the ancestor of the hexaploid *Spartina* lineage, 3-6 MYA. Candidate genes specific to the *Spartina* DMSP biosynthesis pathway were also retrieved from *Spartina* transcriptomes, and provide a framework for future investigations to decipher the molecular mechanisms involved in this plant phenotypic novelty that has major ecological impacts in saltmarsh ecosystems.

Key words: Cordgrass, Gene phylogeny, Genome duplication, Phenotypic novelty

1. Introduction

Elucidating the genomic determinants of physiological and phenotypic innovations is a major challenge of ecological and evolutionary genomics. Such innovations need to be explored in a historical context toward comparative and integrative approaches (Byers et al., 2016). The history of plant diversification is punctuated by recurrent whole genome duplication (polyploidy) events and subsequent diploidization and genome fractionation, which shaped modern plant genomes (Wendel et al., 2016). These processes undoubtedly have helped increase the physiological and adaptive potential of many lineages. Comparative approaches conducted in a well-understood phylogenetic context help inform the mechanisms involved in the evolution of new functions. Here, we aim to understand the evolution of an ecologically important function, namely DMSP synthesis in the polyploid genus *Spartina* Schreb.

DMSP (dimethylsulfoniopropionate) is one of the major sulfonium compounds in marine environments. It has long fascinated plant biologists and ecologists, thanks to its uneven distribution across specific eukaryotic lineages, and because of its ecological role in marine ecosystems (reviewed in Stefels, 2000). The DMSP released by producer organisms is a key molecule in marine food webs, where microorganisms use it as a main sulphur source (Yoch, 2002). DMSP catabolites, such as the volatile DMS (dimethylsulfide), also have an ecological impact, being involved in the global sulfur cycle, in acid precipitation and in climate regulation (Andreae and Raemdonck, 1983; Aneja, 1990; Barnard et al., 1982; Charlson et al., 1987; Malin et al., 1992; Nguyen et al., 1978; Nriagu et al., 1987). This gas is also implicated in cloud nuclei formation, increasing cloud reflectance and thus albedo (Charlson et al., 1987; Yoch, 2002).

DMSP is commonly produced in marine algae, including green and red macro algae, or coccolithophores, diatoms and dinoflagellate. It is also found in some corals (Raina et al., 2013), but occurs only rarely in higher plants (reviewed in Dickschat et al., 2015). In monocots, significant amounts of DMSP have only been reported in *Spartina* and in a few *Saccharum* L. species, including sugarcane (*S. officinarum* L., Paquet et al., 1994). In *Spartina*, DMSP was first discovered in *Spartina anglica* C.E. Hubb. (Larher et al., 1977), but later in *Spartina alterniflora* Loisel (Colmer et al., 1996; Dacey et al., 1987; Husband et al., 2012; Kiehn and Morris, 2010; McFarlin and Alber, 2013; Otte and Morris, 1994), *Spartina foliosa* Trin. (Otte and Morris, 1994) and *Spartina maritima* (Curtis) Fernald (Otte and Morris, 1994). The capacity to produce DMSP was also tested in *Spartina patens* (Aiton) Muhl. and *Spartina cynosuroides* (L.) Roth, but no DMSP was detected in these species (Dacey et al., 1987). In eudicots, DMSP has only been found in *Wollastonia biflora* (L.) Wild., Asteraceae (Storey et al., 1993). DSMP has mostly been studied in leaves, where it is believed to be synthesized in chloroplasts (Trossat et al., 1996).

While the pathways and genes involved in the DMSP catabolism are well characterized (reviewed in Curson et al., 2011), little is known about the genetics and evolutionary origin of DMSP biosynthesis. As implied by the phylogenetic distribution and rarity of DMSP production in flowering plants, synthesis must have evolved independently at least three times (Stefels, 2000). In a series of studies, Hanson and co-workers (Hanson et al., 1994; James et al., 1995; Kocsis et al., 1998) showed that DMSP synthesis proceeds by a distinct route in angiosperms compared to marine algae and dinoflagellates, and involves different intermediate steps in the Asteraceae versus the Poaceae. In *Wollastonia*, DMSP synthesis is performed via three enzymatic steps (resulting in two intermediate metabolites: S-methylmethionine (SMM) and DMSP-amine), whereas the *Spartina* pathway involves four enzymatic steps with three intermediate compounds (SMM, DMSP-amine and DMSP-aldehyde). Whereas the first and last enzymatic steps are performed in *Spartina* species that do not produce

DMSP (and are common to all angiosperms), the second and third steps are specific to DMSP-producing *Spartina* species (Kocsis et al., 1998). The first step, catalyzed by the MMT enzyme, corresponds to the conversion of methionine into SMM (Bourgis et al., 1999) and the fourth step, catalyzed by BADH enzyme, converts DMSP-aldehyde into DMSP (Trossat et al., 1997; Vojtěchová et al., 1997). The intermediate specific steps involve (i) the conversion of SMM to DMSP-amine by an S-methyl-Met decarboxylase (SDC) related to an ornithine decarboxylase, and (ii) the conversion of DMSP-amine to DMSP-aldehyde by an oxidase (DOX) related to a diamine oxidase (Kocsis et al., 1998; Kocsis and Hanson, 2000). The protein sequences of these enzymes, as well as their corresponding genes, are unknown.

Various DMSP functions have been proposed, but remain unproven. As most DMSP producing species live in marine environments or exhibit salt-tolerance, an osmoregulatory role was suggested early-on (Colmer et al., 1996; Dickson et al., 1980; Diggelen et al., 1986; Karsten et al., 1992; Kirst, 1996; Mulholland and Otte, 2002; Otte et al., 2004; Otte and Morris, 1994; Storey et al., 1993). DMSP may also have other functions, such as antioxidant (Husband et al., 2012; Husband and Kiene, 2007; McFarlin and Alber, 2013; Raina et al., 2013; Sunda et al., 2002) or cryoprotectant (Karsten et al., 1996) properties. Relationships between DMSP and the salicylic acid pathway (involved in pathogen resistance) were suggested in *Spartina* (Kiehn and Morris, 2010). Some authors also detected a negative effect of dieback on DMSP concentrations in leaves or stems (Kiehn and Morris, 2010; McFarlin and Alber, 2013). These studies indicate that DMSP function is not fully elucidated, and that it may vary among species.

Here we report the first exhaustive survey of DMSP production in the genus *Spartina*, analyzed in the light of phylogenetic history. *Spartina* (Poaceae, Chloridoideae) is a monophyletic, polyploid perennial grass genus embedded in the paraphyletic genus *Sporobolus* R.Br.(Peterson et al., 2010,

2014); this led Peterson et al., (2014) to propose (i) inclusion of *Spartina* in a large, monophyletic genus *Sporobolus*, and (ii) conservation of the name *Sporobolus* over the older generic names *Spartina*, *Calamovilfa*, *Crypsis*, and *Thellungia* In this study, we kept the former *Spartina* names (that were used in the relevant literature of DMSP), and we provide a synonym list referring to the new nomenclature in Table 1 . Several *Spartina* species play an important ecological role in the coastal saltmarsh sedimentary dynamics, where they are considered "ecosystem engineers" (Ainouche et al., 2009). *Spartina* includes two main lineages, one tetraploid (2n=4x=40), which mostly diversified in the New World (subsect. Ponceletia and subsect. Spartina, Peterson et al. 2014, table 1), and one hexaploid (2n=6x=60, 62 which are now included in subsect. Alterniflori (Peterson et al., 2014, table 1), containing two American and weakly divergent sister species, *S. alterniflora* and *S. foliosa,* and a third Euro-African species, *S. maritima* (Baumel, 2002, Peterson et al. 2014). No diploid *Spartina* species are known, which suggests that *Spartina* evolved from an already polyploid ancestor (as the most closely related species are also polyploid), about 12-20 MYA (Rousseau-Gueutin et al., 2015). Hybridization within and between these two lineages resulted in various ploidy levels (7x, 8x, 9x, 12x, Ainouche et al., 2012; Strong and Ayres, 2013). Of particular interest is the reticulate history involving hybridization between *S. alterniflora* (as maternal parent) and *S. maritima* (as paternal parent) in Western Europe during the 19th century, which resulted in the independent formation of two sterile hybrids: S*partina x neyrautii* Foucaud (in southwest France) and *Spartina x townsendii* H&J Groves (in southern England). Genome duplication in *S. x townsendii* resulted in a fertile new allododecaploid species (*S. anglica*), which rapidly expanded in range and has now invaded several continents (Ainouche et al., 2009). This system represents a classical model of recent allopolyploid speciation (Gray *et al.*, 1991), and is used in various studies to explore the short-term consequences of hybridization and genome duplication in a well-understood historical frame (Ainouche et al., 2012, 2004; Ainouche and Wendel, 2014).

Previous studies indicate that *Spartina* contains both DMSP-producing and non-producing species (reviewed in Otte et *al.*, 2004), but there remains a need for a more exhaustive survey. Here, we addressed the following questions: (i) What are the DMSP producing (DMSP+) and non-producing (DMSP-) species in *Spartina*? (ii) Does DMSP production have single or multiple origin? (iii) Is the ability to produce DMSP related to polyploidy *per se*? (iv) What are the effects of hybridization and polyploidy on DMSP amounts in the DMSP+ species? (v) What are the *Spartina* genes putatively involved in the DMSP biosynthesis pathway? We hypothesized that polyploid speciation would have played a central role in the DMSP production ability and concentration, and that this function is phylogenetically constrained.

To answer these questions, DMSP amounts were determined in species representing all *Spartina* lineages, in different tissues and conditions, using ^{1}H NMR spectroscopy and UPLC-MS, and the DMSP production ability was examined in the light of Spartina phylogeny. Effects of increased salinity in species that usually do not produce DMSP are explored for the first time. Genes involved in the DMSP biosynthesis pathway are retrieved from *Spartina* transcriptomes and gene phylogenies and comparative analyses with model grass genomes are performed.

2. Materials and Methods

2.1. Plant material and sampling

Fourteen *Spartina* taxa were used in this study (Table 2), encompassing the various ploidy levels (tetraploid, hexaploid, heptaploid, octoploid, nonaploid and dodecaploid) known in this group and verified using flow cytometry analyses (Ainouche M. unpublished). Specimens are conserved either as herbarium or dried leaf samples or maintained in the greenhouse at University of Rennes 1 (table 2). The nonaploid plant introduced in this study was described in Renny-Byfield et al. (2010) and is

believed to result from a backcross between *S. anglica* and its maternal parent *S. alterniflora*. Plant materials were collected in the field and maintained in a growth chamber under controlled freshwater conditions (14h light at 20°C/10h dark at 18°C with 80% of hygrometry). Plant tissues (leaves, roots or inflorescences, see below) were harvested and immediately frozen in liquid nitrogen and stored at -80°C. They were then lyophilized and ground.

2.2. DMSP quantification

For ^{1}H NMR spectroscopy analyses, 100 mg of each sample were extracted with 1.5 ml of 50% methanol, vortexed quickly, agitated gently for 20 minutes and centrifuged at 4 000 *g* for 5 minutes. These extraction steps were performed two more times after dissolving the obtained pellet in 1 ml of 50% methanol. The supernatants were collected after each extraction, gathered and dried with a rotary evaporator. For UPLC-MS, the same protocol was applied except that 50 mg of sample was used and the added volumes were adjusted accordingly. For this method, 1% formic acid was incorporated to the 50% methanol solution and the supernatant was not dried. Heptafluorobutyric acid (HFBA) at 50 mM was added to the supernatants.

DMSP production was first explored among the different *Spartina* species using ^{1}H NMR spectroscopy. It was tested in leaves, and when possible in roots or inflorescences. Extracts were dissolved in 800 µl of D_2O. A BRUKER Avance 400 spectrometer equipped with a 5 mm inverse TBI 1H/{BB}/31P probe was used. The spectra were performed with a 30° pulse using a 10 s delay at 25°C. Calculation of DMSP concentrations was based on integration of NMR peaks, and areas of peaks corresponding to this metabolite were compared to the trimethylsilyl propionate (TSP) peak of known concentration.

A UPLC-MS method, which is more sensitive than ^{1}H NMR (Pan and Raftery, 2007), was also performed on the extracts of all *Spartina* species grown in the growth chamber (Table 2). For each

species, at least two biological and technical replicates were performed. Because DMSP is commercially unavailable and to generate a standard, DMSP was synthesized using the protocol described by Zhang et al., (2007) for the synthesis of 3-(Sulfonium-1-yl)propanoic acid. Dimethylsulphide (Sigma Aldrich) (1.03 ml, 13.94 mmol) and acrylic acid (957 µl, 13.94 mmol) were dissolved in 7.4 ml of acetone at 0°C. HCl 10 M (2.9 ml) was added and the mixture was stirred at room temperature for 27 h. The excess HCl was removed by evaporation under reduced pressure and the residue was washed twice with 7 ml ethyl acetate. After decanting, DMSP was identified in the aqueous layer that was evaporated under vacuum at 40°C. The grey solid obtained was crystallized from diethyl ether/methanol (1:1; V/V) (Zhou et al., 2009). Filtration led to a white powder identified as DMSP (999 mg, 42% yield). Its purity was determined using ^{1}H NMR spectrometer (Avance 300 MHz; Bruker) and was found to be higher than 95%.

For UPLC separation, extracts were filtered through PTFE membranes (0,45 µm; 25 mm) before injection. The UPLC Acquity system (Waters) used for separation and quantification of DMSP contained a binary gradient pump where solvent A was 1 mM HFBA + 0,1% propionic acid and solvent B was acetonitrile + 0,1% propionic acid. This system was equipped with a C18 BEH 1,7 µm 2,1x100 mm (Waters) column. The separation started by an isocratic step from 0 to 1 min at 10% B, from 1 to 9 min linear gradient until 90% B, from 9 to 9.5 min isocratic 90% B, from 9.5 to 11 min linear gradient until 10% B, the last step was from 11 to 12 min isocratic 10% B. The flow rate was 0.7 ml min^{-1} during all the run time. 1 µl of sample solution was injected. The column temperature was kept at 45°C. Detection of compounds was performed using a TripleQuadrupole detector (Waters) with the ESI (ElectroSpray Ionisation) positive mode by MultiReactionMonitoring (MRM). MS parameters to detect DMSP compound were as follows: capillary voltage 4500 V, source temperature 150°C, desolvation temperature 450°C, desolvation gas flow rate 800L hour^{-1}. The extraction cone voltage was 26V; the collision energy voltage was 17 V. Data were collected using the QuanLynx software (Waters).

2.3. Salt treatment

Three *Spartina* species, namely *Spartina pectinata* Link cv. Aureomarginata), *Spartina versicolor* Fabre (Kouali, Algeria) and *Spartina anglica* (La Richardais, France) were subjected to a salt treatment. Modified Hoagland solutions (Table S1) with three different sodium chloride concentrations were prepared (0 g L^{-1}, 10 g L^{-1} and 35 g L^{-1}). For *S. pectinata* and *S. versicolor*, two biological replicates per condition were performed whereas triplicates were used for *S. anglica*. The plants were first washed to remove sediments, placed into plastic jars filled with sand and acclimated for four weeks in a climatic chamber. The pots were then submerged in the prepared NaCl solution for 15 min to saturate the substrate, as described in Otte and Morris (1994). This treatment was renewed every two days. Leaves were harvested after two weeks of treatment. The plant DMSP content was then analyzed using the UPLC-MS method.

2.4. Statistical analyses

Statistical analyses were conducted using the R software (R Development Core Team. 2012). To detect potential significant differences between species, a one-way ANOVA was performed on log transformed DMSP concentrations values. Data obtained from the salinity experiments were also analyzed using a one-way ANOVA. The α level for significance was $P \leq 0.05$ for all tests.

2.5. Evolutionary dynamics of genes involved in the DMSP pathway

The DMSP biosynthesis pathway in *Spartina* involves four enzymes (Fig. 1): (i) Methionine S-methyltransferase or MMT, (ii) SDC, (iii) DOX, and (iv) DMSP-aldehyde dehydrogenase corresponding to the betaine aldehyde dehydrogenase (BADH) (Trossat et al., 1997; Vojtěchová et al., 1997). This latter enzyme is a member of the ALDH (aldehyde dehydrogenase) superfamily. Genes encoding these enzymes were searched in Viridiplantae databases and compared to *Spartina* sequences.

To explore the evolutionary dynamics of the *mmt* and *aldh* genes, all *mmt* (one copy) and *aldh* (14 copies) sequences in *Arabidopsis* were retrieved from TAIR (www.arabidopsis.org). BLASTP (e-value threshold of 10^{-50}; (Altschul et al., 1997)) of *A. thaliana* copies for these genes were performed against 41 fully sequenced plant genomes available on Phytozome (www.phytozome.net) for *mmt* and on fewer species for *aldh,* considering the high copy number of this gene family: *Physcomitrella patens*, and various Angiosperm lineage representatives (Chase et al. 2016): all Poaceae species available in Phytozome (*Sorghum bicolor, Zea mays, Setaria italica, Panicum virgatum, Oryza sativa* and *Brachypodium distachyon*), one Asterid (*Mimulus guttatus*) and five Rosids, including two Eurosid I (*Medicago truncatula*, *Populus trichocarpa*), two Eurosid II (*Arabidopsis thaliana*, *Gossypium raimondii*) and *Vitis vinifera*. These genes were also searched in *Spartina* by performing a tBLASTn (e-value threshold of 10^{-6}; Altschul et al., 1997) of the *Arabidopsis mmt* and *aldh* gene sequences against the leaf and root transcriptomes of four hexaploid (*S. maritima*, *S. alterniflora, S. x townsendii* and *S. x neyrautii)* and one allododecaploid *Spartina* species (*S. anglica*) (Boutte et al., 2016; Ferreira et al., 2013). Amino-acid sequences of either the *mmt* or the *aldh* genes were aligned using Geneious (version 6.1.8; Biomatters). The accession numbers or *Spartina* contigs of the *mmt* and *aldh* sequences considered in the phylogenetical analyses are provided in Table S2 and S4, respectively. Moreover, the *Spartina mmt* and *aldh* amino acid sequences are given in Table S3 and S5, respectively. The BADH4 sequence (*Beta vulgaris*), which was shown to be involved in the conversion of DMSP-aldehyde into DMSP (Trossat et al., 1997), was also included in the *aldh* analysis. After manually adjusting the alignments, the best model of evolution was identified using Modeltest implemented in MEGA 5.2.1 (Tamura et al., 2011) for both data matrices: JTT + G and WAG + G for *mmt* and *aldh* genes respectively. Maximum Likelihood (ML) phylogenetic analyses (1000 bootstrap replicates) were performed using MEGA with *Physcomotrella patens* as outgroup. The nonsynonymous/synonymous rate ratio (dN/dS) was also studied for both genes using Yang (2007).

These ratios were calculated using either nucleotide sequences corresponding to the entire CDS, the functional domains, or the genic region used in phylogenetic analyses.

To explore whether the different ALDH copies have retained their function, we examined the amino acids corresponding to the catalytic site (presence of a cysteine in active enzymes: Farres et al., 1995; Kopečný et al., 2011) and those involved in substrate specificity in all sequences used in the phylogeny. We also examined the amino acids implicated in ALDH10 substrate specificity, allowing the specific binding of ω-aminoaldehydes, which correspond to an aspartic acid (D) and a serine (S) at the position 110 and 292 of the *Spinacia oleracea* sequence, respectively (Riveros-Rosas *et al*., 2013). We also looked at the amino-acid at position 441 in *Spinacia oleracea* that is believed to be determinant for substrate specificity of BADH: high substrate affinity for betaine aldehyde in presence of an alanine (A) or a cysteine (C), or low affinity in the presence of an isoleucine (I) (Diaz-Sanchez et al., 2012).

Putative *sdc* and *dox* genes were first searched in *O. sativa*, *Z. mays* and *S. bicolor* from Phytozome using keyword search and BLASTP (e-value threshold of 10^{-6}; Altschul *et al.*, 1997). Only genes presenting a decarboxylase or amine oxidase domain using Pfam (e-value threshold of 10^{-3}; (Finn et al., 2014) were retained. A tBLASTn (e-value threshold of 10^{-6}; Altschul *et al.*, 1997) of these remaining sequences was then performed against the five DMSP+ *Spartina* transcriptomes (Boutte et al., 2016). A few other putative *Spartina sdc* and *dox* sequences were additionally identified using functional annotations of *Spartina* contigs (Boutte et al., 2016). To determine if the same *sdc* and *dox* genes are present in the different *Spartina* transcriptomes, the homologous relationship between *O. sativa* and *Spartina sdc* and *dox* sequences was identified by BLASTn (≥70% of identity, ≥60pb of overlap) and represented graphically using Circos (Krzywinski et al., 2009).

3. Results

3.1. DMSP content analysis

The ability of the different *Spartina* species to produce DMSP (DMSP+) was first screened using ^{1}H NMR analyses. *S. maritima, S. alterniflora, S. foliosa* and their hybrid (*S. x neyrautii* and *S. x townsendii*) or allopolyploid derivatives (*S. anglica* and the 9x hybrid) were all found to produce DMSP (Fig. 2) in all tested tissues (leaves, roots and inflorescences). In all other *Spartina* species (*Spartina argentinensis* Parodi, *S. pectinata*, *S. gracilis* Trin., *S. bakeri*, *S. versicolor*, *S. arundinacea* (Thouars) Carmich.) no DMSP was detected, including the *S. pectinata* individuals presenting different ploidy levels (tetraploid, hexaploid or octoploid); these species are hereafter termed "DMSP-". The heptaploid species *Spartina densiflora* Brongn., which derives from the hexaploid *S. alterniflora* (DMSP+) and the tetraploid *S. arundinacea* (DMSP-), has inherited the ability to produce this compound.

The concentration of DMSP was quantified in several organs of *S. alterniflora* and *S. anglica* (using ^{1}H NMR), and was found to be higher in leaves than in roots or inflorescences. DMSP concentration in *S. alterniflora* was 19.53 µmol g-1 MS in leaves, compared to 2.27 and 3.31 µmol g-1 MS in roots and inflorescences, respectively. Similarly, *S. anglica* exhibits 11.92 µmol g-1 MS of DMSP in leaves compared to 3.89 and 6.90 µmol g-1 MS in roots and inflorescences.

DMSP amounts were further analyzed in *Spartina* leaves using the more sensitive UPLC-MS method. A DMSP standard solution was first characterized to ensure accurate identification in the UPLC-MS analyses. This compound eluted at 0.53 minutes. DMSP ion was observed on MS spectra at 135 m/z and its fragment at 73 m/z corresponding to one of its fragmentation ion, i.e. acrylic acid (Spielmeyer and Pohnert, 2010). The limit of quantification (LOQ) was 0.23 µg ml^{-1} for DMSP. Using this sensitive method, we observed that the *Spartina* species previously detected as DMSP- using ^{1}H NMR were confirmed as DMSP- (Table 2 and Table S6, Supporting Information).

DMSP concentrations in *Spartina* DMSP+ (Table S6, Supporting Information) were compared by performing a one-way ANOVA on log transformed data (F=4.729; p=0.008). This analysis indicates that *S. alterniflora* contains significantly more DMSP in the leaves than do *S. anglica* (p=0.004) and *S. x townsendii* (p=0.030) (Fig. 3). In addition, we noted the following differences, but without statistical significance: (i) the tested *S. alterniflora* individuals tend to produce more DMSP than *S. maritima*; (ii) *S. x neyrautii* genotypes display intermediate DMSP production compared to its parents *S. alterniflora* and *S. maritima*; (iii) *S. x townsendii* contains a DMSP concentration closer to its paternal parent *S. maritima*; (iv) *S. x neyrautii* tends to produce more DMSP than the other F1 hybrid, *S. x townsendii*; (v) the allododecaploid *S. anglica* presents a similar concentration to *S. x townsendii* despite its genome doubling; and (vi) *S. foliosa* tends to produce less DMSP than its closely related species, *S. alterniflora*..

DMSP concentration was also determined in *S. pectinata* and *S. versicolor* (DMSP-) and in *S. anglica* (DMSP+, as positive control) grown for two weeks in the presence of various NaCl concentrations. Salt treatment had no significant effect on DMSP concentrations in *S. anglica* (ANOVA; F=0.452; p=0.656). DMSP was not detected in any *S. versicolor* and *S. pectinata* individuals, irrespective of NaCl concentration.

3.2. Evolutionary dynamics of genes involved in DMSP biosynthesis

3.2.1. Gene encoding MMT

The *mmt* sequences were retrieved from fully sequenced Viridiplantae genomes (phytozome) as well as from *Spartina* transcriptomes (partial transcripts), corresponding to 44 sequences obtained from 36 species (Table S2, Supporting Information). The resulting data matrix of 411 amino acids, corresponding to exons 6 to 11 of the *O. sativa mmt* gene (Fig. 4), was subjected to Maximum Parsimony (MP) and Maximum Likelihood (ML) phylogenetic analyses. The tree topology (similar in

both analyses) is in agreement with the expected angiosperm phylogeny, with the monocot (represented by Poales) and the eudicot species present in two separate clades (Fig. 5). The *mmt* gene is present in single copy in most Embryophytes, but two *mmt* gene sequences were found in eight species (Fig. 5). In all cases, the two copies present in one species are grouped together. *mmt* contigs were detected in three of the five considered *Spartina* transcriptomes: one in *S. alterniflora*, one in *S. maritima*, and three in *S. x townsendii*. For phylogenetic analyses, only the *Spartina x townsendii mmt* contig that is longer than 400 amino-acids was considered. Pairwise synonymous and non-synonymous substitution rates for the *mmt* gene indicate purifying selection when considering either the region used for phylogeny (mean dN/dS ratios = 0.088) (Table 3), the whole CDS (mean dN/dS ratios = 0.096) or each functional domain (mean dN/dS ratios of 0.177 and 0.043 for methyltransferase and aminotransferase domain respectively).

3.2.2. Genes encoding ALDH

The *badh* gene (4th step of *Spartina* DMSP biosynthesis pathway) belongs to the aldehyde dehydrogenase (*aldh*) family. Within this family, 14 (in *Arabidopsis thaliana*) to 26 copies (in *Panicum virgatum*) were identified per species. In total, 272 sequences were retrieved (Table S4, Supporting Information) and aligned, yielding a data matrix of 403 amino acids for ML analysis. Sequences were grouped into 11 different major clades (denoted as “copy I” to “copy XI”) corresponding to *aldh* paralogous genes (Fig. 6). All species have a copy in each clade, except a few that have lost one or two copies (e.g. no *O. sativa* copy in clade IV). Within clades, additional *aldh* gene duplications were observed in some lineages. For example, additional gene duplications occurred in Monocots within clades II, III, IX, X and XI (Fig. 6). Within *Spartina*, *aldh* transcript sequences were observed in 10 of the 11 major clades (except clades III and X). The BADH4 sequence, which is involved in the conversion of DMSP-aldehyde into DMSP, belongs to clade IX (named ALDH10 in (Kirch et al., 2004) and includes *Spartina* species (Fig. 6).

The structure of all *aldh* genes was compared using *O. sativa* as a reference. Coding sequences range in size from 1272 to 1794 bp, and the number of exons varies from 8 to 20 (Fig. 4). Within a clade, a high conservation of exon synteny is observed even when a different number of exons is observed, presumably from intron loss (Fig. 4). Similarly, the size of intronic sequences may vary among *aldh* sequences (1101 to 7156 bp), even within clades (*i.e.* clade X). This variation is not explained by the presence of repeated elements (Fig. 4). dN/dS ratios were estimated for *aldh* sequences (genic region used in the phylogeny) and indicate purifying selection for each gene copy (dN/dS ranging from 0.001 to 0.758, Table 3) or for all *O. sativa* paralogous copies (dN/dS= 0.23; Table 3). Similar results were observed when considering the entire CDS or the functional domain. To explore whether the different ALDH copies have retained their function, we examined the presence of a catalytic site as well as other amino acids implicated in the substrate specificity of some ALDH enzymes. Among the 293 ALDH sequences studied here, 289 sequences presented an active catalytic site (presence of a cysteine at that position). The four sequences lacking a cysteine at this site were found in species having another copy in which the cysteine was present (i.e. *P. virgatum* in clade IX). We also examined the amino acids implicated in ALDH10 substrate specificity (aspartic acid and a serine at the position 110 and 292 of the *Spinacia oleracea* sequence). We observed that all but two sequences retain these amino acids, the exceptions being species presenting another functional copy. In the ten other clades, we found different amino acids at those positions, suggesting different substrate specificity between ALDH10 and the other ALDH enzymes. Within clade IX (BADH gene), we looked at another amino-acid involved in substrate affinity (high affinity in presence of an alanine or a cysteine at position 441 in *Spinacia oleracea*, or low affinity in presence of an isoleucine). We observed the presence of at least one copy per species with a cysteine, alanine or isoleucine. DMSP+ *Spartina* species have both types of amino-acid implicated in the differential affinity for betaine aldehyde, and thus their BADH enzyme is likely to bind other ω-aminoaldehydes, as DMSP-aldehyde.

3.2.3. Identification of putative sdc and dox genes

The two steps of the *Spartina* DMSP biosynthesis pathway that are specific to DMSP+ species involve a S-methyl-Met decarboxylase (SDC) and a DMSP-amine oxidase (DOX) (Kocsis and Hanson, 2000). Genes encoding proteins with a decarboxylase or amine oxidase activity were retrieved in some Poaceae species: a total of 96, 77 and 117 putative decarboxylase sequences were obtained for *O. sativa*, *S. bicolor* and *Z. mays,* respectively. Similarly, 34, 35 and 65 putative *dox* sequences were found. These putative *sdc* and *dox* sequences (hereafter named *sdc* and *dox* for simplicity) were identified in *Spartina* using tBLASTn against the five DMSP+ *Spartina* transcriptomes (Boutte *et al*., 2016; Ferreira *et al*., 2013). A total of 193 *sdc* contigs for *S. alterniflora*, 200 for *S. maritima*, 170 for *S. x townsendii*, 152 for *S. x neyrautii* and 163 for *S. anglica* were found (Table S7, Supporting Information). Concerning the putative *dox* contigs, 52 sequences were obtained for *S. alterniflora*, 65 for *S. maritima*, 54 for *S. x townsendii*, 55 for *S. x neyrautii* and 53 for *S. anglica* (Table S8, Supporting Information). This contig number is higher than the number of corresponding genes as several contigs in a species may belong to a single gene. Within species, the presence of several *Spartina* contigs belonging to the same *O. sativa* homolog may be visualized in Fig. 7.

Of the 96 *sdc O. sativa* genes, 30 to 41 orthologous genes were identified in *Spartina* species. Similarly, of the 34 *dox O. sativa* genes, 15 to 21 *dox* orthologous *Spartina* genes were found (Fig. 7). When considering all *Spartina* transcriptomes, a higher number of different *sdc* (52) and *dox* (22) genes were obtained (Fig. S1, Supporting Information). Consideration of the *Spartina* transcriptomes enabled us to identify homologous contigs among *Spartina* species and to obtain homologous sequences in *Spartina* from almost all *O. sativa* exons (Fig. 7).

4. Discussion

4.1. Monophyletic origin of DMSP production in Spartina

Our results show that the ability to produce DMSP appeared once in *Spartina*, in the allohexaploid ancestor of *S. maritima*, S*. alterniflora* and *S. foliosa* (hereafter called the "hexaploid clade"), about 2 to 10 million years ago (Rousseau-Gueutin et al., 2015). All taxa derived from these hexaploids have inherited this ability, including the hexaploid hybrids and the allododecaploid *S. anglica*. Interestingly, the alloheptaploid *S. densiflora,* derived from a DMSP+ and a DMSP- species (Fortune et al., 2008) also inherited this ability. Although the metabolic pathway leading to the DMSP synthesis has been elucidated in some plants (Kocsis et al., 1998), little is known about mechanisms involved in DMSP production. Our results indicate that a genetically determined (*i.e.* heritable) mechanism (yet to be elucidated) is involved in DMSP synthesis. The presence of DMSP is not related to ploidy level increase *per se*, as our analyses reveal that DMSP is not produced in autohexaploid and octoploid cytotypes derived from the tetraploid *S. pectinata* (Kim et al., 2010). The tetraploid *S. argentinensis* that represents a distinct lineage in the genus, being placed either as sister of all other *Spartina* species or as sister of the hexaploid clade (Baumel et al. 2002, Fortune et al. 2007, 2008, Peterson et al. 2014) does not produce DMSP. The auto or allopolyploid nature of the hexaploid DMSP+ ancestor is not fully established. Discerning allopolyploidy from autopolyploidy is not an easy task in relatively old polyploids (especially when diploids are extinct), but the presence of divergent homoeologous gene copies in the hexaploid DMSP+ *S. maritima* and *S. alterniflora* (Boutte et al., 2015; Fortune et al., 2007) suggest a reticulate origin of this clade. This history raises the question as to whether gene duplication, combined with merger of divergent genomes, led to the emergence of this new function. It is difficult to know whether this function arose immediately following the formation of the hexaploid DMSP+ ancestor, or occurred following subsequent evolution of this ancestral lineage. Several examples of phenotypic and physiological novelties entailed by the short term and/or the long-term evolution of hybrid and polyploid genomes have been identified, such as

the free threshing character (Q/q locus) and grain hardness (Ha locus) in polyploid wheat (Chantret, 2005; Zhang et al., 2011). In grasses, DMSP amounts have also been detected in some *Saccharum* species, including the domesticated polyploid sugarcane *S. officinarum* and its wild ancestor *S. robustum* (Paquet et al., 1994). Much lower quantities were reported in *S. spontaneum*, although amounts may vary among different varieties. DMSP+ *Spartina* and *Saccharum* species, which occupy coastal marsh and cultivated areas respectively, appear to be the main terrestrial source of atmospheric sulfur produced via DMSP degradation (Dacey et al., 1987). It would be of interest to perform comparative analyses to determine whether the ability to synthesize DMSP, which evolved independently in polyploid *Spartina* and *Saccharum*, involved similar genetic and/or regulatory mechanisms. Sugarcane and related species have a complex genetic history, involving various ploidy levels, aneuploidy and hybridization (Grivet et al., 2006, 2004), and an exhaustive screening of DMSP amounts in the various cytotypes is lacking.

4.2. Reticulation, polyploidy, and variation in DMSP amounts

DMSP is unevenly distributed among organs of the DMSP+ *Spartina* species. We found that DMSP content is higher in leaves than in roots or inflorescences. This result agrees with previous studies (Dacey et al., 1987; Mulholland and Otte, 2002, 2000; Otte and Morris, 1994). Trossat *et al.* (1996) have explored the subcellular localization of the different DMSP biosynthesis steps in *Wollastonia biflora*, and found that the first step (conversion of methionine into SMM by the MMT enzyme) is cytosolic, whereas the other steps leading to DMSP are performed in the chloroplasts, which may explain the higher DMSP content in leaves than in roots or inflorescences.

As mentioned in the introduction, an osmoprotectant or osmoregulatory role for DMSP has been hypothesized, and this role is still under debate for DMSP producing plants (Otte et al., 2004). Within *Spartina*, it appears that all DMSP+ species naturally grow in low marsh zones and tolerate several

hours of immersion in sea water at high tides, whereas DMSP- species preferentially occupy high marsh or inland zones (Mobberley, 1956). Salinity was previously found to have no effect on *S. alterniflora* and *S. anglica* (Colmer et al., 1996; Diggelen et al., 1986; Otte and Morris, 1994), and we obtained similar results in this study for *S. anglica*. Our analyses of DMSP- species (*S. versicolor* and *S. pectinata*) also reveal that the presence of NaCl does not induce DMSP production.

We also explored the effect of recent hybridization and polyploidy on DMSP. When comparing the F1 hybrids *S. x townsendii* and *S. x neyrautii* to their parents (i.e. *S. alterniflora* and *S. maritima*), we found that *S. x neyrautii* exhibits an intermediate level of DMSP production compared to its parents (parental additivity), whereas the other F1 hybrid *S. x townsendii* has a DMSP production similar to *S. maritima* (paternal dominance). Thus, independent hybridization events involving the same maternal and paternal species (Baumel et al., 2003) appear to have different effects on DMSP amounts. These different effects of hybridization are also conspicuous at the morphological and gene expression levels in *S. x townsendii* and *S. x neyrautii* (Baumel et al., 2003; Foucaud, 1897; Hubbard et al., 1978): comparative global transcriptomic studies found both additive and non-additive parental gene expression in these hybrids, with maternal (similar to *S. alterniflora*) expression dominance most frequently encountered (and more pronounced in *S. x townsendii*), and a series of differentially expressed genes between these two hybrids (Chelaifa et al., 2010; Chelaifa *et al.*, unpublished).

The effects of recent genome duplication on DMSP amounts were explored by comparing the hexaploid *S. x townsendii* to its dodecaploid derivative *S. anglica*: no effect on DMSP amounts was observed, which suggests dosage compensation on the genetic system involved in this case. Moreover, we found that the hexaploid *S. alterniflora* produces significantly more DMSP than the hexaploid *S. x townsendii* and the dodecaploid *S. anglica*. S. *alterniflora* also produces more DMSP than its closely related sister hexaploid species *S. foliosa*. The nonaploid *Spartina* hybrid (deriving

from *S. alternifora* and *S. anglica*) and the heptaploid *S. densiflora* seem to produce less DMSP than *S. alterniflora*. Thus, DMSP concentration seems not to be affected by increasing ploidy level *per se*.

4.3. Evolutionary dynamics of genes involved in the DMSP pathway

We explored genes encoding the enzymes involved in the first (MMT) and last (BADH) steps of DMSP synthesis. We found that the MMT enzyme is present in single copy in most Embryophytes, but that in a few species two *mmt* sequences were observed, likely arising from relatively recent polyploidization events. For example, *P. virgatum*, which exhibits two *mmt* copies, underwent a whole genome duplication 0.5 to 1 MYA (Garsmeur et al., 2014). This gene most likely plays an important function: indeed, the product of the *mmt* enzyme (namely the SMM compound) is implicated in sulfur transport in plants and may be involved in the grain protein synthesis (Bourgis et al., 1999). It is then not surprising that this low copy number gene is under high selective constraint.

In contrast to *mmt*, the *badh* gene belongs to a complex *aldh* multigene family with 14 to 26 copies in the angiosperm species considered here. The proteins encoded by these different copies seem to be active, with intact open reading frames, conserved putative catalytic sites, and with evidence of purifying selection. Similar examples of duplicated genes retention through numerous polyploidization events have already been observed for genes involved in protein complexes, including genes encoding ribosomes, proteasomes (Freeling, 2006) or genes involved in the photosystem II protein complex (Coate et al., 2011). Within the *aldh* gene family, the retention of the different copies may be related to different substrate specificities or tissue specificities. Different *aldh* genes are involved in multiple functions related to the oxidation of amino aldehydes. Kirch *et al.* (2004) noted that different ALDH genes are involved in diverse roles, such as male sterility, plant defense and abiotic stress tolerance. Moreover, some aminoaldehyde dehydrogenases were shown

to oxidize various substrates but with different catalytic efficiencies (Kopecny et al., 2013; Riveros-Rosas et al., 2013).

The ALDH10 clade, containing the BADH enzyme, is the only ALDH family present in the 20 studied species that is thought to convert ω-aminoaldehydes. This is bioinformatically confirmed here by the analysis of the two amino acids conferring ω-aminoaldehyde specificity (D110, S292), which are only present in sequences of this clade. Different copies of ALDH10 may have been maintained partly because BADH is able to bind different substrates, such as betaine aldehyde, DMSP aldehyde or other ω-aminoaldehydes, with different efficiencies (Kopecny et al., 2013; Trossat et al., 1997). It has been shown that a single amino acid is responsible for the substrate specificity of the BADH enzyme (Diaz-Sanchez et al., 2012). Interestingly, *Sorghum bicolor,* known to accumulate glycine betaine in response to stress, exhibits a cysteine whereas an isoleucine is present in *Arabidopsis thaliana* and *Oryza sativa*, which do not accumulate glycine betaine (Rhodes and Hanson, 1993). In *Spartina*, our results suggest that DMSP+ species may bind different ω-aminoaldehydes substrates, including DMSP-aldehyde.

In addition to the analysis of known genes involved in the non-specific steps of DMSP production, we performed exploratory analyses of sequences annotated as decarboxylase or amine oxidase to target genes encoding the SDC and DOX enzymes, which were found specific to DMSP production in *Spartina* (Kocsis and Hanson, 2000). Candidate genes were identified in the transcriptomes of five DMSP+ species, opening new perspectives on the possibility of performing comparative transcriptomic analyses between DMSP- and DMSP+ species.

In summary, the present study provides a clear view of the phylogenetic context and the genetic transmission of DMSP production in polyploid *Spartina* species, and provides a framework for future

investigations to decipher the molecular mechanisms involved in this plant phenotypic novelty that has major ecological impacts in saltmarsh ecosystems.

Acknowledgements

We thank the Chemical Sciences Institute of Rennes (UMR CNRS 6226) for DMSP synthesis, as well as the Metabolic and Metabolomic Profiling (P2M2, INRA Le Rheu, France) and the NMR-EPR-MS Technology (Brest, France) platforms for DMSP detection and quantification. J. Keller is thanked for his help during the salt treatment experiment, F. Nassur and T. Fontaine for the maintenance of the *Spartina* plant collection in the greenhouse. We thank D.K. Lee, S. Kim, D. Strong, S. Rinehart, E. Chow, R. Klinger and T. Columbus, who kindly helped us obtain American *Spartina* samples.

Fundings

We thank the ‘‘Région Bretagne’’, the Partner University Fund and the European Union Seventh Framework Program [FP7-CIG-2013 – 2017; grant no. 333709 to M.R.-G.], the CNRS and University of Rennes 1 (LIA ECOGEN) for financial support.

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Table 1. *Spartina* revised nomenclature

New nomenclature: *Sporobolus* sect. *Spartina* (Schreb.) P.M. Peterson & Saarela, **com. & stat. nov.**	**Former nomenclature**
Sporobolus subsect. ***Alterniflori*** P.M. Peterson & Saarela, **subsect. nov.**	
Sporobolus alterniflorus (Loisel.) P.M.Peterson & Saarela, **comb. nov.**	*Spartina alterniflora* Loisel.
Sporobolus anglicus (C.E.Hubb.) P.M.Peterson & Saarela, **comb. nov.**	*Spartina anglica* C.E.Hubb.
Sporobolus foliosus (Trin.) P.M.Peterson & Saarela, **comb. nov.**	*Spartina foliosa* Trin.
Sporobolus maritimus (Curtis) P.M.Peterson & Saarela, **comb. nov.**	*Spartina maritima* (Curtis) Fernald
Sporobolus ×townsendii (H.Groves & J.Groves) P.M.Peterson & Saarela, **comb. nov.**	*Spartina ×townsendii* H.Groves & J.Groves
Sporobolus subsect. ***Ponceletia*** (Thouars) P.M. Peterson & Saarela, **comb. & stat. nov.**	
Sporobolus mobberleyanus P.M.Peterson & Saarela, **nom. nov.**	*Spartina arundinacea* (Thouars) Carmich.
Sporobolus spartinus (Trin.) P.M.Peterson & Saarela, **comb. nov.**	*Spartina spartinae* (Trin.) Merr., syn. *Spartina argentinensis*
Sporobolus subsect. ***Spartina*** (Schreb.) P.M. Peterson & Saarela, **comb. & stat. nov.**	
Sporobolus bakeri (Merr.) P.M.Peterson & Saarela, **comb. nov.**	*Spartina bakeri* Merr.
Sporobolus densiflorus (Brongn.) P.M.Peterson & Saarela, **comb. nov.**	*Spartina densiflora* Brongn.
Sporobolus hookerianus P.M.Peterson & Saarela, **nom. nov.**	*Spartina gracilis* Trin.
Sporobolus michauxianus (Hitchc.) P.M.Peterson & Saarela, **comb. nov.**	*Spartina pectinata* Link
Sporobolus versicolor (Fabre) P.M.Peterson & Saarela, **comb. nov.**	*Spartina versicolor* Fabre

Table 2. Origin and growth conditions of the *Spartina* samples analyzed using either ^{1}H NMR spectroscopy or UPLC-MS: the ploidy level of the different samples as well as the organs harvested and the number of replicates performed per species (between brackets) are also indicated.

Species	Ploidy level (x=10)	Origin	Sample conditions	^{1}H NMR analysis	UPLC-MS analysis
S. alterniflora Loisel	6x	Finistère, France (Le Faou); Hampshire, England (Hythe)	growth chamber	leaves (3), roots (2), inflorescences (2)	leaves (3)
S. anglica C.E. Hubb.	12x	Ille-et-Vilaine, France (La Guimorais); Morbihan, France (Le Hezo); Côtes-d'Armor, France (Mordreuc Pleudihen-sur-Rance)	growth chamber	leaves (8), roots (1), inflorescences (2)	leaves (3)
S. argentinensis Parodi	4x	Argentina, Santa Fe	herbarium (Univ. Rennes 1)	leaves (1)	
S. arundinacea (Thouars)	4x	Amsterdam Island, France	herbarium (Univ. Rennes 1)	leaves (1)	
S. bakeri Merr.	4x	Florida, USA	growth chamber	leaves (3), roots (1)	leaves (3)
S. densiflora Brongn.	7x	Andalusia, Spain (Odiel)	growth chamber	leaves (2)	leaves (2)
S. foliosa Trin.	6x	California, USA (Dog Beach, Chula Vista)	growth chamber	leaves (5)	leaves (2)
S. gracilis Trin.	4x	California, USA (Bischop)	growth chamber		leaves (3)
S. maritima (Curtis) Fern.	6x	Morbihan, France (Le Hezo, Quenouille)	growth chamber	leaves (3)	leaves (3)
S. x neyrautii Foucaud	6x	Pyrénées-Atlantiques, France (Hendaye)	growth chamber	leaves (5)	leaves (3)
S. pectinata Link	4x	commercialized Aureomarginata cultivar	growth chamber	leaves (2)	leaves (2)
S. pectinata Link	6x	Illinois, USA	greenhouse	leaves (1)	
S. pectinata Link	8x	South Dakota, USA	greenhouse	leaves (1)	
S. x townsendii H&J Groves	6x	Hampshire, England (Hythe, Hayling Island)	growth chamber	leaves (5)	leaves (3)
S. versicolor Fabre	4x	Hérault, France (Frontignan-Aresquiers); Var, France (Vieux Salins); Algeria (Kouali)	growth chamber	leaves (3), roots (2)	leaves (2)
Hybrid 9x	9x	Hampshire, England (Marchwood)	growth chamber	leaves (2)	leaves (2)

Table 3. Comparison of non-synonymous (dN) and synonymous (dS) ratios obtained for *mmt* and *aldh* sequences. This ratio was studied for different sequence alignments, i.e. using the coding region used for phylogenies, using all CDS or the functional domains of *mmt* and *aldh* sequences. For *aldh*, these comparisons were performed within each major clade for the coding region used for phylogeny, whereas only the clade IX was analyzed on all CDS and functional domain. dN/dS ratio was calculated for the three types of regions for *mmt* and *O. sativa aldh* paralogs.

Sequences analyzed	Mean dN/dS (Min-Max)	Coding region analyzed
mmt	0.088 (0.016-0.718)	Same as phylogeny
mmt	0.096 (0.030-2.245)	All CDS
***mmt* methyltransferase domain**	0.177 (0.004-0.723)	Functional domain
***mmt* aminotransferase domain**	0.043 (0.009-0.228)	Functional domain
***aldh* clade I**	0.095 (0.031-0.758)	Same as phylogeny
***aldh* clade II**	0.095 (0.030-0.276)	Same as phylogeny
***aldh* clade III**	0.082 (0.002-0.500)	Same as phylogeny
***aldh* clade IV**	0.075 (0.028-0.657)	Same as phylogeny
***aldh* clade V**	0.034 (0.012-0.098)	Same as phylogeny
***aldh* clade VI**	0.072 (0.022-0.253)	Same as phylogeny
***aldh* clade VII**	0.099 (0.023-0.377)	Same as phylogeny
***aldh* clade VIII**	0.087 (0.023-0.279)	Same as phylogeny
***aldh* clade IX**	0.082 (0.020-0.465)	Same as phylogeny
***aldh* clade IX**	0.092 (0.024-0.217)	All CDS
aldehyde dehydrogenase domain (clade IX only)	0.122 (0.012-1.095)	Functional domain
***aldh* clade X**	0.045 (0.001-0.172)	Same as phylogeny
***aldh* clade XI**	0.065 (0.007-0.657)	Same as phylogeny
All *aldh* copies *(O. sativa* only)	0.230 (0.007-0.693)	Same as phylogeny
All *aldh* copies *(O. sativa* only)	0.295 (0.008-1.016)	All CDS
aldehyde dehydrogenase domain (*O. sativa* only)	0.414 (0.008-1.363)	Functional domain

Figure Legends

Fig. 1 DMSP biosynthesis pathway in *Spartina*, as proposed by Kocsis and Hanson (2000). Enzymes are indicated in italic. The first and the last steps are widely distributed among angiosperms whereas the intermediate steps (2 and 3) are specific to DMSP-producing species.

Fig. 2 Detection of DMSP in *Spartina* species. Phylogenetic relationships are redrawn from Ainouche *et al*. (2012). *Spartina* species that are able and unable to produce DMSP (using either ^{1}H NMR or UPLC-MS) are indicated in red and blue, respectively. The ploidy level of each tested species is indicated with brackets. Molecular dating of the tetraploid and hexaploid clades is obtained from Rousseau-Gueutin *et al*. (2015).

Fig. 3 Mean DMSP concentrations (in µmol g^{-1} MS) in *Spartina* leaves: dots with different associated letters correspond to values statistically different at the 5% level. Standard errors are indicated.

Fig. 4 Structure of *mmt* and *aldh* genes in *Oryza sativa*. Exons are represented by boxes. Colored boxes correspond to functional domains: blue to methyltransferase, orange to aminotransferase and pink to aldehyde dehydrogenase. Hatched boxes represent coding regions used for phylogenetic analyses. Within clades, corresponding exon regions from different *aldh* sequences are indicated with numbers. Transposable elements (DNA transposons and retrotransposons) and simple repeats are represented with diamonds.

Fig. 5 ML analysis of the *mmt* gene in Viridiplantae (JTT+G model; bootstrap 1000). *Physcomitrella patens* was used as outgroup. Bootstrap values (%) are noted above branches; the number of substitutions per site is indicated below branches. The *Spartina mmt* sequence is indicated in bold. Polyploidy events (as reported by Garsmeur *et al*., 2014) are represented by stars.

Fig. 6 ML analysis (50% consensus tree) of the *aldh* gene family in Viridiplantae (WAG+G model; bootstrap 1000). *Physcomitrella patens* (sequences indicated by black diamonds) was used as outgroup. Bootstrap values (%) are shown for nodes of the different BADH copies. ALDH copies (roman letters) and Monocots/Eudicots clades are indicated. Within a clade (*aldh* gene copy), duplications specific to Monocots are represented by capital letters. *aldh Spartina* sequences, retrieved from *Spartina* transcriptomic data, are represented by red dots. The position of the *Beta vulgaris* BADH4 sequence that was shown to perform the conversion of DMSP-aldehyde into DMSP (Trossat *et al*. 1997) is indicated (copy IX clade).

Fig. 7 Physical localization of the *O. sativa sdc* (96) and *dox* (34) genes and identification of their homologs in *Spartina* transcriptomic data. The first outer circle represents the twelve *O. sativa* chromosomes. *Oryza sativa* chromosomes are drawn to scale and their sizes are indicated between brackets. *sdc* and *dox* genes are represented by red and blue triangles, respectively: triangles facing the inside or outside of the circles denote sense or antisense strands in the *O. sativa* genome. The exon number of each *O. sativa* gene is indicated above each triangle outside the outer circle. On the five other inner circles are represented the *Spartina* contigs homologous to the *sdc* and *dox O. sativa* genes: the *O. sativa* exons present in each *Spartina* contig are noted above each triangle.

Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1. Hoagland solution used in the experiments

Table S2. Accession or *Spartina* contig numbers for the different Angiosperm *mmt* sequences used in this study.

Table S3. Sequences of the *Spartina mmt* contigs used in the phylogenetical analysis.

Table S4. Accession or *Spartina* contig numbers for the different Angiosperm *aldh* sequences used in this study.

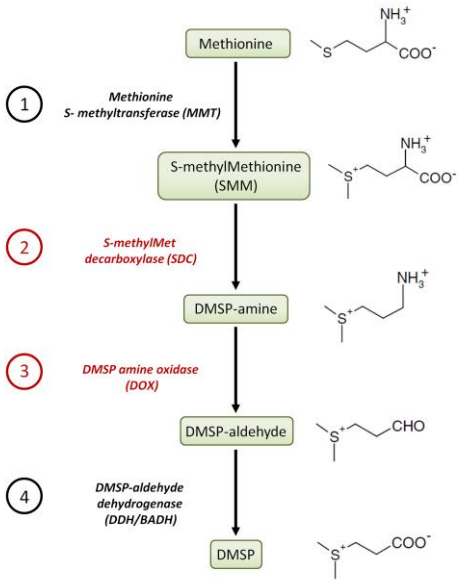
Table S5. Sequences of the *Spartina aldh* contigs used in the phylogenetical analysis.

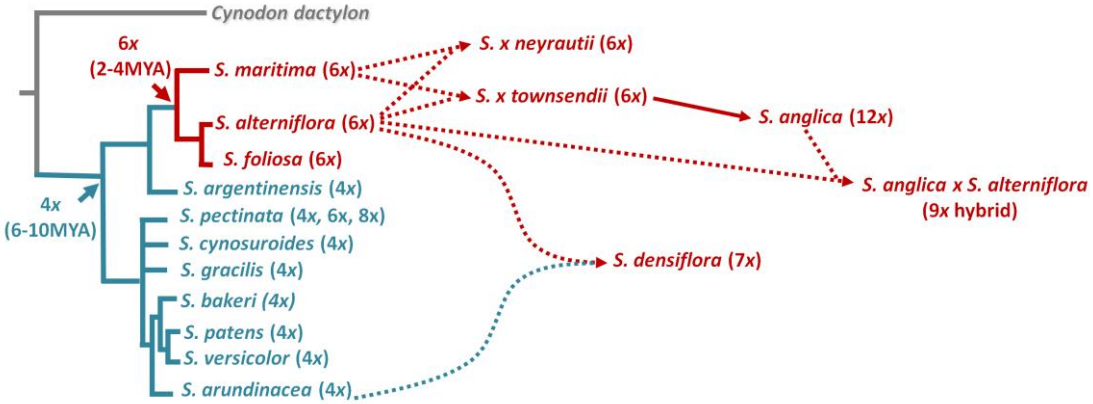
Table S6. DMSP concentrations obtained for each *Spartina* individual using UPLC-MS. The values presented for each individual result from mean values of two technical replicates.

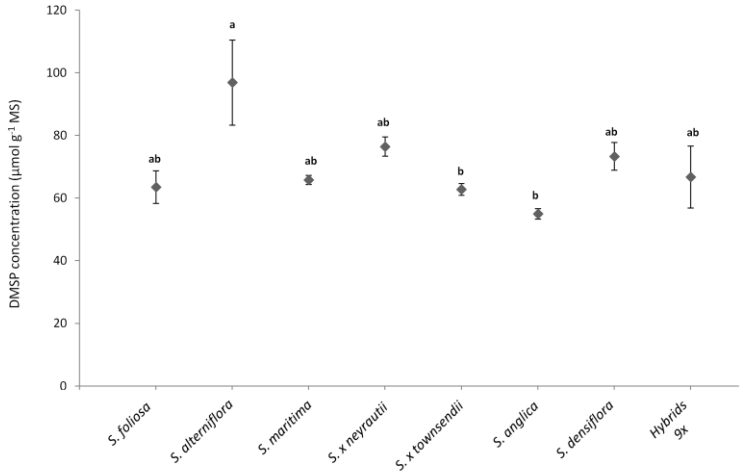
Table S7. Putative *sdc* contigs identified in *Spartina* transcriptomes.

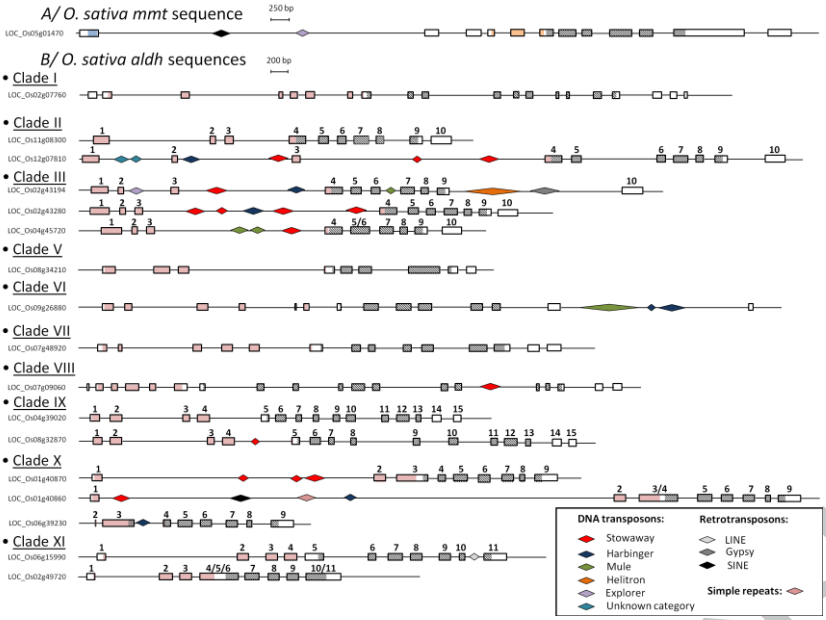
Table S8. Putative *dox* contigs identified in *Spartina* transcriptomes.

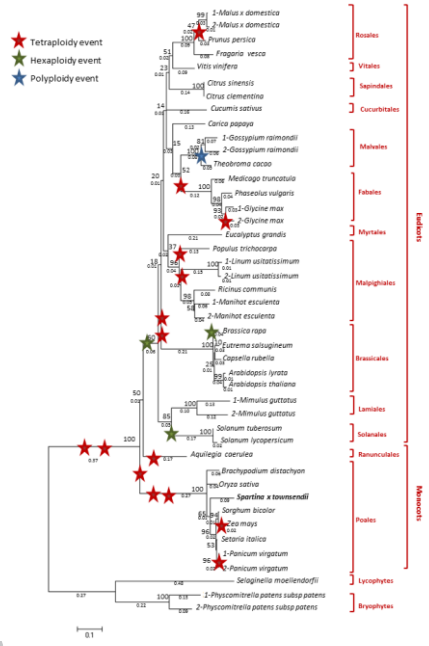
Fig. S1 Physical localization of the *O. sativa sdc* (96) and *dox* (34) genes in the *O. sativa* genome and identification of their homologs in *Spartina* transcriptomic data.

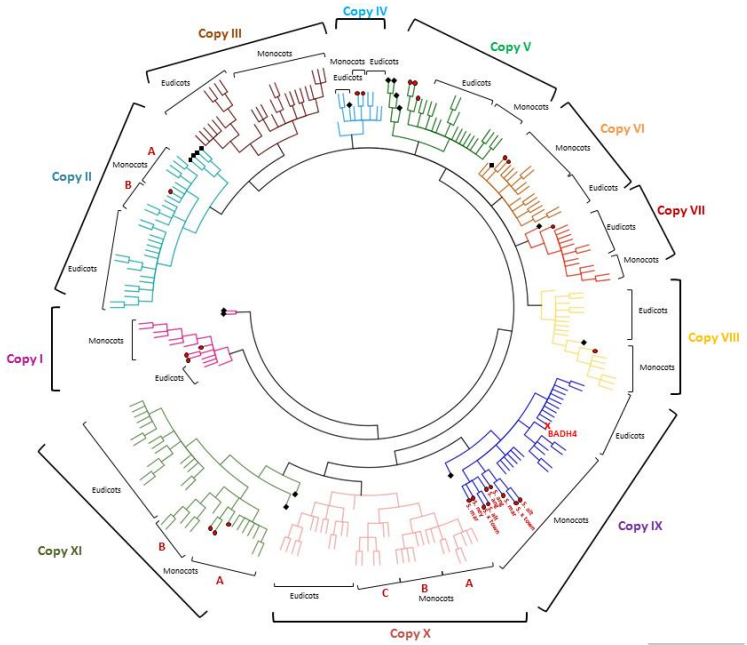


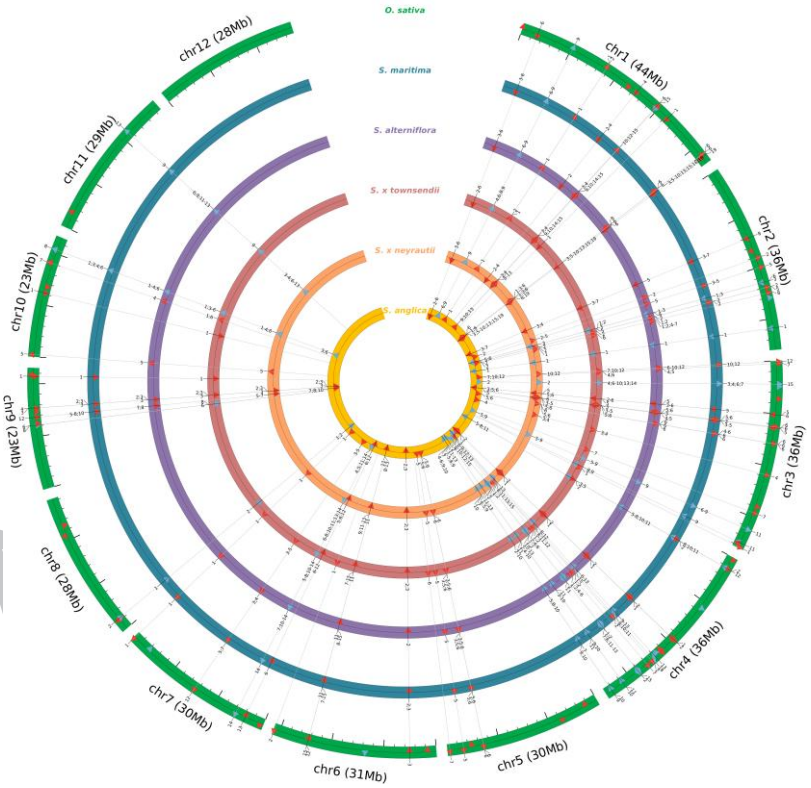








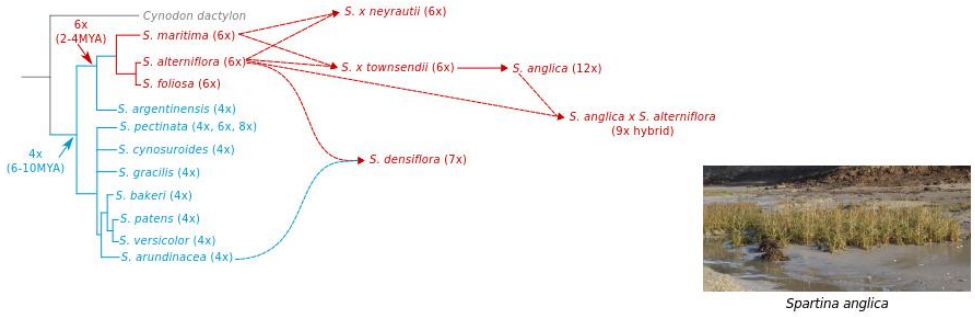




Highlights

- The ability to produce DMSP was explored in *Spartina* in the light of the species phylogeny and ploidy
- DMSP synthesis evolved once in a hexaploid lineage and was transmitted to derived species
- Candidate genes involved in the DMSP biosynthetic pathway were identified in *Spartina*
- Comparative analyses of the genes involved in the DMSP biosynthesis pathway
- Hypotheses regarding DMSP synthesis and role in plants are discussed

Graphical abstract



Spartina anglica