

Genetic association of toxin production in the dinoflagellate *Alexandrium minutum*

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Abstract

Dinoflagellates of the genus *Alexandrium* are responsible for harmful algal blooms and produce paralytic shellfish toxins (PSTs). Their very large and complex genomes make it challenging to identify the genes responsible for toxin synthesis. A family-based genomic association study was developed to determine the inheritance of toxin production in *Alexandrium minutum* and identify genomic regions linked to this production. We show that the ability to produce toxins is inheritable in a Mendelian way, while the heritability of the toxin profile is more complex. We developed the first dinoflagellate genetic linkage map. Using this map, several major results were obtained: 1. A genomic region related to the ability to produce toxins was identified. 2. This region does not contain any polymorphic *sxt* genes, known to be involved in toxin production in cyanobacteria. 3. The *sxt* genes, known to be present in a single cluster in cyanobacteria, are scattered on different linkage groups in *A. minutum*. 4. The expression of two *sxt* genes not assigned to any linkage group, *sxtI* and *sxtG*, may be regulated by the genomic region related to the ability to produce toxins. Our results provide new insights into the organization of toxicity-related genes in *A. minutum*, suggesting a dissociated genetic mechanism for the production of the different analogues and the ability to produce toxins. However, most of the newly identified genes remain unannotated. This study therefore proposes new candidate genes to be further explored to understand how dinoflagellates synthesize their toxins.

DATA SUMMARY

The RNA-Seq sequences of the different strains can be found at this address: <https://www.ebi.ac.uk/ena/browser/view/PRJEB52370> (ENA accession number: PRJEB52370). The reference transcriptome is available on Seanoe dataportal: <https://doi.org/10.17882/45445>.

INTRODUCTION

Genetic association studies aim to link phenotypic variations to genomic regions. The first attempts to understand the genetic bases of phenotypic variability were based on linkage maps developed after characterizing inheritance between parents and F1 offspring resulting from artificial sexual crosses [1, 2]. Following the development of molecular markers in the 1980s, quantitative trait locus (QTL) mapping studies were developed, mostly in plants, where numerous offspring may be obtained from a couple of parents [3–7]. In micro-eukaryotes, such approaches were limited by both the ability to perform sexual crosses *in vitro* and to phenotype the individuals, yeasts being a notable exception [8–10]. Since the explosion of genomic resources, the focus shifted from family- to population-wide association studies, mostly in humans and pathogenic bacteria [11–13].

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Keywords: *Alexandrium minutum*; Genetic linkage map; Paralytic Shellfish Toxins (PSTs); *sxtG*; *sxtI*; Toxin inheritance.

Abbreviations: eQTL, expression quantitative trait loci; HAB, Harmful algal bloom; PSP, paralytic shellfish poisoning; PST, paralytic shellfish toxin; SNP, single nucleotide polymorphism; STX, saxitoxin.

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Data statement: All supporting data, code and protocols have been provided within the article or through supplementary data files. Seven supplementary tables are available with the online version of this article.

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Impact Statement

Dinoflagellates are micro-eukaryotes of primary ecological importance. Of special interest, some species produce toxins that bio-accumulate in the trophic network. Being non-model with huge genomes, very few genomic resources and studies have been developed for these organisms. By developing the first genetic linkage map and genomic association study based on sexual crosses in dinoflagellates, the present study takes a major step toward a better understanding of the genetic basis of Paralytic Shellfish Toxin (PST) production. So far, PST production in dinoflagellates has been investigated by focusing on candidate genes homolog to genes known to be involved in PST production in cyanobacteria (*sxt* genes). Here, we show that the ability to produce toxins is inherited in a Mendelian manner and that the types of toxins produced follows a more complex pattern. We identified a genomic region associated to PST production and genes potentially regulated by this region. Altogether, we identified new candidate genes related to toxin production and discussed the importance of some genes considered as markers of toxicity.

However, in non-model micro-eukaryotes, genetic association studies using either linkage map or genome sequences are still scarce. For instance, the SAR supergroup (a clade containing Stramenopiles, Alveolata, and Rhizaria) is a deep branching eukaryote clade encompassing brown macro-algae, as well as numerous micro-eukaryotes such as ascomycetes, ciliates, radiolarians, and dinoflagellates. Within this clade, about ten genetic maps have been developed, mostly in parasites [14–23]. There is no genetic map available for dinoflagellates, which may be explained by the difficulties of obtaining hundreds of descendants [24] to analyse quantitative traits. However, a smaller number of offspring may be sufficient to investigate the genetic linkage of many qualitative traits.

Dinoflagellates are aquatic microorganisms displaying numerous peculiar genetic and molecular characteristics. They have multiple copies of genes arranged in tandem [25], numerous chromosomes in a permanent semi-condensed state and lack histones [26–28]. Only a few of their genes seem to be transcriptionally regulated [29–33], and transcription appears to be driven by a trans-splicing mechanism controlled by a specific sequence, the splice leader [34, 35]. Moreover, dinoflagellates have very large genomes (3 to 245 Gbp [36]), strongly limiting the feasibility of sequencing the entire genome. As a result, only genomes a few Gbp long have been sequenced, e.g. the coral symbiont *Breviolum minutum* [37] and *Polarella glacialis* [38].

Among dinoflagellates, four genera are known to produce Paralytic Shellfish Toxins (PSTs): *Gymnodinium*, *Pyrodinium*, *Centrodinium*, and finally *Alexandrium*, for which at least 11 species are known to be PST-producers [39, 40]. PSTs are non-protein toxins, produced by cyanobacteria and dinoflagellates, responsible for the Paralytic Shellfish Poisoning (PSP) syndrome in humans following the consumption of contaminated seafood. PSTs correspond to more than 57 analogues of saxitoxin (STX) classified into different groups, based on the nature of the substitutions on radicals R1 to R5 (Fig. 1; Table 1; [41]).

The first identified precursors of PSTs in cyanobacteria and dinoflagellates were arginine, acetate, and S-adenosyl methionine (SAM) [42] and several biosynthetic intermediates were confirmed experimentally by LC-MS analyses [43]. Some intermediates were also found in the dinoflagellate *Alexandrium tamarense* [44, 45], notably the two first products of the cyanobacterial biosynthetic pathway Int-A' and Int-C'2 [46]. The ability to produce toxins (qualitatively and quantitatively) shows both intra- and inter-specific polymorphisms in dinoflagellates, as well as cyanobacteria [47, 48].

So far, the genetic basis of PST production has mainly been studied in cyanobacteria. The first gene identified as involved in PST production was a carbamoyltransferase, named *sxtI*, whose role is to transfer a carbamoyl group to STX precursors in the PST biosynthetic pathway [49]. Using a genome walking approach upstream and downstream of *sxtI*, a cluster of 26 genes was identified

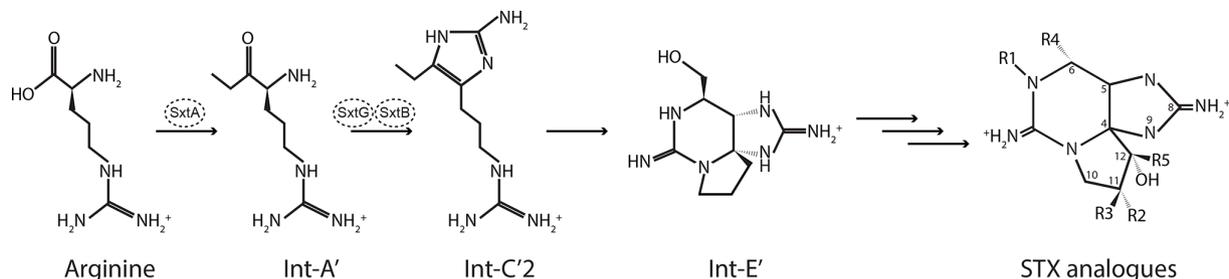


Fig. 1. Intermediates involved in the PST biosynthetic pathway in dinoflagellates [45, 111]. The *sxt* gene products involved in the biosynthetic pathway in cyanobacteria, and potentially involved in dinoflagellates, are indicated by dotted circles.

Table 1. Radicals of different saxitoxin analogues

Type of molecule	Analogue	R1	R2	R3	R4	R5	Type of toxin
Non-sulphated	STX	H	H	H	OCNH2	OH	Carbamate
Mono-sulphated	GTX1	OH	H	OSO3-	OCNH2	OH	
	GTX2	H	H	OSO3-	OCNH2	OH	
	GTX3	H	OSO3-	H	OCNH2	OH	
	GTX4	OH	OSO3-	H	OCNH2	OH	
	GTX5	H	H	H	OCNHSO3-	OH	N-sulfocarbamoyl
	GTX6	OH	H	H	OCNHSO3-	OH	
Di-sulphated	C1	H	H	OSO3-	OCNHSO3-	OH	
	C2	H	OSO3-	H	OCNHSO3-	OH	
	C3	OH	H	OSO3-	OCNHSO3-	OH	
	C4	OH	OSO3-	H	OCNHSO3-	OH	
Decarbamoylated	dcSTX	H	H	H	OH	OH	Decarbamoyl
	dcGTX1	OH	H	OSO3-	OH	OH	
	dcGTX2	H	H	OSO3-	OH	OH	
	dcGTX3	H	OSO3-	H	OH	OH	
	dcGTX4	OH	OSO3-	H	OH	OH	

as responsible for the synthesis, regulation, modification, and transport of these toxins in several species of cyanobacteria [49–51]. The genes *sxtA*, *sxtG*, *sxtH*, and *sxtI* were systematically present in PST-producing cyanobacteria [43]. The first intermediate in the PST biosynthetic pathway, Int-A', is thought to be produced by SxtA, and Int-C'2 by SxtG and SxtB. The various core genes (*sxtDIJKHTSUVW*) lead to saxitoxin formation and its analogues are obtained by the action of tailoring genes (*sxtLN XO*, as well as *sxtSUL* and *GxtA*), while the remaining *sxt* genes are dedicated to toxin regulation and transport [43].

Following these pioneer studies, homologues of cyanobacterial *sxt* genes were identified in dinoflagellates, with a particular focus on *sxtA* and *sxtG* [52–55]. In dinoflagellates, *sxtA* consists of four catalytic domains and has two isoforms: the short form, consisting of domains A1 to A3, and the long form, consisting of domains A1 to A4. Although the gene *sxtG* and the *sxtA4* domain first appeared to be present in PST-producing strains only, they were also identified in non-producing strains of species that usually produce PST [47, 53, 56]. Conversely, there is no published data showing a PST-producing strain lacking *sxtA4*, as reported in [57, 57]. In addition, a strain of *A. affine*, a usually non-toxic species, producing low levels of PST has not been investigated for the presence of *sxt* genes [58].

The functional role of cyanobacterial SxtA, GxtA, SxtSUL, SxtT, SxtH, SxtN in the biosynthesis of STX and analogues was recently confirmed *in vitro* [59–61], and *in vivo* after SxtA was expressed in *E. coli* [62]. In dinoflagellates, *sxt* genes have indeed been found [52–55], but experimental evidence of their implication in toxin production is still lacking. Current knowledge about this biosynthetic pathway in dinoflagellates is summarized in Fig. 1.

So far, a single study specifically investigated PST synthesis inheritance from parents to recombinant offspring in dinoflagellates [63]. The authors crossed *A. catenella* strains with different toxin profiles and obtained offspring. After analysing the toxin profile and mating type of the offspring, the authors concluded that the genes responsible for toxin synthesis are encoded in the nucleus and that the toxin profile is inherited in a Mendelian manner, i.e. the F1s inherit the toxin composition of one of the parent strains.

The present study aimed at identifying genomic regions associated with the ability to produce PSTs in the dinoflagellate *A. minutum*, one of the PST-producing species of the genus *Alexandrium*, responsible for harmful algal blooms (HABs) worldwide. A classic genetic approach, involving sexual crosses of four *A. minutum* clonal strains (i.e. two PST-producing and two non-producing) was performed in order to obtain F1 offspring. The PST quota and profile of parental strains and F1s were determined to investigate the inheritance of PST production and test the hypothesis of a Mendelian segregation suggested by Sako *et al.* [63] in terms of: 1. ability to produce the toxins, 2. types of analogues produced and 3. quantity of toxins. The genome size of *A. minutum* (24 to 28 pg [47]) precludes whole genome sequencing and assembly. As an alternative, RNA-Seq was used to focus on the coding regions of the expressed genes of the genome and to identify thousands of SNPs among parental strains [64]. A genetic map based on the SNP markers recombination in the F1s was developed. Using these genomic resources, we investigated:

Table 2. Characteristics of the four parental strains

	T1	T2	NT1	NT2
Sampling site	Bay of Brest, France	Bay of Cork, Ireland	Bay of Concarneau, France	Bay of Concarneau, France
Year of isolation	2013	2010	2010	2010
Mating Type	-	+	+	-
Toxic profile	C1/C2/GTX3/GTX2/dcGTX2/ dcGTX3/STX	GTX3/GTX2/STX	Non toxic	Non toxic

1. whether *sxt* genes form a gene cluster, 2. the genomic regions associated with toxicity and if they contain *sxt* genes, 3. the genes, whose expression seems to be controlled by the genomic regions linked to toxicity and if these genes correspond to *sxt*.

METHODS

Strains, crosses, and cell cultures

The dinoflagellate *A. minutum* has a haplontic life cycle [39] with alternance of asexual haploid cell multiplication and sexual reproduction, leading to the formation of a diploid resting cyst. This cyst enters a dormancy phase after which germination associated with meiosis may occur, leading to new haploid vegetative cells [65]. The dinoflagellate *A. minutum* appears to be heterothallic, which means that sexual reproduction is only possible between strains with a different mating-type [65, 66]. Four clonal parental strains (Table 2; Table S1, available in the online version of this article) were crossed considering previously established sexual types [33]: mt+ strains were crossed with mt- strains, resulting in four crosses, namely T1xT2, NT1xNT2, T1xNT1, and T2xNT2.

Unless stated otherwise, all strains were grown in K medium containing Na₂SiO₃ [67], referred hereafter as ‘K medium’, and under a 12/12 L:D cycle, 18 °C and 80 μmol photons m⁻² s⁻¹.

Parental strains were grown for 10 days and diluted in their own culture filtrate (0.2 μM) to obtain an initial cell density of 5000 cells per millilitre. Pairwise crosses were performed by mixing 1 ml of each strain in 24 well plates. Resting cysts resulting from gamete fusion produced mucus and bound to the bottom of the plates. After ten days, microplates were washed twice using sterile sea water (to remove vegetative cells), and stored at 4 °C in the dark for 8 months. After this dormancy period, microplates were washed with sterile sea water and placed for 5 days in an algal incubator after addition of 1 ml of K/2 (K diluted in *A. minutum* filtrate (0.2 μM)) per well. The presence of vegetative cells was confirmed and quantified under a microscope. Single cell isolation was then performed using serial dilution in K medium. Growth was assessed regularly and culture volume progressively increased to 30 ml of K medium, leading to a total of 62 F1 strains that were confirmed to be recombinant and monoclonal using SNP markers (see below).

Toxin extraction and analysis

Toxin quantification was performed across three sets of experiments (PST1, PST2, and PST3). The PST1 analysis was performed in 2020 on 52 strains collected at the end of the exponential phase, including all four parents, with one biological replicate per strain. The PST2 analysis was performed later in 2020, on three parent strains (NT1, NT2, T1), 18 F1s from the T2xNT2 cross and two F1s from the T1xNT1 cross, all harvested in stationary phase, with one biological replicate per strain. The PST3 analysis was performed in 2021, focusing on the T2xNT2 cross, with five biological replicates harvested at the end of the exponential phase. Cells were pelleted and stored at -20 °C before extraction.

The samples were prepared and analysed according to Shin *et al.* [40] but from non-lyophilised cell pellets. Briefly, they were resuspended in 1 ml of 1% acetic acid and lysed in an ultrasonic bath for 15 min before SPE clean-up on ENVI-carb cartridges (Supelco, Bellefonte, Pennsylvania, USA), diluted 1/4 in acetonitrile and finally analysed by LC-MS/MS. The method includes 19 PST analogues, and limits of detection and quantification are provided in Shin *et al.* [40].

RNA extraction, library preparation and sequencing

For mRNA extraction, parental and F1 strains were grown for 10 days in 50 ml and cells were pelleted by centrifugation (4500 r.p.m. for 8 min at 4 °C). Just after centrifugation, cell pellets were sonicated on ice for 30 s in LBP buffer (Macherey-Nagel, Duren, Germany). Extraction was performed using NucleoSpin RNA Plus kit (Macherey-Nagel) following the manufacturer’s protocol. Extracted RNA was quantified using a Biotek Epoch. Library preparation was performed using the Illumina mRNA TruSeq stranded kit starting from 0.5 μg of total RNA. Library quality was assessed on a Bioanalyzer using high-sensitivity DNA analysis chips. Paired-end sequencing was performed using 2x150 bp cycles on Illumina Hiseq 3000 at the GeT-PlaGe France Genomics sequencing platform (Toulouse, France).

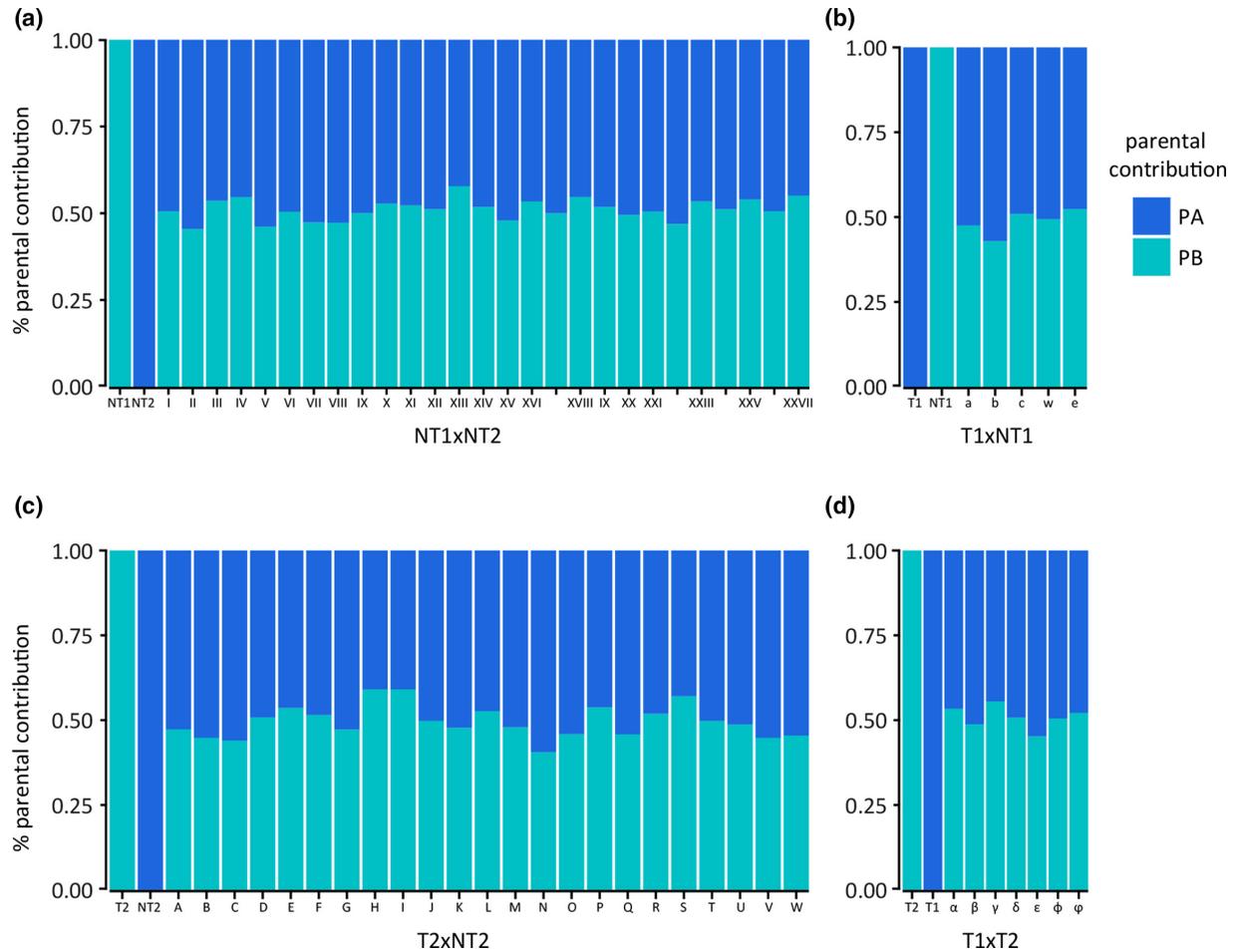


Fig. 2. SNP parental contribution for each F1 of NT1xNT2 (a), T1xNT1 (b), T2xNT2 (c), T1xT2 (d) crosses.

Bioinformatics analysis

Alignment and genotyping

Ambiguous, low quality reads, sequencing adapters and spliced-leader sequences were trimmed from raw reads using Trimmomatic version 0.33 [68] with parameters ILLUMINACLIP:Adapters.fasta:2:30:10, LEADING:3, TRAILING:3, MAXINFO:80:0.8, and MINLEN:70.

Trimmed reads were aligned to a reference transcriptome developed previously [64] using BWA-MEM (Burrows-Wheeler Alignment Tool [69]). Only reads with a mapping score >10 were retained. Pairs for which the two reads did not map concordantly on the same transcript were removed. Samtools was used to sort and index bam files, as well as to generate a raw count table for gene expression analysis [70]. Freebayes [71] was used to genotype the strains using 457368 SNPs previously validated [64]. Freebayes was first run enforcing diploidy. As *A. minutum* vegetative cells are haploid, monoclonal F1 strains had to be homozygotes at the various SNP positions. Non-monoclonal strains were identified and discarded based on the presence of numerous heterozygous SNPs. Freebayes was then run enforcing haploidy. Nucleotide divergence between the parental strains was calculated by dividing for each pair of strains the number of polymorphic SNP sites by the total number of sites covered by more than ten reads using BEDtools [72]. The genotype of the F1 strains was compared to the parental strains to infer the recombination pattern of the parental strains. Out of the 79 initial F1 cultures, 62 strains were identified as clonal and recombinant (Fig. 2), and used to build the genetic linkage map. Among them, ten strains were lost, thus 52 strains were considered to assess toxicity (Table S1).

Annotation

The reference transcriptome was initially annotated based on homology with the UNIPROT/SWISSPROT database [64]. To improve the annotation of *sxt* genes, a tblastn [73] analysis was performed against the reference transcriptome using as queries *sxt* genes from dinoflagellates and cyanobacteria extracted from GenBank (*sxt A* to *Z* and *sxtSUL*, *DIOX*, *PER*, *ACT*) (e -value < $1e^{-10}$). An additional blastx was performed with the transcripts identified by tblastn against the nr database (e value < $1e^{-5}$) with these

candidate transcripts. TransDecoder version 5.5.0 [74] was used on the transcriptome to select the longest ORFs and generate the associated protein sequences. These sequences were searched against the HMM library pfam-A.hmm [75] using the `hmmsearch` function of the HMMER programme (version 3.1b2 [76]).

Genetic linkage map

Transcripts presenting genetic variability (polymorphic at one or several SNP positions) among the four parents were considered to develop a genetic linkage map (38690 transcripts). Transcripts presenting multiple recombination events in the F1 strains, possibly reflecting chimeric transcripts (1798 transcripts), as well as transcripts displaying missing data in at least one strain (23172 transcripts) were removed from the initial dataset. The remaining 13720 transcripts were used to develop a genetic linkage map using the `mstmap` function of the R package `Asmap` [77–79], with default parameters except for `bychr` parameter (set to 'false', no preconception about the partition of transcripts in different chromosomes) and a p-value threshold set to $1e^{-7}$.

Genetic association and eQTL analysis of toxicity

Hereafter, a strong focus is made on the T2xNT2 cross. The genetic linkage between toxicity (considered as a qualitative trait: toxic, non-toxic) and the 13720 transcripts used to build the map was tested using Fisher's exact test, focusing on the T2xNT2 cross only, followed by a false discovery rate (FDR) correction, with significance set at 0.05. Transcripts displaying genetic variation between T2 and NT2, but not among the four parental strains and displaying a recombination pattern matching the identified region of interest were manually assigned to this region. Such an approach only focuses on genes present in the genomes of all the strains, but displays genetic variation among them.

An eQTL analysis was performed to identify SNP variants associated with gene expression. Raw read counts of all F1 RNA-Seq libraries were normalized using the `vsd` function of the `Deseq2` package [80]. Still focusing on the T2xNT2 cross, these expression data were used to perform the eQTL analysis on the 31 transcripts of the gene cluster linked to toxicity (see Results) using the R package `MatrixEQTL` [81]. This analysis could indirectly identify genes underlying the ability to produce toxins due to a presence/absence pattern, as such genes would not be expressed if absent from the genome of a given strain.

RESULTS

Recombinant F1 strains

Four parental strains (T1, T2, NT1, and NT2; Table 2, Table S1) differing genetically (pairwise nucleotide divergence ranging from 0.24–0.35% based on $15e+06$ bp) and in terms of toxin profiles were crossed. A total of 62 monoclonal F1s were obtained; 27 from the NT1xNT2 cross (named I to XXVII, Table S1), 23 from the T2xNT2 cross (named A to W, Table S1), seven from T1xT2 (named α , β , γ , δ , ϵ , Φ , and φ) and five from T1xNT1 (named a, b, c, e, and w). Considering the genetic peculiarities of dinoflagellates and since recombination patterns have never been investigated in *A. minutum*, the parental contribution was calculated for each of the 62 clonal F1s (Fig. 2). The mean SNP contribution of each parent did not differ from a 0.5/0.5 expectation (t-test: p-values between 0.27 and 0.71), confirming that all F1s are recombinant from the parental genotypes. The contribution averaged 0.503/0.497 overall (standard deviation 0.038), and 0.506/0.494, 0.518/0.482, 0.505/0.495, 0.505/0.495, for NT1xNT2 cross (Fig. 2a), T1xNT1 cross (Fig. 2b), T2xNT2 (Fig. 2c), and T1xT2 (Fig. 2d), respectively (standard deviations: 0.03, 0.03, 0.05, and 0.03, respectively). Moreover, the contribution of the two parents did not differ between crosses (Fisher Exact test, p-values=1).

Toxin inheritance

Toxin profiles and quotas were assessed at different growth phases. The analogues detected for a given strain are the same across analyses and replicates, except for analogues present in very small quantities, which are not always detected. The quantities of toxins produced vary between replicates (Table S2).

T1 parental strain produced eight toxins at a total toxin content of 40 pg per cell (Fig. 3a) and composed of 62% of C1 and C2 analogues, 30% of GTX3, 5% GTX2 and traces of dcGTX2, dcGTX3, GTX5, and STX (<1%). T2 parental strain produced only three analogues: GTX3 (55%), GTX2 (26%), and STX (19%) for a toxin content of 1.4 pg per cell. The two parental strains NT1 and NT2 did not produce any of the 19 screened toxins at a detectable level. The toxin content in toxic F1s varied from 0.025 to 10 pg/cell.

No toxin was detected in any of the 27 F1s from the NT1xNT2 cross (Table S2). All the F1s from T1xT2 cross produced toxins (Fig. 3b). Of the four T1xNT1 F1s, only one, strain e, produced toxins (Fig. 3c). Of the 18 F1s from the T2xNT2 cross, 13 produced toxins (Fig. 3d). To test whether the ability to produce toxins followed Mendelian proportions in the progeny, a Fisher test was performed considering F1s of toxic x non-toxic crosses. This trait did not deviate significantly from the expected value of 50% of toxic F1s (p-value 0.54 for both crosses taken together, p-value=0.3 for the T2xNT2 cross).

When considering analogues individually, C1 and C2 were produced in large amounts (32 and 31%, respectively) by T1 but were never found in its progeny. The same applied to GTX5, although this analogue was produced by T1 at low amount. STX was

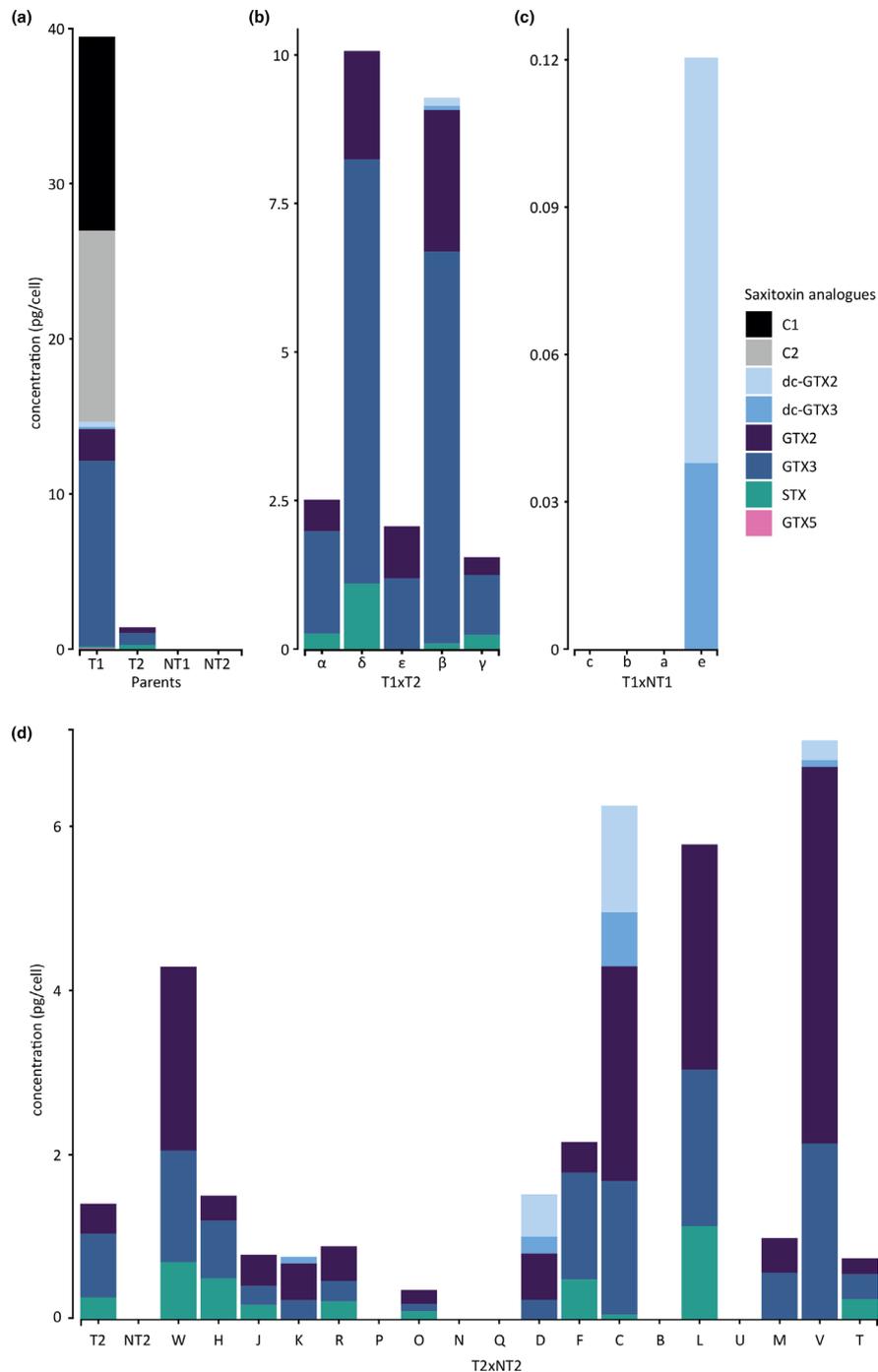


Fig. 3. Quantities of different PST analogues measured in parents (a) and offspring of T1xT2 (b), T1xNT1 (c), and T2xNT2 (d) crosses in pg/cell. NT1xNT2 offspring are not represented as they are all non-toxic.

present in the two toxic parents (albeit at trace level in T1, i.e. 0.2% of toxin content) but not always detected, neither in T1xT2, T2xNT2 nor T1xNT1 F1s. In the T1xNT1 cross, dcGTX2 and dcGTX3 were the only analogues detected, in low amounts (0.08 and 0.03 pg/cell, respectively), in a single descendant (strain e), while the profile of the toxic parent consisted of eight toxins. These two analogues were also found in four toxic F1s (strains C, D, K and V) of the T2xNT2 cross, whereas they were not detected in either parent. These results showed a complex toxin profile inheritance that cannot be explained by Mendelian segregation at a single locus.

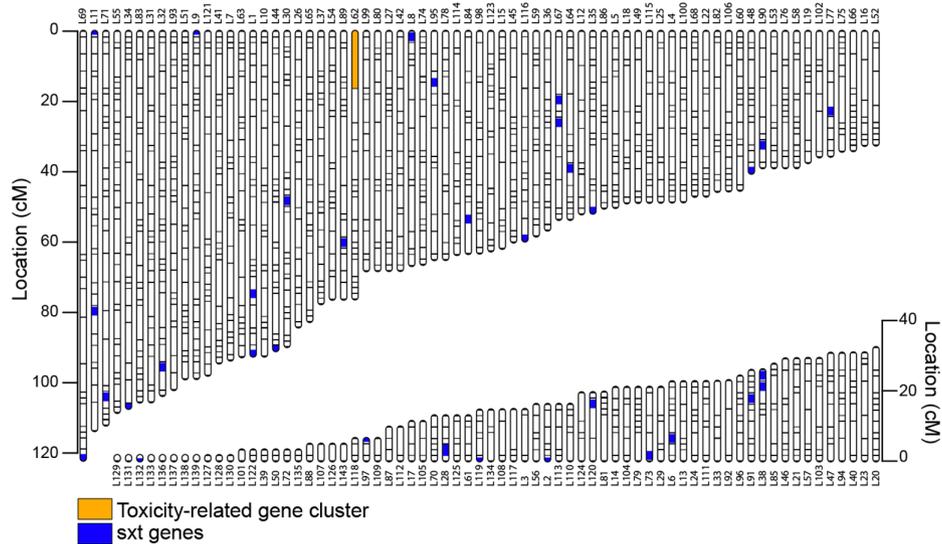


Fig. 4. Genetic map: 139 linkage groups ordered according to their size in cM. Orange area represents the toxicity-related gene cluster (l62). Blue areas are not on scale and each one represents the location of one *sxt* homologue in the transcriptome.

Linkage map

A linkage map was obtained by analysing the recombination pattern of 13720 transcripts in the 62 clonal recombinant F1s. These transcripts were clustered into 1685 bins (a bin encompassing a set of co-localized markers) distributed across 139 linkage groups (Fig. 4), corresponding to a total length of 5727 centimorgans (cM). Linkage groups had an average length of 41.2 cM (minimum 0, maximum 122.09 cM). The smallest linkage group contained one transcript and the largest 275, distributed in 28 bins. The density (cM/number of transcripts) per transcript averaged 0.43 cM/transcript (minimum 0, maximum 1.88). The distance between two adjacent markers averaged 361 cM (minimum 1.61, maximum 16.7 cM) and this distance was below 5 cM in 82.9% of cases.

Genetic linkage

To investigate the possibility of a genomic region associated with the ability to produce toxins (qualitative trait), the focus was placed on the 18 F1s (five non-toxic, 13 toxic) from the T2xNT2 cross. A region correlated to this toxin-producing ability was identified (Fig. 5a). This region was located on the L62 linkage group, between positions 0 and 16.2 cM (Fig. 5b). It consisted of 31 transcripts. This region was then manually enriched (see Methods) up to 50 transcripts, of which 25 could be annotated (Table 3,

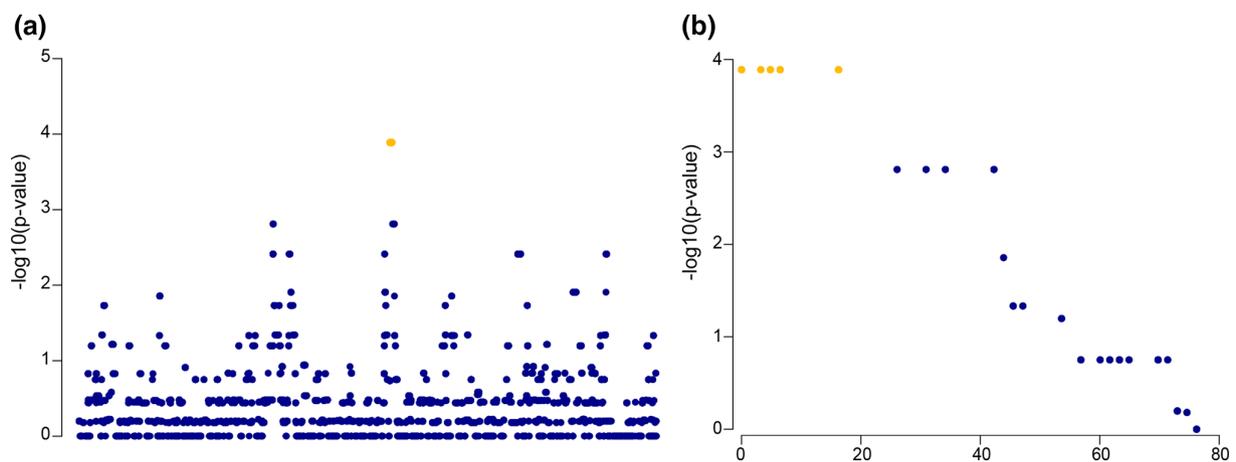


Fig. 5. Genomic association of the ability to produce toxins (a) Results of the Fisher test associating linkage groups with the toxin-producing capacity of F1s from the T2xNT2 cross; (b) Focus on linkage group 62 containing the region (0 to 16.2 cM) significantly related to the ability to produce toxins in T2xNT2 F1s.

Table 3. L62 transcript annotation. Only the pfam domain with the best overall score was shown for each transcript. The list of all pfam domains, as well as the list of GO terms associated with each transcript, are available in Table S3

Transcript	Position in L62	Putative function	Best pfam domain hit	Pfam evalue	Uniprot hit	Uniprot evalue
comp104658_c0_seq1	0 cM	Sulfotransferase	Sulfotransfer_3	1.4e-06	/	/
comp99374_c0_seq1	0 cM	Diphthamide biosynthesis / Chaperone DnaJ	DnaJ	1.7e-13	DPH4_ASHGO	1,00e-08
comp103051_c0_seq1	0 cM	/	/	/	/	/
comp111463_c0_seq2	0 cM	/	/	/	/	/
comp32533_c0_seq1	0 cM	/	/	/	/	/
comp65252_c0_seq1	0 cM	/	/	/	/	/
comp74963_c0_seq1	0 cM	/	/	/	/	/
comp128798_c0_seq1	3.2 cM	EGF-like domain / Tyrosine protein-kinase	Ephrin_rec_like	3.6e-06	SCUB1_HUMAN	3,00e-06
comp106553_c0_seq1	3.2 cM	/	/	/	/	/
comp111972_c0_seq1	4.8 cM	EF-hand	EF-hand_1	0.019	/	/
comp117087_c1_seq1	4.8 cM	Tetratricopeptide repeat	TPR_12	1.8e-21	TTC28_MOUSE	1,00e-09
comp130842_c0_seq1	4.8 cM	EamA-like transporter family	EamA	6.4e-06	/	/
comp94939_c0_seq1	4.8 cM	/	/	/	/	/
comp105001_c0_seq1	6.4 cM	Leucyl/phenylalanyl-tRNA--protein transferase	Leu_Phe_trans	8.8e-24	/	/
comp121729_c0_seq1	6.4 cM	Ankyrin repeat	Ank_4	1.9e-24	ANR50_HUMAN	3,00e-13
comp125039_c0_seq1	6.4 cM	Glycosyltransferase	Glyco_tranf_2_3	0.0079	/	/
comp129241_c1_seq1	6.4 cM	/	/	/	/	/
comp131122_c0_seq1	6.4 cM	/	/	/	/	/
comp101486_c0_seq1	16.2 cM	Potassium channel / Ion channel	Ion_trans_2	7.7e-23	TOK1_YEAST	8,00e-11
comp102705_c1_seq4	16.2 cM	NAD-binding domain	NAD_binding_10	2.5e-10	/	/
comp102814_c0_seq1	16.2 cM	GTP-ase	MMR_HSR1_Xtn	3.3e-34	DRG2_ARATH	9,00e-125
comp109406_c0_seq1	16.2 cM	Chlorophyll A-B binding protein	Chloroa_b-bind	2.4e-23	FCPA_MACPY	6,00e-13
comp115397_c0_seq1	16.2 cM	Methyltransferase	Methyltransf_21	1.6e-05	/	/
comp116297_c0_seq1	16.2 cM	Peptidase C14 (Caspase/ Metacaspase)	Peptidase_C14	4.5e-17	MCA2_ARATH	2,00e-17
comp118522_c0_seq1	16.2 cM	Potassium channel / Ion channel	Ion_trans_2	1.5e-21	KCO5_ARATH	3,00e-09
comp123175_c0_seq1	16.2 cM	Amine oxidase	Amino_oxidase	5.6e-36	PPOC_TOBAC	7,00e-121
comp123308_c0_seq2	16.2 cM	Alpha/beta hydrolase	Abhydrolase_3	6.9e-12	/	/
comp124989_c0_seq1	16.2 cM	Pyridine nucleotide-disulphide oxidoreductases	Pyr_redox_2	3.3e-51	/	/

Continued

Table 3. Continued

Transcript	Position in L62	Putative function	Best pfam domain hit	Pfam evalue	Uniprot hit	Uniprot evalue
comp125738_c0_seq1	16.2 cM	Cysteine dioxygenase	CDO_I	3.3e-53	CDO1_HUMAN	3,00e-27
comp125984_c0_seq1	16.2 cM	WEB protein/ Apolipoprotein	Apolipoprotein	0.002	Y5673_ARATH	4,00e-04
comp129241_c0_seq1	16.2 cM	Protein kinase / Cyclic nucleotide-binding domain	cNMP_binding	1.1e-11	KAPR_ASPNG	5,00e-14
comp24034_c0_seq1	16.2 cM	Galactosyltransferase	Galactosyl_T	2,00E-06	/	/
comp74512_c0_seq1	16.2 cM	Plastid lipid-associated protein	PAP_fibrillin	0.00057	/	/
comp125555_c0_seq9	16.2 cM	Myosin-2 heavy chain	/	/	MYS2_DICDI	3,00e-05
comp100745_c0_seq1	16.2 cM	/	/	/	/	/
comp101484_c0_seq1	16.2 cM	/	/	/	/	/
comp113111_c3_seq1	16.2 cM	/	/	/	/	/
comp118918_c0_seq1	16.2 cM	/	/	/	/	/
comp121267_c1_seq1	16.2 cM	/	/	/	/	/
comp122552_c0_seq1	16.2 cM	/	/	/	/	/
comp124016_c0_seq1	16.2 cM	/	/	/	/	/
comp124374_c0_seq1	16.2 cM	/	/	/	/	/
comp129321_c0_seq1	16.2 cM	/	/	/	/	/
comp130665_c0_seq1	16.2 cM	/	/	/	/	/
comp131282_c0_seq1	16.2 cM	/	/	/	/	/
comp25504_c0_seq1	16.2 cM	/	/	/	/	/
comp26886_c0_seq1	16.2 cM	/	/	/	/	/
comp29037_c0_seq1	16.2 cM	/	/	/	/	/
comp29558_c0_seq1	16.2 cM	/	/	/	/	/
comp30077_c0_seq1	16.2 cM	/	/	/	/	/

Table S3 for all pfam domain hits and GO terms). These include, among others, a sulfotransferase, two potassium channels and a caspase domain. None of the transcripts present in L62 matched any *sxt* homologue.

In the T1xNT1 cross, the distribution of SNPs in the L62 region did not match the toxicity pattern observed in the offspring (Table S8): F1s b, c and e all had the SNP of the non-toxic parent in 43 transcripts; offspring 'a' had the SNP of the toxic parent in only 16 transcripts (from 0 to 6.4 cM); finally, in seven transcripts there was no polymorphic SNP between the two parents.

Identifying *sxt* homologues

A total of 68 transcripts homologues to cyanobacterial *sxt* genes were identified in the reference transcriptome (Table S4). Of these, 29 are monomorphic and do not display any SNP, 35 do not form a gene cluster and are distributed on 30 different linkage groups (Fig. 4).

Four linkage groups contained two *sxt* genes: region L1 contained one *sxtA* and one *sxtU* in positions 74.6 and between 90.8 and 92.4 cM respectively; region L11 contained two *sxtU* in positions 79.6 and between 58.3 and 59.9 cM; region L28 contained two *sxtU* in position 3.23 cM; region L67 contains one *sxtU* and one *sxtA* between positions 13.1 and 19.5 cM and in position 26.02 cM respectively. Only three of the 12 putative *sxtA* homologues could be assigned to linkage groups. Neither of them was in the same linkage group. Finally, four *sxt* homologues could not be assigned to a particular linkage group, despite having differentiated SNPs between parents. Among them, an *sxtA* -long isoform- (comp112540_c0_seq1) and an *sxtU* (comp108760_c0_seq1)

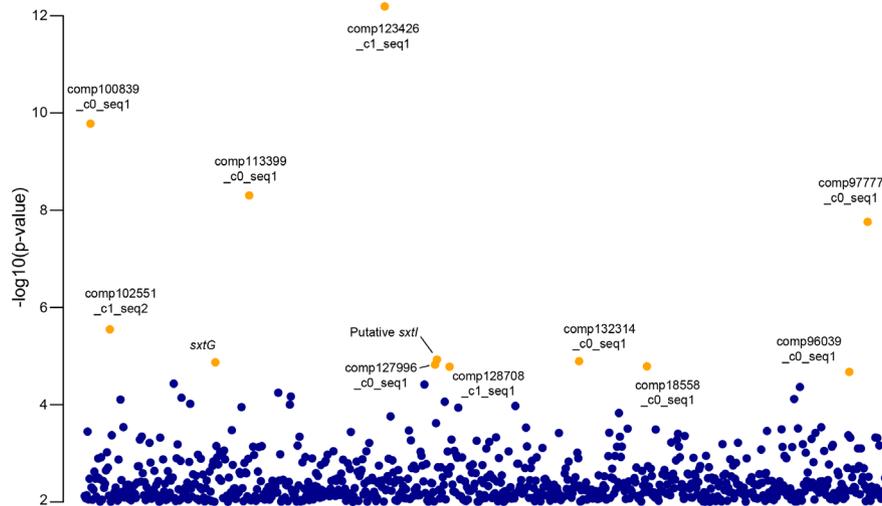


Fig. 6. eQTL analysis associating the L62 region (0 to 16.2 cM) with the expression of certain transcripts (in orange the transcripts, whose expression is significantly linked to this region).

could not be genotyped for all offspring (Table S5) but displayed a recombination pattern incompatible with any linkage group (including L62). The other two appeared to be chimeric transcripts.

eQTL

The genetic linkage analysis allowed us to detect inherited differences on coding sequences. However, genetic differences between strains may be carried by non-coding elements, such as regulatory sequences, to which our genetic linkage analysis does not give access (as only the expressed sequences are considered). An eQTL analysis was performed to link the L62 region associated with toxin production to gene expression. This analysis revealed 12 transcripts, whose expression is linked to the 0–16.2 region of the L62 linkage group (Fig. 6, FDR<0.2). One, comp97777_c0_seq1, belonged to the toxicity-related L62 cluster, six belonged to other linkage groups, and five monomorphic transcripts could not be assigned to any linkage group (Table S6).

Six of these 12 transcripts are annotated, two of which correspond to *sxt* homologues. The first one, comp110949_c1_seq1 (Fig. 6, Fig. 7b) is a *sxtG* homologue (Table S4) catalysing the second step of the PST biosynthetic pathway in cyanobacteria. The second one, comp127996_c2_seq1 (Fig. 6, Fig. 7a) corresponds to the *sxtI* gene, a carbamoyltransferase involved in the last step leading to complete formation of STX in cyanobacteria. Both of these transcripts are overexpressed in toxic F1s compared to non-toxic ones. The other identified transcripts are poorly annotated (Table S6).

The expression of the 12 transcripts identified by the eQTL analysis was then examined in all F1s of the four crosses (Fig. 7). The *sxtI* homologue (Fig. 7a) and comp127996_c0_seq1 (Fig. 7f) showed the same expression pattern, with a clear demarcation between toxic and non-toxic individuals. The only notable exception being the toxic T1xNT1 e strain, displaying contrasting expression levels compared to the other toxic strains. It should be noted that this specific strain displayed both very low toxicity levels and a singular toxic profile (Fig. 3c). The *sxtG* (Fig. 7b) homologue, comp123426_c1_seq1 (Fig. 7d), comp100839_c0_seq1 (Fig. 7e) and comp102551_c1_seq2 (Fig. 7g) followed the same pattern of demarcation between toxic and non-toxic F1s, but with a less clear delimitation than *sxtI* and comp127996_c0_seq1. The six remaining transcripts displayed differential expression only in the T2xNT2 F1s with high variability for the NT1xNT2 F1s (Fig. 7c and h-l).

DISCUSSION

The present study developed the first genomic association study in dinoflagellates to identify genetic regions associated with toxin production. Following crossing experiments and F1 phenotyping, the ability to produce PST appeared inherited as a simple Mendelian trait compatible with a single locus genetic determinism. However, toxin profile inheritance, i.e. the types of PST analogues produced, was not compatible with Mendelian inheritance and was probably governed by a more complex genetic determinism involving several genes. SNP genotyping and subsequent recombination analyses were used to obtain the first dinoflagellate genetic map. Contrasting with PST-producing cyanobacteria, in which the *sxt* genes responsible for toxin production were grouped in a single gene cluster, dinoflagellate *sxt* genes appeared scattered in numerous linkage groups. In accordance with the simple Mendelian inheritance hypothesis, a single genomic region was associated with the ability to produce PSTs. This genomic region did not carry any polymorphic *sxt* gene. An eQTL analysis identified several transcripts, including

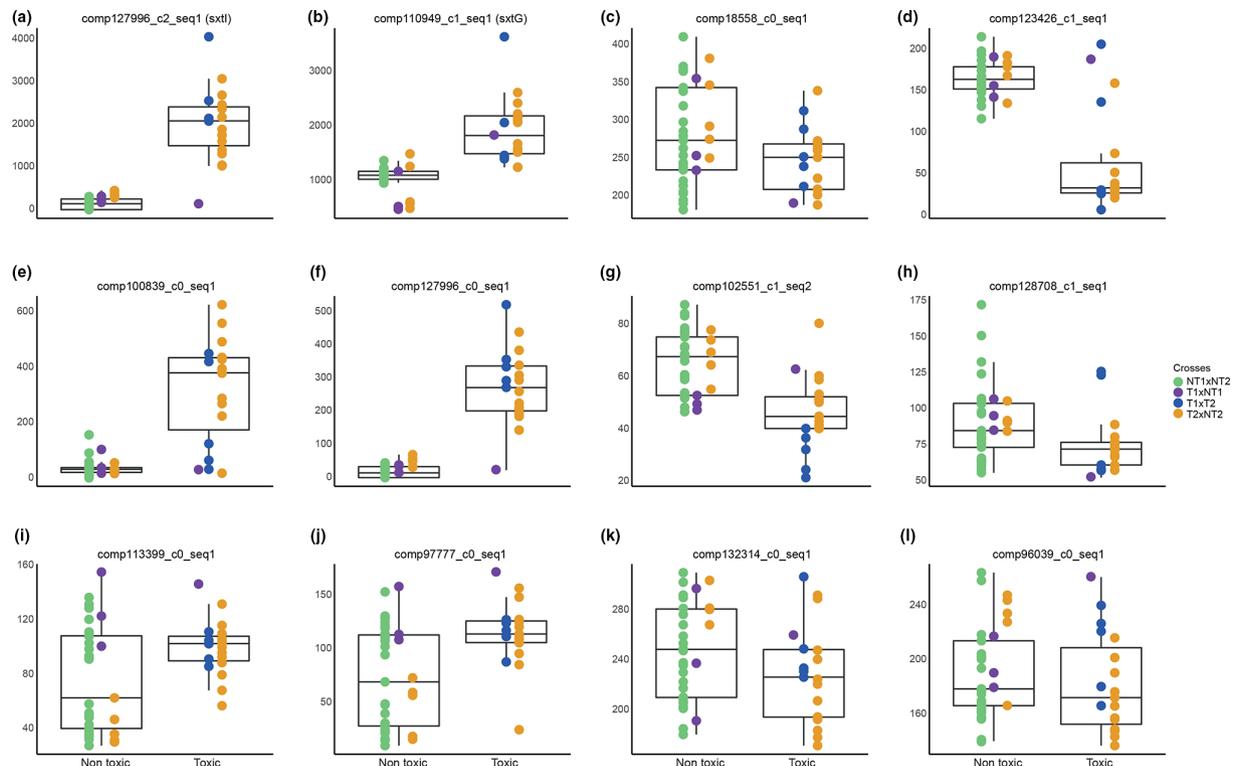


Fig. 7. Normalized expression of transcripts found by eQTL analysis, in F1s of all crosses according to their phenotype (toxic or non-toxic).

two *sxt*, whose expression seemed to be controlled by the genomic region associated with the ability to produce PSTs. These results are discussed below.

Linkage map and genomic association study in dinoflagellates

Very few linkage maps were developed for members of the SAR clade. Previous maps were obtained from species with genome sizes ranging from 23 to 560 Mbp [17, 20–23, 82], while *A. minutum* genome is estimated to be ~25 Gbp [47]. Despite this huge difference in genome size, the *A. minutum* linkage map was only slightly larger than previous ones, with 139 linkage groups spanning 5727 cM, compared to fewer than 60 linkage groups spanning 1782.7 to 2883.9 cM in previous studies. The average distance between two markers is 3.6 cM, compared to the mean distance ranging from 0.3 to 6.6 cM in previous studies, indicating that the *A. minutum* map is resolute. Finally, although linkage groups do not necessarily correspond to chromosomes, as a matter of comparison, 143 chromosomes were observed in *A. fundyense* [83].

Recent genomic association studies tend to focus on quantitative traits, requiring hundreds or even thousands of individuals [24]. However, traits of biological interest are not necessarily quantitative. This is especially the case for the ability to produce toxins or disease susceptibility [84]. The present study clearly demonstrates that a few tens of offspring may be sufficient to be resolute for genomic association studies focusing on such qualitative traits.

Toxicity inheritance

Mendelian inheritance of the ability to produce toxins

In agreement with previous studies [85–88], the present study showed that the PST profile, i.e. the ability to produce toxins and the types of analogues produced, may be considered as a character rather stable for a given strain. The only marginal exceptions were for analogues produced at concentrations close to the detection limits, which may rather reflect technical limitations (already noticed in [88]). The higher variability of quantities and ratio of analogues is also perfectly in agreement with previous studies showing that environmental factors (e.g. biotic, abiotic, nutrients, physiological state) can modulate the toxin level [89–94].

None of the F1s obtained from crosses between the two non-toxic strains were toxic, all of the F1s obtained from crosses between the two toxic strains were toxic and the ability to produce toxin did not deviate from the 50% expectation when toxic and non-toxic parental strains were crossed. As vegetative dinoflagellates cells are haploids, there is no dominance involved. These results suggest that the ability to produce toxins may be inherited as a simple mono locus Mendelian trait. This finding was in agreement with

the genomic association analysis that identified a single genomic region correlating with the ability to produce toxins. Martins *et al.* [95] reported a culture of *A. minutum* that lost its ability to produce toxins, while a subculture was still able to produce PSTs. Since this loss was a non-transient feature, a mutation in the PST biosynthetic pathway was considered rather than differential regulation at the gene level. This is consistent with this study, where the ability to produce is indeed controlled by a single genetic region in T2xNT2 cross. However, there was no association between the distribution of SNPs and the ability to produce toxins in the four offspring of the T1xNT1 cross, suggesting an alternative mechanism for this cross.

Multigenic bases of the toxin profiles

When examining the types of analogues produced, inheritance tends to be more complex. It deviated from Mendelian expectations and probably involved several loci. The best illustration concerned C1 and C2 analogues, produced in large amounts by T1, but never found in F1s. If the transmission was indeed Mendelian, the probability of finding C1 and C2 analogues in the five F1s of the T1xT2 cross would be 1/2 per F1, leading to a probability of finding no F1 with the T1 profile of 3%. These results indicated that the types of analogues produced have a multigenic basis. The partial inheritance of the toxin profiles contrasted with previous work [63] in *A. catenella* that highlighted a strict Mendelian inheritance of the toxic profiles of the parental strains in F1, without any modification of the parental profiles. The strains used by Sako *et al.* [63] belonged to a different species and displayed toxic profiles extremely different from the ones analysed in the present study. Altogether, these results illustrate that depending on the genetic make-up of the parental strains and on the produced analogues, parental profiles may sometimes be inherited as a whole and sometimes strongly modified by sexual reproduction.

This finding is perfectly compatible with the *sxt* genes being spread-out in the genomes of dinoflagellates rather than regrouped in a single cluster as in cyanobacteria. Considering that *sxt* genes are involved in PST production in dinoflagellates, this may have major consequences regarding the ability to produce the various PST analogues and on gene expression. First, in perfect accordance with the complex pattern of PST analogues inheritance identified in the present study, meiosis following gamete fusion would lead to new combinations of alleles with potential impacts on the ability to produce the various PST analogues. Second, in gene clusters the same *cis* module may co-regulate several genes, while independent *cis* modules would be required to regulate the expression of *sxt* genes spread out in various genomic regions. Since each of these independent modules can diverge genetically, this would imply the possibility of independent regulation of all these genes, even with a single *trans*-acting regulator.

In line with this hypothesis, a major result was the absence of genetically variable *sxt* genes in the region of interest. Indeed, such genes have long been associated with toxin production in cyanobacteria [43, 50, 51], and the identification of numerous homologues in dinoflagellates ([52]; present study) make them ideal candidates to explain toxin production in dinoflagellates. Here, 68 *sxt* homologues were identified. None of the 39 *sxt* homologues displaying genetic variability among parental strains was present in the genomic region associated with toxicity. The 29 remaining were genetically monomorphic in the parental strains and could not be assigned to a linkage group. As these genes are monomorphic, genetic variation of their coding region cannot be involved in the ability to produce toxins. However, genetic variation in the *cis* regulatory region could translate into gene expression differences. Out of the 29 monomorphic *sxt* homologues, two displayed over-expression in the toxic strains. The two *sxt* homologues, whose expression were upregulated in toxic strains, were *sxtG* and *sxtI*. These two genes were previously reported as candidates involved in toxicity in dinoflagellates. The *sxtG* homologues were reported in PST-producing dinoflagellate species only [56] and *sxtG* expression was upregulated in experimental conditions favouring toxin production [96]. Similarly, *sxtI* expression was upregulated in conditions favouring toxin production, and downregulated when toxin synthesis was stopped [96, 97]. Although identifying the molecular bases of the production of the various analogues is beyond the scope of the present study, the case of the strain e (T1xNT1 cross) might be worth being mentioned. In this strain, only dcGTX2 and three analogues are present and *sxtI* expression is extremely low. Based on the dinoflagellate putative biosynthetic pathway [98], carbamoylated analogues (GTX1 to 6, C1 to C4, STX, and NeoSTX) seem to be catalysed by SxtI, whereas decarbamoylated analogues ('dc'-type analogues) appear to be produced from dc-STX without involving SxtI. The absence of C1 and C2 in strain e could be explained by such a mechanism.

Gene expression regulation of *sxtI* and *sxtG* by the toxicity-related L62 region adds additional evidence that these two genes are related to toxin production in dinoflagellates and could be compatible with the above-mentioned hypothesis of *trans*-regulation by L62 of the genes responsible for the toxin profile.

Candidate genes

No evidence for *sxtA* implication in the ability to produce PST

The *sxtA* gene is by far the most studied when investigating the production of PSTs. This gene presents two isoforms in dinoflagellates: a short form comprising three domains (A1 to A3) homologue to cyanobacterial *sxtA* domains and a fourth domain without any known homology [64] and a long form with the four cyanobacterial domains (A1 to A4 [53]). It was postulated that this long form would be the cause of toxicity in dinoflagellates as it was repeatedly found in toxic strains, and rarely in non-toxic ones [52, 53, 56, 99, 100]. A recent study identified the presence of the long isoform in both toxic and non-toxic parental strains used in the present study among others [47]. At least two homologues of the long form of *sxtA* were identified in the present

study. Neither of them appeared to be related to the ability to produce toxins. One could be assigned to the L1 linkage group. The other one could not be assigned to any group, but distribution of parental markers in the progeny was clearly incompatible with L62. Similarly, *sxtA* expression was not controlled by this region. This is consistent with Wiese *et al.* [101] who showed that *sxtA4* expression in *A. catenella* does not vary when the amount of PSTs changes between different growth phases. However, in the same species, *sxtA4* expression has been shown to vary between toxic and non-toxic mutants [100]. In addition, a strain of *A. catenella*, containing both toxic and non-toxic cells, has been studied multiple times to elucidate the mechanisms responsible for the loss of toxicity of part of these cells [102–105]. In the non-toxic subclone, *sxtA4* expression was significantly reduced. It was postulated that changes in the 3'UTR region of *sxtA4* were responsible for the loss of toxicity via post-transcriptional regulatory mechanisms [105]. In our study, the mechanism is probably distinct, as *sxtA4* expression did not change between toxic and non-toxic individuals. However, a distinct post-transcriptional or translational mechanism, potentially regulated by the L62 region, cannot be excluded to explain the loss of toxicity in F1 strains.

At an evolutionary scale, it has been shown that of the three *sxtA1* isoforms detected in dinoflagellates, only one is thought to be specific to STX-producing species [56]. In the present study, genetic variation of the *sxtA1* isoforms could not be related to toxicity. The evolution of *sxtA* by acquisition of the specific domain *sxtA4* has also been suggested in toxin-producing cyanobacteria and dinoflagellates [106]. In the present study, and as mentioned above, the polymorphism of *sxtA4* does not correspond to the variations in toxicity.

Altogether, our study and previous ones suggest that depending on the precise genetic make-up of the strains, there may be multiple ways to lose the ability to produce PSTs in dinoflagellates.

Other genes involved in toxin production

The implication of L62 or of differentially expressed genes without homology with *sxt* in PST production cannot be excluded. It is however difficult to identify clear candidates. A major reason is the lack of homology with genes of identified functions for a great majority of dinoflagellate genes [64]. In the L62 region of interest, only 25/50 genes had a putative homology with a protein domain. The putative functions may be attributed to many different cellular processes (e.g. methyltransferase-, carboxylesterase-domains). Nevertheless, it is worth mentioning some of the identified homologues since their functions have previously been associated with toxin production in different marine dinoflagellates. For example, comp104658_c0_seq1 displays a domain homologous to a sulfotransferase (L62, 0 cM). In *Gymnodinium catenatum*, O-sulfotransferases are responsible for the transformation of STX into GTX 2 and 3 [107].

A second candidate might be a gene encoding a metacaspase (L62, 16 cM). This metacaspase is a specific arginine/lysine protease. Caspases, as well as their counterparts in bacteria and plants, orthocaspases and metacaspases, are involved in programmed cell death, however these enzymes might have other functions in plants, algae and cyanobacteria such as cell homeostasis, culture ageing [108] or even roles in survival pathways and cell cycle progression as suggested in *A. tamarensis* [109]. A study on metacaspases from environmental samples from the Baltic Sea also indicates that several metacaspases were in the same expression cluster than the genes related to toxin production in *Nodularia spumigena*, suggesting a link between toxin synthesis, metacaspase expression and programmed cell death [110].

Finally, of the 12 transcripts identified by eQTL analysis, two were not annotated, including comp127996_c0_seq1, for which the expression pattern is identical to that of *sxtI*. Further studies are needed to discover or confirm the putative functions of the identified genes, and their implication in the PST biosynthetic pathway in dinoflagellates, e.g. by genetic knock-out, long read sequencing of part of the genome (L62 region for example), or by screening natural populations or a larger number of offspring.

CONCLUSION

The present study showed that the ability to produce toxins in *A. minutum* is an inheritable Mendelian character and seems to be controlled by a single genetic region. This region does not contain homologues of *sxt* genes identified in cyanobacteria. However, it seems to control the expression of *sxtI* and *sxtG* gene homologues. Unfortunately, it was impossible to determine whether these two homologues are physically present in the genomic region associated with toxicity, if they displayed genetic polymorphism in their regulatory region (*cis*-regulation), or if they are in another linkage group regulated by a gene present in L62 (*trans*-regulation). A starting point to test these hypotheses would require the development of specific sequencing strategies targeting the *cis* regulatory or the entire L62 regions.

The present study also showed that toxin profiles seem to be controlled by several genes, since these profiles are not transmitted as a whole in progeny. This multigenetic origin of the profiles would be in accordance with the *sxt* genes being spread out in multiple linkage groups.

The *sxtA* homologues were not found to be related to toxin production in this study, as they were absent from the identified genetic region and were not differentially expressed between toxic and non-toxic strains. Caution has nevertheless to be taken, as gene expression regulation in dinoflagellates is believed to be mainly driven by post-transcriptional mechanisms involving

trans-splicing [34]. Finally, most of the newly discovered candidate genes are not annotated and therefore represent new perspectives to unravel the mechanisms that govern the synthesis of PSTs in dinoflagellates.

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Author contribution

Conceptualization: M.L.G., Data curation, Formal analysis: L.M., M.L.G., Funding acquisition: M.L.G., Investigation: J.Q., M.L., G.A.R., D.R., M.L.G., Methodology, Project administration, Resources, Software, Supervision: M.L.G., D.R., H.H., Validation, Visualization: L.M., Writing – original draft: L.M., Writing – review & editing: M.L.G., L.M., D.R., H.H.

Conflicts of interest

The author(s) declare that there are no conflicts of interest.

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