Cell Response to Extracellular DNA and Self-DNA Inhibition

Thesis

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Cell response to extracellular DNA
and self-DNA inhibition

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To my grandmother, whose strength will always be a guiding light for me.
### Table of contents

Abstract ........................................................................................................................................... 7  
Acknowledgments .......................................................................................................................... 9  
List of Figures .................................................................................................................................. 11  
List of Tables ................................................................................................................................... 12  
List of abbreviations ....................................................................................................................... 13  

Chapter 1: INTRODUCTION ........................................................................................................... 15  
  1.1 The extracellular DNA: a general overview ............................................................................. 15  
  1.1.1 Features and fate .................................................................................................................. 15  
  1.1.2 ExDNA in the soil ................................................................................................................. 15  
  1.1.3 ExDNA in marine and fresh water ....................................................................................... 16  
  1.1.4 ExDNA in multicellular organisms .................................................................................... 17  
  1.1.5 ExDNA in microbial biofilm ............................................................................................... 20  
  1.2 Structural roles and bioavailability of exDNA ......................................................................... 21  
  1.2.1 Protective function .............................................................................................................. 22  
  1.2.2 Nutritive function ................................................................................................................. 22  
  1.2.3 Genetic material .................................................................................................................. 22  
  1.2.4 Signalling molecule ............................................................................................................. 25  
  1.3 Novel findings on exDNA ....................................................................................................... 26  
  1.4 Hypothesis of sensing and mechanism of action of exDNA .................................................. 29  
  1.5 Potential pharmacological and biotechnological applications .............................................. 31  

AIM OF THE PROJECT .................................................................................................................. 33  

Contribution statement .................................................................................................................. 36  

Chapter 2: exDNA AND MULTICELLULAR AUTOTROTROPHS: EFFECTS ON 
ARABIDOPSIS THALIANA ............................................................................................................. 37  
  2.1 Preface to the work on A. thaliana ............................................................................................ 37  
  2.1.2 Arabidopsis: The Model Plant ............................................................................................. 37  
  2.2 Materials and Methods .......................................................................................................... 38  
  2.2.1 DNA extraction and preparation ......................................................................................... 38  
  2.2.2 Plant materials and treatments ........................................................................................... 39  
  2.2.3 Transcriptome and bioinformatics analyses ........................................................................ 40  
  2.2.4 Confocal microscopy experiment ....................................................................................... 41  
  2.2.5 QRT-PCR validation .......................................................................................................... 41  
  2.3 Results .................................................................................................................................... 42  
  2.3.1 Differential gene expression self and nonself-exDNA exposure ........................................ 42  
  2.3.2 DEGs associated to enriched GOs ..................................................................................... 46  
  2.3.3 One hour post treatment ..................................................................................................... 47
4.1.2 Human cell line as model system for heterotroph organisms ........................ 49
4.1 ExDNA in multicellular organisms ................................................................ 50

3.5 Future perspectives ......................................................................................... 62

Chapter 3: exDNA AND UNICELLULAR EUKARYOTIC ORGANISMS: EFFECTS ON
MICROALGAE ........................................................................................................ 64

3.1. Microalgae: a model system for photosynthetic organisms .............................. 64
3.1.2 Chlamydomonas reinhardtii: a model for freshwater microalgae ...................... 65
3.1.3 Nannochloropsis gaditana: a model for marine microalgae ............................... 65

3.2 Materials and methods ..................................................................................... 66
3.2.1 DNA extraction and fragmentation.................................................................... 66
3.2.2 Algal growth and growth inhibition assay ........................................................ 67
3.2.3 Microscopic analysis of cell structure and chlorophyll distribution .................... 68
3.2.4 DNase treatment and cell vitality assays .......................................................... 69

3.3. Results .............................................................................................................. 69
3.3.1 Self-DNA inhibits growth of freshwater and marine algae ............................... 69
3.3.2 Morphological changes after treatment with exDNA ....................................... 72
3.3.3 Microscopic evaluation of aggregates formation after treatment with self-exDNA .................................................................................................................. 73

3.4. Discussion ....................................................................................................... 77
3.5 Future perspectives and impact ......................................................................... 79

Chapter 4: exDNA AND MULTICELLULAR HETEROTROPHS: EFFECTS IN
HUMAN CELLS .......................................................................................................... 80

4.1 ExDNA in multicellular organisms ..................................................................... 80
4.1.2 Human cell line as model system for heterotroph organisms .......................... 81

4.2 Materials and methods ...................................................................................... 82
4.2.1 DNA extraction and fragmentation ................................................................. 82
4.2.2 Evaluation of cellular viability ......................................................................... 83
4.2.3 Video microscopy time-lapse ......................................................................... 84

4.3 Results .............................................................................................................. 86
4.3.1 ExDNA treatment decreases human cell viability ......................................... 86
4.3.2 The treatment with exDNAs affects cellular physiology .............................................. 87
4.4 Discussion ......................................................................................................................... 90
4.5 Future perspectives .......................................................................................................... 91
Chapter 5: exDNA AND MULTICELLULAR HETEROTROPHS: EFFECTS ON CIONA ROBUSTA .......................................................... 93
  5.1 Advantages of Ciona robusta as model system ................................................................. 93
  5.2 Materials and methods..................................................................................................... 95
    5.2.1 DNA extraction and fragmentation .............................................................................. 95
    5.2.2 Animals and embryos ................................................................................................. 96
    5.2.3 Sample preparation and treatments .......................................................................... 97
  5.3 Results ............................................................................................................................... 98
  5.4 Discussion ......................................................................................................................... 103
  5.5 Future perspectives .......................................................................................................... 105
Chapter 6: Final discussion .................................................................................................... 106
Chapter 7: Future perspectives and applications ................................................................. 111
References ............................................................................................................................... 112
List of publications with contributions .................................................................................. 152
Conference attendance and presentations .......................................................................... 153
List of non-book component ............................................................................................... 153
Abstract
The discovery of DNA beyond the confinement of cell boundaries, generally defined as extracellular DNA, poses questions on its alternative roles that may differ from its being the main repository of genetic information within a cell. DNA can be extruded from cells by active or passive release, remaining in the extracellular context, that can be either the body of multicellular organisms or the natural environment, as free molecule or complexed with inorganic and organic particles, and/or organized in extracellular structures.

The presence of extracellular DNA (exDNA) outside cells, either in multicellular organisms or in the environment, has been initially discussed in terms of horizontal gene transfer, nutrient, and then as protective and/or signalling molecule. Recently, a novel and interesting research line revealed for the exDNA additional roles depending on its nature, environmental location and structural organization. Indeed, in 2015, Mazzoleni and his colleagues demonstrated for the first time that the exposure to fragmented self-exDNA (conspecific or similar/homologous DNA) differently from the exposure to nonself-exDNA (heterologous, DNA from phylogenetically unrelated species, or, more in general, distant in sequence similarity terms), inhibits root growth and seed germination in plants in a concentration dependent manner. Later, it has been also demonstrated that it induces intracellular calcium signalling and plasma membrane depolarization and also triggers well-known markers of the immunological response, e.g. H₂O₂ production and MAPK activation. Following the results on plants, the inhibitory effect of self-exDNA on other species was also demonstrated by Mazzoleni and his group and, on the basis of these findings, they suggested the generality of the observed phenomenon which opens new perspectives in the context of self-inhibition processes.

Nevertheless, the mechanism underlying the specific recognition of either self or nonself-exDNA and subsequent responses in plants as well as in other organisms is still poorly understood.

The presented project aims at investigating the effects of the exposure of organisms from model species to nonself- and self-exDNA. In particular, one of the main objectives, is to confirm the inhibitory role of self-exDNA when compared to the exposure to nonself-exDNA, as highlighted for the first time in plants and then also in other species by Mazzoleni and colleagues in 2015. In this regard, it will be explored the effect of nonself- and self-exDNA on both terrestrial and aquatic species including representatives of unicellular and multicellular organisms from animals and plants with the purpose of confirming the generality of the phenomenon observed by Mazzoleni et al. in 2015. In particular, as representative of multicellular autotrophs, the early response to either nonself- and self-exDNA will be adressed in the model plant Arabidopsis thaliana through the evaluation of gene expression changes and exDNA spatial distribution at root and cellular levels. Then, to confirm the differential responses also in aquatic environments,
it will be also evaluated the effect of nonself- and self-exDNA on cell density and on the
morphology of two microalgae *Nannochloropsis gaditana* and *Chlamydomonas reinhardtii*,
examples of unicellular autophots, living in marine and freshwater, respectively. Furthermore,
aiming at highlighting potential differences and similarities among the responses of cells from
different phyla, the effects of nonself- and self-exDNA will be evaluated on the vitality,
morphological and physiological features of human cells, considered as a simplified system
representing non-photosynthetic species. Finally, to further characterize the phenomenon in
multicellular organisms living in marine environment, the chordate *Ciona robusta*, a model
system which is considered the closest living relatives of vertebrates, will be used to investigate
the effects of exDNAs on the main stages of the chordate embryos development and, thus, to infer
on its potential role on vertebrates developmental processes.

The findings of this project will contribute to shed light on the differential responses of organisms
of different clades to either self- or nonself-exDNA. This will have relevant ecological
implications and will support the unexpected new functional roles of exDNA in species
interactions at community and ecosystem levels also deserving high potentiality for the
development of biotechnological and industrial applications.
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I strongly believe that the advantage of a collaborative teamwork is the possibility to exchange and integrates different ideas, advises but also constructive criticisms that only enriches the value of the individual work.

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Emanuela Palomba
List of Figures

Chapter 1
Figure 1.1. Inhibitory effect of fragmented self-exDNA on plants (a) and other organisms of different taxonomic group (b).
Figure 1.2. Physicochemical responses induced by self-exDNA in plants.
Figure 1.3. Potential pharmacological and biotechnological applications of self-exDNA.

Chapter 2
Figure 2.1. Arabidopsis thaliana: a plant model system.
Figure 2.2. Schematic representation of the experimental design.
Figure 2.3. Number of differentially expressed genes in treatment with self-exDNA (A) and nonself-exDNA (B) vs control, comparisons and transitions per stage (1, 8 and 16 hpt).
Figure 2.4. Summary of GO enrichment analysis on filtered DEGs.
Figure 2.5. Number of either specific or common filtered DEGs in the main GO groups according to different self- and nonself-exDNA treatments (at 1, 8 and 16 hpt).
Figure 2.6. Fluorescence microphotography (confocal images) of early response (1 h) to self- vs. nonself-exDNA treatments.
Figure 2.7. Summary of the main differences in response to extracellular self- and nonself-exDNA.
Figure 2.8. Model of Extracellular DNA Associated Pathways (EDAP) in plants.
Supplementary Figure 2.2. Correlation analysis between log2FC RNA-seq and log2 ΔΔCt of QRT-PCR.
Supplementary Figure 2.1. Correlation analysis (A) of gene expression (RPKM) in all replicates.

Chapter 3
Figure 3.1. Structure of Chlamydomonas reinhardtii (a) and Nannochloropsis gaditana (b) cells.
Figure 3.2. Schematic overview of the experimental design.
Figure 3.4. Microscopy analysis of C. reinhardtii (a) and N. gaditana (b) samples in controls and after treatments with nonself- and self-exDNA at 30 ng/µL.
Figure 3.5. Additional pictures from microscopy analysis of C. reinhardtii (a) and N. gaditana (b) samples in controls and after treatments with nonself- and self-exDNA.
Figure 3.6. Fluorescence analysis of C. reinhardtii (a) and N. gaditana (b) samples in the controls and after treatments with self-exDNA at 30 ng/µL. Cellular aggregates were analysed using DAPI staining.
Figure 3.7. Fluorescence analysis of C. reinhardtii after treatment with self-exDNA at 30 ng/µL: focus on cellular aggregates formation.
Figure 3.8. Microscopy analysis of C. reinhardtii aggregates after treatment with DNase.

Chapter 4
Figure 4.1. Schematic representation of a human cell.
Figure 4.2. Schematic overview of the experimental design.
DNA PREPARATION: extraction, RNase treatment and sonication of DNA from human cell line PNT2 (self-DNA) and from Ailsa Craig (tomato) (nonself-DNA), according to Mazzoleni et al., 2015a.
Figure 4.3. Tracking analysis of a subset of cells after the treatment with nonself- and self-exDNA and in the control.
Figure 4.4. PNT2 cell viability after the exposure to nonself- and self-exDNA.
Figure 4.5. Microscopy analysis of cell death events after the treatment with nonself- and self-exDNA at 0.3 ng/µL at 24 hrs compared with control.
Figure 4.6. Number of mitotic events during the 24 hrs after the treatment with nonself- and self-exDNA at 0.3 ng/µL.
Figure 4.7. Timing of mitosis after the treatment with self-exDNA and in the control.
Figure 4.8. Mean squared displacement of isolated and clustered cells after the treatment with nonself- or self-exDNA at 0.3 ng/µL.
Supplementary Figure 4.1. PNT2 cells observed by microscope analyses.
Supplementary figure 4.2. Isolated (blue) and clustered (yellow) cells MSD (τ=1) after the treatment with nonself- and self-exDNA compared with the control.
Supplementary Figure 4.3. Clustered and isolated cells distribution in bidimensional space (a) and velocity (µm*s⁻¹) vs time (h) (b).

Chapter 5
Figure 5.1. The ascidian Ciona intestinalis: (a) C. intestinalis tadpole larva and (b) C. intestinalis adult.
Figure 5.2. Schematic overview of the experimental design.
Figure 5.3. Evaluation of *C. robusta* embryos development after the treatment with different concentrations of nonself- and self-exDNA at 24 hrs.

Figure 5.4. *C. robusta* embryos development (early gastrula period at 110 cells, neural plate, tailbud formation and larval stage) after treatment with nonself- and self-exDNA at 200 ng/µL.

Supplementary Figure 5.1. The effect of nonself-exDNA (a) and self-exDNA (b) at 200 and 250 ng/µL on *C. robusta* embryos development.

List of Tables

Chapter 1
Table 1.1. Species selected as models for unicellular and multicellular organisms in the presented work.

Chapter 3
Table 3.1. Average cell density (x 10^4 cells*ml^-1), generation time (Tg) and growth rate inhibition (% of control) (GRI (%)) of *C. reihnardtii* (A) and *N. gaditana* (B) at 168 hpt at different concentrations of nonself- and self-exDNA.

Chapter 4
Table 4.1. Cell count of the total number of cells, dead cells and mitotic events.

Chapter 5
Table 5.1. Evaluation of *Ciona robusta* embryos development after the treatment with different concentrations of nonself- and self-exDNA at 24 hrs.
List of abbreviations

ATP adenosine triphosphate
ABA abscissic acid
ASW artificial seawater medium
BBM bold’s basal medium
BMP bone morphogenetic protein
CRISPR-Cas clustered regularly interspaced short palindromic repeats-CRISPR-associated protein
CTAB hexadecyltrimethylammonium bromide
DAMP damage-associated molecular pattern
DAPI 4′,6-diamidino-2-phenylindole
DDX41 DEAD-box helicase 41
DEGs differentially expressed genes
DNA deoxyribonucleic acid
DOM dissolved organic matter
DUS DNA uptake sequences
EDAP extracellular DNA associated pathways
EDTA ethylenediamine tetra-acetic acid
EPS extracellular polymeric matrix
FBS foetal bovine serum
EST expressed sequence tag
exDNA extracellular DNA
FDR false discovery rate
FGF fibroblast growth factor
FITC fluorescein isothiocyanate
GO gene ontology
HGT horizontal gene transfer
hpf hrs post-fertilization
hpt hours post-treatment
JA jasmonic acid
log2(FC) log2-fold change
MAPK mitogen-activated protein kinase
MRN MRE11/RAD50/NBS1 complex
MSD (τ) mean square displacement
MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NETs neutrophil extracellular traps
NO nitric oxide
PAMP pathogen-associated molecular pattern
PARA-MOPS 3-(N-morpholino) propanesulfonic acid
PNT2 human prostatic epithelial cell line
PRRs pattern recognition receptors
PUFAs poly-unsaturated fatty acids
PVP polyvinylpyrrolidone
QPCR quantitative PCR
RETs root extracellular traps
ROS reactive oxygen species
RPMI roswell park memorial institute
RPKM reads per kilobase of transcript, per million mapped reads
SIB sperm isolation buffer
SDS sodium dodecyl sulfate
SLE systemic lupus erythematosus
TLR9 toll like receptor 9
Tris tris (hydroxymethyl) aminomethane
Wnt wingless-related integration site
1.1 The extracellular DNA: a general overview

The extracellular DNA (exDNA) is defined as “the DNA located outside the cell and originating from intracellular DNA by active or passive extrusion mechanisms or by cell lysis” (Ceccherini et al., 2009). It can be located in different contexts, like varied natural habitats (e.g. soil, sediments, oceans and freshwater), independently from the originating cells, or still associated to them (like in extracellular matrices or biofilms), and/or remain in the body of multicellular organisms (Nagler et al., 2018). ExDNA can be free or bound with either inorganic and/or organic particles (Gahan & Stroun, 2010; James Cleaves et al., 2011; Saeki et al., 2011; Torti et al., 2015; Gardner & Gunsch, 2017; Wood et al., 2020), that can be either present in the environment or be extruded from living beings, either from the native species or not. Specifically, it can be part of well-defined structures, for example matrices of organic compounds released by native organisms, e.g. defensive structures represented by neutrophil extracellular traps (NETs) (Brinkmann et al., 2004) and root extracellular traps (RETs) (Hawes et al., 2011). Moreover, the exDNA can also become part of heterologous organic material (e.g. bound to soil humic acids (Crecchio & Stotzky, 1998) or part of dissolved organic matters (DOM) found in marine ecosystem (Hansell & Carlson, 2014). Furthermore, it can be also enclosed in organic organelles, such as native extracellular vesicles of different type (e.g. microvesicles, apoptotic bodies and exosomes) (Thakur et al., 2014; Malkin & Bratman, 2020; Elzanowska et al., 2021) or presumably heterologous ones.

1.1.1 Features and fate

Once released, exDNA may undergo biotic degradation (mainly due to ubiquitous extracellular and cell-associated DNases) and abiotic decay (physical and chemical) or environmental long-term preservation (Levy-Booth et al., 2007; Nielsen et al., 2007; Torti et al., 2015; Nagler et al., 2018). It can persist as both double (Ascher et al., 2009; Breitbach et al., 2012; Torti et al., 2015) and single stranded (Torti et al., 2015; Burnham et al., 2016; Pathan et al., 2020) and more or less fragmented molecules (Levy-Booth et al., 2007; Ceccherini et al., 2009; Thierry et al., 2016; Nagler et al., 2018).

1.1.2 ExDNA in the soil

In the soil, the exDNA may derive from local or transient fauna or flora, including the microbiota (bacteria, archaea, fungi, protozoa and soil invertebrates) (Levy-Booth et al., 2007; Nielsen et al., 2007). Its persistence is influenced by soil pH, cation content, soil mineralogy and content of
humic substances (Gulden et al., 2009). Moreover, it can be found adsorbed on sand, clay and other soil colloids (Paget et al., 1992; Gardner & Gunsch, 2017) which can protect exDNA from nucleases mediated degradation allowing its long term persistence in the environment (Paget et al., 1992). Moreover, it is also an important component of RETs (Hawes et al., 2011; Wen et al., 2017; Driouich et al., 2019) which have been revealed to be plant defensive systems whose components (i.e. sugars, proteins, amino acids and DNA) are actively produced by root cap cells and released in the external part of the root apex (Vincent et al., 2020).

The analyses of exDNA in the soil even when fragmented, is currently being widely exploited to get information on soil microbial communities composition and ecosystem (Pietramellara et al., 2008; Nagler et al., 2020; Nannipieri et al., 2020; Nkongolo & Narendra-Kotha, 2020), and because of its long term persistence, the exDNA is also widely exploited to recall the historical biodiversity of the investigated environment (Cristescu & Hebert, 2018; Orwin et al., 2018; Taberlet et al., 2018) and for this reason it is commonly referred to as “ancient” DNA (Andersen et al., 2012; Epp et al., 2012).

1.1.3 ExDNA in marine and fresh water

In marine and in freshwater ecosystems, the exDNA is present either free in the water column or in sediments, in case complexed with colloids and other particles heavy enough to sink it to the bottom of the sea (Deflaun et al., 1986; Karl & Bailiff, 1989; Dell’Anno & Danovaro, 2005; Herndl & Reithaler, 2013; Torti et al., 2015). Moreover, despite less characterized than terrestrial biofilms, the exDNA has also been detected in the extracellular polymeric matrix (EPS) of aquatic microbial biofilms (Passerini et al., 2019). In these contexts, considering that marine biofilms are generally composed of a mixed community including marine bacteria and algae (mainly diatom), protozoa and fungi (Railkin, 2003; Briand et al., 2012; Pochon et al., 2015) it would not be unexpected to find DNA of different organisms (e.g. algae) as additional component of microbial biofilms EPS. However, the structure of these biofilms remains largely investigated (Mueller et al., 2006; Cole et al., 2014; Fanesi et al., 2019; Le Norcy et al., 2019) as does their EPS composition (Fanesi et al., 2019).

Particularly in marine ecosystems, the DNA not enclosed in living cells represents 90% of the total DNA pool (Herndl & Reithaler, 2013) and includes DNA released in situ (autochthonous) from sediment microbial communities or local living species, as well as DNA of pelagic and terrestrial origin transiently deposited to the seafloor (allochthonous) (Zafra et al., 2012).

The exDNA adsorption onto mineral matrices physically protects it from degradation allowing its long persistence (Lorenz & Wackernagel, 1987; Agnelli et al., 2007). Consequently, the analysis of exDNA content and variability in water samples allows the characterization of microbes and
other species shedding light on their dynamic changes, their possible interaction and evolutionary patterns (Corinaldesi et al., 2018). Similarly to soil, also in the case of marine ecology, the analyses of long term persisting exDNA in the sediments and in the water column allows investigation of the current and past biodiversity populating the marine environment in the observed contexts, providing useful hints on its evolutionary history (Coolen & Overmann, 1998; Coolen et al., 2004; Agnelli et al., 2007; Boere et al., 2011; Lejzerowicz et al., 2013; Armbrecht et al., 2019) and also interesting tools for biomonitoring surveys (De Schepper et al., 2019), shedding light on possible roles it could exert in the local ecosystem functioning. Moreover, another consequence of the exDNA long persistence is the increased possibility of being transported in flouting water and consequently, be detected in a location where organisms from which the exDNA derives, are not currently present (Shogren et al., 2017; Carraro et al., 2018).

1.1.4 ExDNA in multicellular organisms

In multicellular organisms the exDNA can be of endogenous origin, originated from apoptotic or necrotic cells (Grabuschnig et al., 2020) or actively released from living cells, as clearly highlighted by highly proliferating cells (Stroun et al., 1977) of plants (Wen et al., 2009, 2017) and vertebrates, such as cultured auricles of frog hearts (Stroun & Anker, 1972), cells from rabbit spleen tissues (Olsen & Harris, 1974), human lymphocytes (Anker et al., 1975) and activated neutrophils of different phyla (Hoessli et al., 1977; Neumann et al., 2020).

In the body of multicellular organisms, exDNA can also be of exogenous origin, i.e. derive from death or alive microbes, such as bacteria, viruses or fungi, either symbionts or pathogens, as widely reported in several species such as healthy rumens (beef steers) (Fernando et al., 2010), infected goats, bovines, and diseased humans (Leal-Klevezas et al., 1995), as well as healthy people (Hoffmann et al., 2013). This also happens as a result of pathogens infection (Zhang et al., 1998) or simply upon symbiotic relationships in plants (Clapp et al., 1995), as well as in invertebrates (Drezen et al., 2017), and among them in marine organisms, such as corals (Ziegler et al., 2019) and jellyfish (Peng et al., 2021).

Concerning its specific localizations in multicellular organisms, exDNA has been found freely in body fluids such as plasma, urine and saliva (O’Driscoll, 2007), or bound to cytoplasm constituents (e.g. lipids and proteins and other nucleic acid molecules, such as RNAs) (Gahan & Stroun, 2010), or to extracellular vesicles (Anker et al., 1975; Stroun et al., 1977; Thierry et al., 2016; Elzanowska et al., 2021) of different type, such as microvesicles (Balaj et al., 2011), apoptotic bodies (Xu et al., 2019) and exosomes (Thakur et al., 2014). Moreover, there is evidence of its presence on the outer leaflet of the plasma membrane as observed in human monocytes and B lymphocyte (Meinke et al., 1973; Bennett et al., 1986).
Furthermore, the exDNA has been also found as component of extracellular defensive structures known as NETs, which consist in aggregates secreted by mature and activated neutrophils of different phyla (e.g. Chordata, Arthropoda and Mollusca) (Neumann et al., 2020) which are composed by actin, histone, peroxidases, proteins and DNA (Brinkmann et al., 2004; Tamkovich & Laktionov, 2019; Sofoluwe et al., 2019) of both nuclear and mitochondrial origin (Yousefi et al., 2009).

Interestingly, the concentration of the exDNA circulating in blood has also been shown to correlate with the severity of several diseases such as cancers (Hawes et al., 2015), inflammatory (Fuchs et al., 2010) and autoimmune disorders (Vakrakou et al., 2018), hypertension (McCarthy et al., 2015), Parkinson and Alzheimer diseases (Lowes et al., 2020a). These paved the way for the non-invasive diagnosis of pathological conditions, therapy monitoring and follow-up tests (Thierry et al., 2016). Of note, free circulating exDNA can both be causative or a consequence of pathological conditions.

For example, considering its role in favouring the establishment of diseases, an improper recognition, clearance and degradation of exDNA (of both mitochondrial and nuclear origin) released into circulation following apoptotic processes (Hochreiter-Hufford & Ravichandran, 2013) or defects of systems operating in its removal (Stetson et al., 2008), causes uncontrolled inflammation and eventually autoimmune diseases, particularly when cross react with other proteins or when oxidized following oxidative stress (Lood et al., 2016).

The inflammation is the consequence of the exposure to self-antigens (i.e. exDNA) which can be recognized by macrophages that activate cytotoxic T cells and consequently prime an immune response. Among the autoimmune diseases, the Systemic Lupus Erythematosus (SLE) is well known for being associated with the production of autoantibodies against many macromolecules and particularly relevant are those against nucleic acids. Currently, the model for SLE development recognizes both the massive cell death and the impairment of clearance mechanisms as causes for the increase of the amount of nuclear material in the extracellular space which triggers marked inflammation and damage of multiple organ systems (Pisetsky, 2019).

In the context of NETs, once microbes have been removed, these extracellular matrices are degraded by DNases and macrophages. Of note, in case of DNases deficiency, mice die within few days after neutrophil activation because of blood vessels occlusion caused by persistent NET structures (Jiménez-Alcázar et al., 2017).

Moreover, Savchenko et al. demonstrated that in the early response to myocardial ischemia/reperfusion injury, DNA fragments released within the NETs contribute to worsen cardiomyocyte dysfunction, possibly by acting as DAMPs (Savchenko et al., 2014).
Consistent with the observation in mice, the persistence of NETs can have serious negative consequences in humans, leading to several diseases such as cardiovascular, lung and eye diseases, atherosclerosis, rheumatoid arthritis, thrombosis, diabetes, cancer, and severe COVID-19 (Demers et al., 2012; Arazna et al., 2013; Brinkmann, 2018; Erpenbeck et al., 2019; Daniel et al., 2019; Leppkes et al., 2020).

However it is possible also that already established pathological conditions favour the release of exDNA. For instance, NETs themselves are produced following a microbial infection activating neutrophils that release, among the other components, DNA into the extracellular space having here an active role in the disruption of bacterial cell leading to cell lyses (Halverson et al., 2015).

Also in case of autoimmune disease such as rheumatoid arthritis, the high rate of apoptotic processes lead to an increased release of DNA in the synovium of patients with arthritis (Firestein et al., 1995). This, putatively occurring also in other autoimmune diseases, may trigger an auto-amplifying phenomenon characterized by cell damage, followed by the release of cytoplasmic and nuclear content (containing also DNA) that, being present in an anomalous compartment and concentration, activate the immune system leading to an overall worsening of the pathological condition.

Moreover, sterile inflammatory conditions (i.e. not caused by microbial infection but stimulated in case of injury or trauma) and processes involved in tissue repairs have been shown to increase the level of exDNA (Rock & Kono, 2008). For example, the sterile inflammation characterizing obesity induces adipose tissue cell degeneration and turnover has been demonstrated to enhance the release of DNA into the plasma of mice and humans (Nishimoto et al., 2016).”

Despite the lower concentration if compared with that in patients affected by diseases, it is possible nowadays to detect the exDNA also in healthy subject (Kustanovich et al., 2019). For instance, the fetal DNA was found in the maternal plasma and thus it could be useful for non-invasive detection of fetal genetic disorders (Lo et al., 1997). Moreover, since present on the surface of cells (Meinke et al., 1973; Bennett et al., 1986) it is quantitatively detectable and spatially mapped by the technique of atomic force microscopy on palm and buccal cells at the single cell level (Wang et al., 2017). Wang et al., presented the atomic force microscopy as a promising tool for the evaluation of the spatial distribution and structural properties of exDNA on epithelial cells from different donors determining their characteristic surface profile and allowing the comparison across each cell type by using samples obtained from different individuals. Since surface DNA profiles have been shown to be particular for different donors, they could be a potential tool for forensic identification at the single cell level (Wang et al., 2017).

As also discussed above for soil and aquatic environments, the DNA extracted from archaeological or fossil remains provides valuable information on our evolutionary and
demographic history and on our genetic relationship with extinct human populations or closest relatives (Gilbert et al., 2005; Patterson et al., 2006; Slatkin & Racimo, 2016). Indeed, recent advances in high-throughput sequencing analysis allowed the generation of draft genomes sequences of our extinct hominin relatives, the Neanderthals (Green et al., 2010) and also an almost complete list of genetic changes occurring in modern humans since their divergence from Denisovans (Meyer et al., 2012), an extinct relative of Neanderthals. These findings provide valuable information on genomic regions that may have been positively selected in ancestral modern humans, (e.g. genes involved in metabolic processes and in the development of nervous and skeletal systems). Moreover, the recent analysis of the ancient human microbiomes or pathogens that were present in the organism of the individual at the time of death (mainly found in frozen or mummified tissues but also in coprolites and dental calculus) could shed light on ancient human life style and diseases and potentially contribute to the advance of modern medicine (Kazarina et al., 2019). In plants, the presence of exDNA located outside plants cells is little discussed and mostly unknown (Bronkhorst et al., 2021). It has been demonstrated that the DNA could be in part present outside the nucleas within the cytoplasm of meristematic root cells (Chayen & Norris, 1953) and that the DNA from exogenous sources, such as from bacteria, is released into plant cells (Stroun et al., 1970) and is putatively involved in the pathogenesis of plant disease (i.e. crow gall disease caused by Agrobacterium tumefaciens) (Stroun et al., 1971). Nevertheless, most of the recent literature focused the attention on exDNA as structural component of the plant root defensive structures similar to NETs, i.e. RETs (Hawes et al., 2011; Wen et al., 2017; Driouich et al., 2019). RETs are produced by highly proliferating cells, located in the external part of the root apex, adjacent to the apical meristem. These cells secrete a mucilaginous substance surrounding the plant root cap which is mostly composed by carbohydrates, proteins, amino acids and also DNA of mainly nuclear origin (Hawes et al., 2012; Driouich et al., 2019) ranging in size from 150 bp to approximately 5 kb and largely composed of repetitive sequences (Wen et al., 2009; Hawes et al., 2012). Many roles have been described for RETs. For example, they have a lubricant protecting role of the root tips and are involved in propagation of gravitropic signals (Moore et al., 1990) from the root cap to the root tip, they are involved in the protection of roots from the toxicity deriving from ions and also function as pathogen trap (Driouich et al., 2019).

1.1.5 ExDNA in microbial biofilm

The extracellular polymeric substance of both terrestrial and marine biofilms (Flemming & Wingender, 2010; Flemming et al., 2016a; Decho & Gutierrez, 2017; Abada & Segev, 2018), as well as in biofilms of clinically relevant microorganisms such as Staphylococcus spp., Streptococcus spp., Candida spp., Pseudomonas aeruginosa (Mann et al., 2009), is mainly
composed of carbohydrates, lipids, proteins and nucleic acids including fragmented or intact exDNA mostly derived from genomic DNA (Panlilio & Rice, 2021) (from 400 up to 10000 bp (Zafra et al., 2012; Oyama et al., 2016)). The exDNA found in microbial biofilms can come from lysed cells (Sutherland, 2001; Barnes et al., 2012; Brockson et al., 2014; Okshevsky & Meyer, 2015; Rose & Bermudez, 2016) or be actively secreted by microorganisms that produce the biofilm itself (Kadurugamuwa & Beveridge, 1995; Peterson et al., 2013; Grande et al., 2015; Gallo et al., 2015; Kim et al., 2018; Kassinger & van Hoek, 2020).

In these contexts, it functions as structural component during biofilm formation and development (Ascher et al., 2009). In particular, it is required during the initial surface adhesion and aggregation of microbial cells (Das et al., 2010; Tang et al., 2013), it helps the extracellular matrix gelification (Svein et al., 2019), the maintenance of specific cell orientations (Gloag et al., 2013). Moreover, it controls the viscoelastic relaxation of the biofilm preventing mechanical stress conditions (Peterson et al., 2013) and it is required for the induction of morphological changes such as the transition from yeast to hyphal growth during Candida albicans biofilms development (Payne & Boles, 2016). Since the exDNA is a structural component of biofilms, it represents a suitable target for the treatment of biofilms produced by human pathogens in order to damage their structural integrity and thus increase susceptibility of the biofilm to antibiotic agents (Ye et al., 2017; Rocco et al., 2017; Koo et al., 2017).

1.2 Structural roles and bioavailability of exDNA

As mentioned, the presence of exDNA outside cells, either in multicellular organisms or in the environment, reveals bioactive roles beyond the confinement of cells. Indeed, it has been initially described as source of organic phosphate in soil (Levy-Booth et al., 2007) and in aquatic environments (Dell’Anno & Danovaro, 2005) and also as building blocks for newly synthetized nucleic acids (Morrissey et al., 2017). Moreover, exDNA has a protective role in extracellular structures produced by animal neutrophils, plant root cap cells and bacteria, i.e. NETs (Brinkmann et al., 2004), RETs (Hawes et al., 2011) and biofilms (Mulcahy et al., 2008), respectively. Of note, it has demonstrated to contribute to the phenomenon of horizontal gene transfer (HGT) in bacteria and archaea (Lorenz & Wackernagel, 1994; Merod & Wuertz, 2014; Orwin et al., 2018) but also in eukaryotes (Boto, 2010). Interestingly, exDNA functions as a signaling molecule enhancing the lateral roots and root hairs growth in plant (Paungfoo-Lonhienne et al., 2010) and as an endocrine signaling molecule in vertebrates, where alone, or enclosed within RNA/lipoprotein complexes, is able to modify the biology of the recipient cells (Gahan & Stroun, 2010). Recently, it has been demonstrated that exDNA can also negatively affect the growth of
conspecific organisms of different phyla by producing inhibitory effects on their growth (Mazzoleni et al., 2015a,b). The following sections will be dedicated to each of these roles.

1.2.1 Protective function

In animals, the exDNA has a demonstrated protective role as a component of NETs. Indeed, because of its phosphodiester backbone, it chemotactically attracts and immobilises pathogens promoting the disruption of microbial membrane integrity and leading to cell lyses (Brinkmann et al., 2004; Hawes et al., 2015).

In plants, the exDNA has protective role in the extracellular structures of RETs (Hawes et al., 2011) considered as pathogen traps protecting the root tip meristem (Driouich et al., 2019). The exDNA located in RETs functions as scaffold for the adhesion of microbes, preventing their spreading throughout the organisms, also exerting a direct bactericidal function (Halverson et al., 2015), resulting a relevant component in the plant innate immunity response to pathogen invasion (Hawes et al., 2011, 2016; Wen et al., 2017; Driouich et al., 2019).

The exDNA plays a protective function also in biofilms. Being negatively charged, exDNA chelate cationic antimicrobials (Mulcahy et al., 2008) and protects against aminoglycosides (Chiang et al., 2013).

1.2.2 Nutritive function

In soil and in the seawater, exDNA has nutrient functions and may act as energy source especially in environment with low nutrient input (Levy-Booth et al., 2007; Hawes et al., 2016). For example, exDNA represents approximately 10% of the organic phosphate pool available in soil (soil organic phosphorus) (Dalai, 1977; Nagler et al., 2018). Moreover, it has been shown that after its breakdown by extracellular and cell-associated DNases, smaller exDNA molecules are taken up by microbial cells, where they either serve as building blocks for newly synthetized nucleic acids or are further broken down to essential nutrients such as phosphate, carbon, nitrogen and oxygen (Morrissey et al., 2017). In marine ecosystems, the exDNA, is important for deep-sea benthic microbial metabolism, where eventually, it can substantially contribute to phosphate cycling (Dell'Anno & Danovaro, 2005).

1.2.3 Genetic material

During the twentieth century, the concept of HGT via transformation was revealed by eminent scientists (Griffith, 1928; Avery et al., 1944), even before the discovery of the DNA structure, as
the process by which unicellular and/or multicellular organisms can uptake and incorporate exogenous genetic material from their surroundings. Indeed, these authors demonstrated that DNA in the environment can transform bacteria so that they can express a different phenotype. The role of exDNA in the context of HGT in marine and terrestrial microbial biofilms has been addressed in several studies (Lorenz & Wackernagel, 1994; Merod & Wuertz, 2014; Orwin et al., 2018). Indeed, biofilms offer ideal conditions for HGT because of the high cell density, a circumscribed environment and the accumulation of exDNA. Despite its binding to various minerals, the long persistence of exDNA in these structure preserves its ability to transform competent microbial cells leading, for example, to antibiotic resistant bacteria, to homologous recombination of foreign DNA into the host chromosome with relevant evolutionary implications (Poté et al., 2003; Levy-Booth et al., 2007). Indeed, homologous recombination of foreign DNA is believed to have played a major role in bacteria evolution (Flemming et al., 2016b). The HGT was initially been described in the microbial world. For example, it is well known that between 10% and 16% of the Escherichia coli chromosome arose through lateral gene transfer (Ochman et al., 2000) and also that B. subtilis has remarkable ability to become naturally competent for transformation (Sisco & Smith, 1979). Later, it was demonstrated to occur not only in Bacteria and Archaea but also in eukaryotes (Boto, 2010). A first example concern the occurred HGT from the chloroplast and mitochondrial genomes to the nuclear genome that according with the endosymbiotic theory, probably originated from bacterial endosymbionts (a cyanobacterium and an alpha-Proteobacterium, respectively) of a progenitor to the eukaryotic cell (Blanchard & Lynch, 2000). Interestingly, cytosolic and plastid glyceraldehyde-3-phosphate dehydrogenase genes have been probably laterally transferred between different lineages of algae (i.e. dinoflagellate and euglenophyte) (Takishita et al., 2003). Moreover, in plants, DNA can be released by plant cells and it can move freely throughout the plant from cell to cell, presumably via the plasmodesmata, entering cells and nuclei and integrate into the genome of host cells, being able to be transcribed and expressed (Gahan & Chayen, 1965; Gahan, 2003). In vertebrates, it was demonstrated that nucleic acids can possibly enter the somatic as well as reproductive cells. For example, Sopikov (Sopikov, 1954) demonstrated that several blood transfusion in birds from black Australorp hens to White Leghorn hens, induced in the progeny of the receivers inheritable acquired characteristics. Benoit confirmed that the injection of pure DNA from a different breed of duck induced inherited morphological characteristics in the recipient one (Benoit et al., 1960).

Particularly in microbial context, it has been explored the mechanism through which the DNA uptake occur. During the process of transformation, the exDNA is recognized and integrated through homologous recombination implicating a mechanism for the discrimination between sequences deriving from different sources. Of note, the mechanism of exDNA uptake appears to mostly occurs via a sequence independent mechanism (Chen & Dubnau, 2004) even though in
some gram negative species (e.g. *Haemophilus influenzae* and *Neisseria* species) the uptake of DNA is favoured by the presence of specific sequences called DUS (DNA uptake sequences). As the genomes of these bacteria are enriched in their respective DUS, uptake of homospecific DNA is favoured (Chen & Dubnau, 2004).

Once the DNA has been taken up, it may become part of the genome of recipient bacterium or, upon recognition of particular characteristics, targeted for degradation.

Indeed, the well-known bacterial DNA restriction-modification (R-M) system is a defensive strategy which modifies the bacterial DNA by transfer methyl groups to cytosines and adenines. Once methylated, the bacterial DNA is protected from sequence-specific restriction endonucleases that recognize and degrade only the unmethylated DNA, or “inappropriately” methylated DNA from the invading phage (Wilson & Murray, 1991; Krieg *et al.*, 1995). A second mechanism for the recognitions of foreign nucleic acids is the CRISPR-Cas system (acronyms for “clustered regularly interspaced short palindromic repeats- CRISPR-associated protein”). This system is based on the integration of short fragments of phage’s DNA, called protospacers, in the bacterial genome. These protospacers are transcribed in short RNAs that are then used by the Cas nuclease as a guides to detect and specifically degrade the phage DNA. The ‘self’ versus ‘nonself’ discrimination operated by CRISPR–Cas system requires specific short sequences (of 2–3 nucleotides), which are collectively known as protospacer adjacent motifs, located adjacent to the targeted protospacers in the invading DNA (Dupuis *et al.*, 2013; Shah *et al.*, 2013; van der Oost *et al.*, 2014). Interestingly, it has been reported a functional synergy between an R–M system and CRISPR–Cas in *Streptococcus thermophilus*, which suggests that fragments of the invader DNA that are generated by the restriction-modification system might be potential substrates for spacer acquisition (van der Oost *et al.*, 2014).

Another cellular machinery is the system *RecBCD* which functions both in the elimination of foreign genomes and in the repair of bacterial DNA through recombination. It distinguishes the bacterial genome from that of the invading phage by recognition of a cis element called *Chi* sequence which is absent in phage’s genome and different among bacteria (Vasu & Nagaraja, 2013). The action of the *RecBCD* machinery depends on the interactions between three genetic elements within the cell, the R-M system, the invading DNA, and the *RecBCD/Chi* system. The R-M complex attacks any unmodified recognition site that could be on the DNA of an invading phage or on the bacterial chromosome. From the restricted double-strand break, the *RecBCD* enzyme starts to hydrolyse the DNA. If the enzyme encounters a *Chi* sequence, which is a marker of bacterial genomes, degradation stops, and a recombinational repair would start (Handa *et al.*, 2009; Vasu & Nagaraja, 2013; Liu *et al.*, 2017).
1.2.4 Signalling molecule

Despite still poorly characterized, there is evidence of the role of exDNA as a signalling molecule. For example, exDNA appears to influence plant physiology. In fact, the presence of exDNA from herring sperm in the plant growth medium enhances the lateral roots and root hairs growth (Paungfoo-Lonhienne et al., 2010). Since the growth medium was fully P-supplied, this phenomenon induced by exDNA cannot be ascribed to its role as source of inorganic phosphate. Indeed, the growth effect was discussed in terms of the triggering of the expression of peptide hormone genes that control root morphology (Paungfoo-Lonhienne et al., 2010).

Interestingly, the role of exDNA in animals and in particular in human biofluids has been also discussed at the light of its function as an endocrine signalling molecule, where alone, or enclosed within RNA/lipoprotein complexes, is able to modify the biology of the recipient cells (Gahan & Stroun, 2010). Of note, it has also been discussed in the transformation of normal cells into cancer cell (Bendich et al., 1965; Gahan & Chayen, 1965; García-Olmo et al., 2000, 2010; Gahan, 2003; Hawes et al., 2015). Accordingly, in 2000 Garcia-Olmo and colleagues proposed the term of “genometastasis”, referring to the occurrence of metastasis via the transfection of cells with dominant oncogenes derived from a primary tumour and circulating in the plasma (García-Olmo et al., 2000; Gahan & Stroun, 2010) inducing cell transformation, tumorigenesis and tumour progression in vivo (Trejo-Becerril et al., 2012).

In multicellular organisms, the exDNA has been discussed in the context of pathogen-associated molecular pattern (PAMP), i.e. molecules originating from pathogens that are recognized by and activate cells of the innate immune system in both plants and animals. For example, it is well-known in animals that cells express different types of receptors able to recognize specific aspects of microbial DNA that, once activated, trigger the immune system and the inflammatory responses (Desmet & Ishii, 2012; Gasser et al., 2017; Wang et al., 2019a; Motwani et al., 2019).

For example, the evolutionary conserved toll like receptor 9 (TLR9) located in the endosomes of specialized cells of the immune system, recognizes the unmethylated CpG-rich DNA, which is more common in bacteria than in the mammalian DNA (Barton et al., 2006). As an example of nuclear systems recognizing DNA, the apparatus for the DNA damage repair (i.e. MRE11/RAD50/NBS1 complex (MRN)) can also recognize foreign nucleic acids in infected cells (Desmet & Ishii, 2012; Pateras et al., 2015). Moreover, the cytosolic DDX41, member of the DExD/H-box helicase superfamily involved in the cell-intrinsic antiviral response, was ubiquitously expressed in various cells (such as dendritic cells and macrophages) and it seems to directly bind dsDNA, bacterial cyclic dinucleotides such as cyclic di-AMP and cyclic di-GMP or viral DNA and to induce interferon mediated pro-inflammatory responses (Desmet & Ishii, 2012; Gasser et al., 2017). In plants, the role of exDNA as a PAMP was also discussed. Indeed, the exDNA of bacterial origin is well-known to trigger the immunological response with the
formation of reactive oxygen species (ROS) and callose deposition (Yakushiji et al., 2009). Interestingly, the treatment with a mixture of different phytopathogenic microbe fragmented exDNA increased phenols and phytotoxic compounds concentrations in chili pepper, which are known to activate the hypersensitive response through the synthesis of salicylic acid. This response induced a protective effect against wilt and root rot disease (Serrano-Jamaica et al., 2021). Interestingly, the application of short sequences of synthetic exDNA with cytosine-phosphate-guanine oligodeoxynucleotide motifs reduced the lesions induced by the pathogenic fungus Zymoseptoria tritici in leaves of wheat plants (Le Mire et al., 2019).

The self-exDNA has been also proposed to act as a "Damage-associated molecular pattern" (DAMP) (Seong & Matzinger, 2004; Duran & Heil, 2018; Roh & Sohn, 2018; Monticolo et al., 2020) that, when present in an anomalous compartment, may trigger the activation of a non-infectious inflammatory response (Seong & Matzinger, 2004; Roh & Sohn, 2018). This occurs in animals, where self-exDNA of nuclear or mitochondrial origin correlates and/or determines various types of diseases (Fuchs et al., 2010; Hawes et al., 2015; McCarthy et al., 2015; Vakrakou et al., 2018), such as cancers (Hawes et al., 2015), hypertension (McCarthy et al., 2015), and autoimmune diseases such as rheumatoid arthritis (Rykova et al., 2017) and systemic lupus erythematosus (Barrat et al., 2005).

In plants, the evaluation of transcriptional response of Arabidopsis thaliana to self-exDNA revealed an upregulation of genes involved in ROS and calcium signalling and, of note, no differential expression in some marker genes commonly known for being regulated by phytohormones and related to plant defence (such as Pathogenesis-related gene 1 regulated by salicylic acid, Plant defensin 1.2, Vegetative storage protein 2 and Jasmonate resistant 1 regulated by jasmonic acid and Ethylene response factor 2 and 5 triggered by ethylene. Moreover, also in the case of self-exDNA treatment A. thaliana showed a phenomenon of resistance against pathogenic bacteria and fungi (Rassizadeh et al., 2021).

1.3 Novel findings on exDNA

Recently, a novel and interesting research line revealed for the exDNA additional roles depending on its nature, environmental location and structural organization.

In 2015, Mazzoleni and his colleagues demonstrated for the first time that fragmented exDNA accumulating in litter during the decomposition process, produces a concentration dependent and species-specific inhibitory effect reducing root growth and seed germination of conspecifics.

In particular, within the same work, they experimentally demonstrated that the exposure to fragmented self-exDNA (conspecific or similar/homologous DNA) differently from the exposure to nonself-exDNA (heterologous, DNA from phylogenetically unrelated species, or, more in
general, distant in sequence similarity terms), inhibits root growth in plants in a concentration dependent manner (Mazzoleni et al., 2015a) (Figure 1.1a). Moreover, they showed that A. thaliana exDNA inhibited the growth of Lepidium sativum seedlings and vice versa, but DNA did not inhibit Acanthus mollis growth (Mazzoleni et al., 2015a). Interestingly, A. thaliana and Lepidium belong to the same order (Brassicaceae), whereas the Acanthaceae belongs to a different order, the Lamiales. Similarly, it was also showed that Capsicum chinense DNA inhibited Lactuca sativa (both Asterales), whereas DNA extracted from Acaciella angustissima (Fabales) did not (Vega-Muñoz et al., 2018), and DNA from lima bean inhibited common bean growth whereas DNA from Acacia farnesiana did not (Duran-Flores & Heil, 2018). These evidences supported the observation that the closer the organism phylogenetic distance, the higher the inhibitory effect of nonself-exDNA on the treated plant (Mazzoleni et al., 2015a).

Later, the same research group demonstrated that the inhibitory effect of self-exDNA, compared to nonself DNA, is a general phenomenon, since the growth inhibition was also demonstrated for other species of different taxonomic groups (Mazzoleni et al., 2015b) (such as algae, bacteria, and fungi) (Figure 1.1b).

Such findings had relevant implications for plant-soil ecological theories, providing a chemical basis for autotoxicity (Mazzoleni et al., 2010), among the mechanisms of plant–soil negative feedback (van der Putten et al., 2016), i.e. the establishment in soil of negative conditions affecting plant growth induced by the plants themselves (Klironomos, 2002) influencing natural plant communities (Van der Putten et al., 1993) and involved in species coexistence (Bonanomi et al., 2005) and unexpected new functional roles of exDNA in species interactions at community (Zhang et al., 2016) and ecosystem (Carteni et al., 2016) levels.

Considering the results of these pioneering studies, to further clarify the novel role of exDNA in plant ecology and physiology, other research groups contributed with subsequent investigations. Indeed, in 2016, the experiments of Barbero and colleagues demonstrated that the exposure to fragments of self-exDNA, differently from nonself-exDNA, induced intracellular calcium signaling and plasma membrane depolarization in Phaseolus lunatus and Zea mays, which characterize the early immune signaling responses in plants (Barbero et al., 2016) (Figure 1.2a). Moreover, in 2018, Duran-Flores and Heil demonstrated that the treatment of Phaseolus vulgaris with self-exDNA inhibits seed germination and triggers events typical of the plant immunological response, e.g. H$_2$O$_2$ production, mitogen-activated protein kinase (MAPK) activation, extrafloral nectar release (Figure 1.2b), in combination with a decreased susceptibility to infection by the bacterium Pseudomonas syringae (Duran-Flores & Heil, 2018).
Interestingly, it was shown that the treatment with both self-exDNA and nonself-exDNA from closely related species caused significant CpG DNA hypomethylation at similar DNA concentrations. This result supports the evidence that the phylogenetic closeness is an important feature for plant responses to exDNA. Moreover, authors pointed out that the extent of the plant response could depend from the degree of damage detected by the plant (the concentration of either self- or nonself-exDNA from closely phylogenetically related species) (Vega-Muñoz et al., 2018). However, to the best of our knowledge, the involvement of epigenetics in the recognition of different genomes in plants and in other multicellular organisms is still largely unknown.”

**Figure 1.1.** Inhibitory effect of fragmented self-exDNA on plants (a) and other organisms of different taxonomic group (b). Bacillus subtilis (B. subtilis), Trichoderma harzianum (T. harzianum), Physarum polycephalum (P. polycephalum), Acanthus mollis (A. mollis), Scenedesmus obliquus (S. obliquus), Sarcophaga carnaria (S. carnaria), Compl. metha (%): completed metamorphosis (%); indicating the % of larvae completing their development; Spore germ (%): Spore germination (%). Adapted from (Mazzoleni et al., 2015a,b).
Figure 1.2. Physiochemical responses induced by self-exDNA in plants. Plasma membrane depolarization (Vm depolarization) and intracellular calcium release after treatment with fragmented self-exDNA (a) and increased ROS production, EFN secretion and MAPK activations after treatment with self-exDNA differently from nonself-exDNA (b). The second panel shows the relative percentage of the Ca\(^2+\) released after treatment with self-exDNA and monitored by using the Calcium orange (CO) dye. Adapted from (Barbero et al., 2016; Duran & Heil, 2018).

1.4 Hypothesis of sensing and mechanism of action of exDNA

The mechanisms behind the differential response of plants (and also other organisms) to self- and non-self-exDNA remains to be understood.

Among the hypothesis, following this first experimental evidences, Mazzoleni and co-workers suggested that the inhibitory effect of self-exDNA, because of its specificity and its occurrence also with DNA of related species, could be the result of a mechanism resembling the “well-known
processes of interference based on sequence-specific recognition of small-sized nucleotide molecules” (Mazzoleni et al., 2015a) and thus, following its uptake, DNA could affect cell functionality in different manners (Carteni et al., 2016).

In particular, once specifically recognized the “homologous” or “highly similar” DNA fragments could either interact with cytoplasmic RNA causing the formation of RNA/DNA hybrids, or directly interact with the genome structure through a mechanisms similar to the Small Fragment Homologous Replacement (Carteni et al., 2016). This could lead to the formation of structures that activate mechanisms of DNA repair allowing the integration of small DNA fragments into the genomic DNA, thus affecting cellular activities (Carteni et al., 2016).

Duran-Flores and Heil suggested other possible mechanisms to explain the inhibitory effect of self-exDNA. Similarly to what suggested in Mazzoleni et al 2015 (Mazzoleni et al., 2015a), one of the proposed mechanisms would consist in a direct uptake of fragmented DNA into cells with subsequent interference with essential biological processes (Duran-Flores & Heil, 2015). Interestingly, this research group (Vega-Muñoz et al., 2018), in line with the previous finding and hypothesis (Mazzoleni et al., 2015a; Barbero et al., 2016; Duran & Heil, 2018), suggested that the intensity of the damage induced by self- and nonself-exDNA could be related to the degree of “self” detected by the plant, and thus depending on the concentration of either self-exDNA or nonself-exDNA and on the phylogenetically distance of nonself-exDNA.

Duran-Flores and Heil in 2015 suggested that, in addition to the interference phenomenon mediated by small fragment of DNA taken up by cells, an additional or alternative hypothesis could be the presence of specific membrane receptor that, once activated by the self-exDNA, triggers an intracellular signalling cascade (Duran-Flores & Heil, 2014, 2015). Nevertheless, they pointed out that, in agreement with what has been proposed by Veresoglou in 2015 (Veresoglou et al., 2015), it would be difficult to have specific receptors for all possible sequences located on plant cell surface. Moreover, Veresoglou suggested that receiver plants would not have any advantages in investing energy to decipher each self-exDNA sequence. Accordingly, Duran-Flores and Heil suggested that self-exDNA may function as a DAMP activating a costly resistance mechanisms to signal a hostile environment causing the death of many conspecifics (Duran-Flores & Heil, 2015). This could ultimately leads the delay in seed germination observed by Mazzoleni in 2015.

Nevertheless, in plants, no specific DNA receptor has been reported yet. (Bhat & Ryu, 2016)

Finally, in 2018, Vega-Muñoz et al. also suggested that the exDNA methylation patterns could explain the mechanism for self-exDNA recognition in plants (Vega-Muñoz et al., 2018).
Despite the attempts to define a putative mechanism cited above, currently, the mechanisms underlying the specific recognition of either nonself- or self-exDNA and subsequent responses in plants is still poorly understood (Bhat & Ryu, 2016).

1.5 Potential pharmacological and biotechnological applications

Collectively, these novel results pave the way for the development of novel pharmacological and biotechnological application. Indeed, the possibility to use the fragmented DNA of a target species can be fundamental in the context of biological control of the same species through the production of selective compounds with pharmacological activity also improving the performance of antibiotic applications (Mazzoleni et al., 2014).

The main advantage of such applications would be the production of a highly selective inhibitory effect on organisms with homologous DNA presumably impairing several cellular functionalities at the same time.

A schematic representation of the steps required for such conceptual self-exDNA treatment is depicted in Figure 1.3 a. The possible applications are shown in Figure 1.3 b-d.
Figure 1.3. Potential pharmacological and biotechnological applications of self-exDNA. Conceptual representation of the use of self-exDNA for biological control (a) and examples of potential practical applications (b). Adapted from (Mazzoleni et al., 2014).
AIM OF THE PROJECT

Mazzoleni et al., (2015a) showed that fragments of self-exDNA, differently from nonself-exDNA, produce a concentration dependent and species-specific inhibitory effect in plants, reducing root growth and seed germination of conspecifics (Mazzoleni et al., 2015a). Moreover, they induce intracellular calcium signalling and plasma membrane depolarization and also triggers well-known markers of the immunological response, e.g. H$_2$O$_2$ production and MAPK activation (Barbero et al., 2016; Duran & Heil, 2018). Following the results on plants, the inhibitory effect of self-exDNA on other species was also demonstrated by Mazzoleni and his group and, on the basis of these findings, they suggested the generality of the observed phenomenon which opens new perspectives in the context of self-inhibition processes (Mazzoleni et al., 2015b).

Nevertheless, several points remain to be addressed. For instance, the confirmation of these effects in aquatic environment, in unicellular and multicellular organisms, the specific processed involved at cellular level and triggered by the exposure to nonself- or self-exDNA.

The presented project aims at investigating the effects of the exposure of organisms from model species to nonself- and self-exDNA. In particular, one of the main objectives, is to confirm the inhibitory role of self-exDNA when compared to the exposure to a nonself-exDNA from a phylogenetically distant species as highlighted for the first time in plants and then also in other species by Mazzoleni and colleagues in 2015 (Mazzoleni et al., 2015a,b), and test its effects in unicellular and multicellular species.

Accordingly, in order to further characterize this phenomenon, we selected different model species including representatives of unicellular and multicellular organisms from animals and plants (Table 1.1).

The presented efforts start with the analysis of the early response to the exposure to nonself- and self-exDNA in multicellular autotrophs. This was assessed by cellular and molecular investigations in the first 16 hours post treatment (hpt). Gene expression analyses and confocal microscopy were used to reveal exDNA spatial distribution at root and cellular levels and provide preliminary insights on the early molecular responses to both type of molecules. The results are included in chapter 2, entitled “exDNA AND MULTICELLULAR AUTOTROTROPHS: EFFECTS ON ARABIDOPSIS THALIANA”.

Then we planned to analyse the effects of the exposure to nonself- and self-exDNA in microalgae, as representatives unicellular autothophs, living in aquatic environments. This effort aimed at testing the acquired knowledge in simpler organisms, while confirming the differential responses also in aquatic environments. In order to evaluate the effects of nonself- and self-exDNA treatments in both marine and freshwater, the two microalgae Nannochloropsis gaditana and
*Chlamydomonas reinhardtii* were considered as model species for the two contexts, respectively. The effects of exDNAs treatments were assessed by evaluating both cellular density and morphology. The analysis and the results are presented in the chapter three titled “exDNA AND UNICELLULAR EUKARYOTIC ORGANISMS: EFFECTS ON MICROALGAE”.

The effects of exDNA were also tested on human cells. The aim was to consider simplified systems representing non-photosynthetic species of general interest, also to highlight potential differences and similarities among the responses from cells from different phyla. In chapter 4, entitled “exDNA ON MULTICELLULAR HETEROTROPHS: EFFECTS IN HUMAN CELL LINES”, we show the response of human cells after the treatment with nonself- and self-exDNAs. Exploiting *in vitro* cell cultures offered the possibility not only to test the universality of the differential effects of exDNA, but also to study how cells of higher multicellular organisms (i.e. humans) respond to external stimuli (Carter & Shieh, 2010; Kaur & Dufour, 2012). For these reasons, the PNT2 cell line (human prostatic epithelial) were analysed under exposure to both nonself- and self-exDNA, in terms of cell viability and changes of morphological and physiological features.

Finally, to further characterize the phenomenon in multicellular organisms living in marine environment, in chapter 5 titled “exDNA ON MULTICELLULAR HETEROTROPHS: EFFECTS IN *CIONA ROBUSTA*”, we report on the effects of exDNA, of either nonself- or self-origin, on the chordate *Ciona robusta* (Asciidaeae). This marine sessile invertebrate is considered as the closest living relatives of vertebrates. For simplicity and advantageous phylogenetic position, ascidians have recently emerged as a model system to investigate the molecular mechanisms underlying cell-fate specification during development (Holland *et al.*, 2004; Delsuc *et al.*, 2006). The model *Ciona robusta* was used to investigate the exposure to exDNAs on the main stages of the chordate embryos development and, thus, to infer on its potential role on developmental processes.

In the last chapter entitled “Final discussion” an evaluation of the results from the overall effort is presented, with the aim to overview on peculiarities or similarities in the responses triggered by the exposure to nonself- and self-exDNA in the biological systems here analysed.
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<th>Organisms/cellular system</th>
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<td><em>Nannochloropsis gaditana</em></td>
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<td><em>Arabidopsis thaliana</em></td>
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*Table 1.1. Species selected as models for unicellular and multicellular organisms in the presented work.*
Contribution statement

Prof. Stefano Mazzoleni¹, Prof. Maria Luisa Chiusano¹, Dr. Pasquale Termolino², Dr. Chiara Colantuono³, Dr Guido Incerti¹, Dr. Giovanna Benvenuto⁵ and Francesco Monticolo¹ contributed to the work of *A. thaliana*. Prof. Stefano Mazzoleni was the group coordinator; Prof. Maria Luisa Chiusano and Dr Pasquale Termolino conceived the experiment and defined its design; Professor Mazzoleni and Professor Maria Luisa Chiusano supervised the analyses and interpreted the results; Pasquale Termolino performed the lab experiments; Chiara Colantuono and Francesco Monticolo performed the bioinformatic analysis supervised by Prof. Maria Luisa Chiusano; Dr Giovanna Benvenuto support the fluorescence microscopy work; Dr. Guido Incerti performed statistical analysis. The author of this thesis contributed in performing the microscopy analysis, in performing the lab experiments and also for the analysis of bioinformatics results.

Dr. Clementina Sansone⁵ and Dr. Christian Galasso⁵ and Dr. Vincenza De Gregorio⁶ and Dr. Giorgia Imparato⁶ contribute to experiments on the human cell line. Dr. Clementina Sansone and Dr. Christian Galasso contributed during the experimental design and to the execution of the viability test. Dr. Vincenza De Gregorio and Dr. Giorgia Imparato performed the video-time lapse experiment. The author of this thesis prepared samples and performed all the viability tests and analysed the results of time lapse acquisitions. The experimental design and the results were discussed and organized with the Director of Studies and supervisors.

Dr. Annamaria Locascio⁵ supported me in the part dedicated to the marine invertebrate *Ciona robusta*. She helped in manually dechorionate embryos and during the microscopy analysis together with Federica Salatiello⁵. The author of this thesis prepared samples and performed the whole experiment. The experimental design and the results were discussed and organized with the Director of Studies and supervisors.

Dr. Pasquale Chiaiese¹ contributed to the planning and execution of the experiments on microalgae. The author of this thesis performed the experiment and collect data and contributed to the results analyses together with the Director of Studies and supervisors.

All other works present in this thesis was performed by the author of the thesis.

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Chapter 2: exDNA AND MULTICELLULAR AUTOTROTROPHS: EFFECTS ON ARABIDOPSIS THALIANA

2.1 Preface to the work on *A. thaliana*

The first evidences of the inhibitory effect of self-exDNA was reported in plants in 2015 by Mazzoleni and co-workers. They demonstrated that the litter autotoxicity can be caused by fragmented exDNA accumulating in litter during the decomposition process (Mazzoleni *et al.*, 2015a). This inhibitory phenomenon was demonstrated to be concentration dependent, species-specific, to reduce root growth and seed germination of conspecifics (Mazzoleni *et al.*, 2015a) and it was also extended to other organisms (Mazzoleni *et al.*, 2015b). These findings bear relevant implications for plant-soil ecological theories, providing a chemical basis for autotoxicity (Mazzoleni *et al.*, 2010) among the mechanisms of plant–soil negative feedback (van der Putten *et al.*, 2016) and a potential role in species interactions at community (Zhang *et al.*, 2016) and ecosystem (Carteni *et al.*, 2016) levels. Furthermore, other studies on the exDNA sensing and response (Barbero *et al.*, 2016; Duran-Flores & Heil, 2018) highlighted unexpected new functional roles at cellular level, with an impact on biomedical and biotechnological applications, and is thus deserving further attention (Mazzoleni *et al.*, 2014; Veresoglou *et al.*, 2015; Bhat & Ryu, 2016).

2.1.2 Arabidopsis: The Model Plant

Arabidopsis is one of the most consolidated model plant for genetic, biochemical, genetic and physiological studies and for synthetic biology application (Holland & Jez, 2018).

*Arabidopsis thaliana* (Figure 3.1) is a small dicotyledonous species, a member of the Brassicaceae or mustard family. Despite the lack of an effective commercial importance (it is often considered a weed) it has proved to be an ideal model organism for studying plant biology for over 40 years (Meyerowitz, 2001).

The main features making *A. thaliana* a model organism are a short generation time, easy laboratory grown, a rapid development, small genome as respect to the plant kingdom (encoding for almost 27,000 genes), transgenic plants that can be made easily obtained as well as mutations can be easily generated. Moreover, it generates approximately 10,000–30,000 seeds that remain viable for two years in seed packets at room temperature or if placed in Nalgene Cryovials, for longer period (https://www.arabidopsis.org/comguide/table_of_contents.html). Furthermore, it can be either self-pollinated (recessive mutations quickly become homozygous and thus expressed) or be cross-pollinated to do genetic mapping and produce strains with multiple mutations (Koornneef & Meinke, 2010; Holland & Jez, 2018).
Figure 2.1. Arabidopsis thaliana: a plant model system

2.2 Materials and Methods

2.2.1 DNA extraction and preparation

DNA was extracted with the same procedure from both A. thaliana young leaves and abdominal muscles of Clupea harengus (common herring) that, because of its relevant phylogenetic distance, has been chosen as source of nonself-exDNA. One gram of tissue was ground in liquid nitrogen and then mixed in hexadecyltrimethylammonium bromide (CTAB) buffer (0.1M Tris-HCl pH 7.5, 0.7 M NaCl, 0.01 M ethylenediamine tetra-acetic acid (EDTA), 1% CTAB). Samples were incubated 45 minutes at 65°C and mixed periodically. Chloroform/Isoamylalcohol (24/1) was added to equal volume of sample mixed and centrifuged 20 minutes at 14,000 g. The upper phase was collected and transferred to a new tube for precipitation with 1 volume of Iso-propanol at -20°C for 1 hour. Samples were centrifuged at 20,000 g, at 4°C for 20 minutes. The upper phase was discarded and pelleted DNA was washed with cold 70% ethanol in a cooled centrifuge at 20,000 g for 20 minutes. Ethanol was discarded and DNA was air dried and resuspended in 200 µl of sterile water. RNase A (Thermo fisher) was added at a final concentration of 0.25 mg/ml and incubated at 37°C for 1 hour. RNase A was inactivated at 70°C for 20 minutes.

The DNA was extracted with the same protocol from different tissue types of each species to randomize and overcome biases due to the methodology employed. Two independent DNA extractions were performed per sample (technical replicate). The DNA samples used as treatments were firstly checked by nanodrop standard quality parameters (260/280 and 260/230 ratio above 1.8) to evaluate DNA purity. The DNA quantity was evaluated using a QUBIT (Thermo Fisher) fluorimeter, and its integrity was evaluated by electrophoresis in 1% agarose gel. The DNA was
sheared using a Bioruptor Plus (Diagenode), in 12 minutes at high power, setting 60 seconds ON and 30 seconds OFF in order to reach an average size ranging from 200 to 700 bp. Such size was selected following previous evidence that this range was that found in decomposing litter and the most effective to produce inhibitory effects in vitro conditions (Mazzoleni et al., 2015a).

2.2.2 Plant materials and treatments

*A. thaliana* (L.) Heynh. Col-0 (186AV) seeds were obtained from the “Centre de Ressources Biologiques” at the “Institut Jean Pierre Bourgin”, Versailles, France (http://dbsgap.versailles.inra.fr/vnat/). Seeds were treated in 70% ethanol for 30 seconds, then transferred in a sodium hypochlorite (1:10 of commercial concentration) and 0.05% tween 20 solution for 10 minutes with occasional vortexing, washed with sterile milli-Q water 4 to 5 times, dried and resuspended in a sterile agar solution (0.2%). Sterilized seeds were vernalized for three days at 4°C and got ready for sowing.

For transcriptome analyses, Petri dishes with a layer of thin Whatman qualitative filter paper, Grade 1 (WHA1001824), were prepared in sterile conditions for each sample. The dishes were wetted with pure sterile water and an adequate number of Col-0 sterilized seeds were sown. The plates were put in the dark for 48 hrs in a growth chamber with 50% controlled humidity. After two days the plates were kept in 16 hrs/ 8 hrs light/dark cycles in controlled humidity conditions. The light intensity and wave length set up were of 120-150 µmol/m2 and 300-700 nm, respectively. If needed, sterile water was added to the plates from time to time, to all samples.

The experimental design for both transcriptome (Figure 2.2) and confocal analyses included two replicates of the following treatments: (1) control: with sterile distilled water; (2) self-exDNA: with *A. thaliana* DNA; (3) nonself-exDNA: with *Clupea harengus* DNA. Both DNA treatments were performed using a concentration of 200 ng/µl (as in Mazzoleni et al., 2015a).

Before both transcriptome and confocal experiments, plants were grown for five days till the two true leaves stage.

For confocal analyses, *A. thaliana* plants (Col-0) were grown vertically on half strength MS basal medium. Five-days-old seedlings were placed on slides. They were treated with self and nonself-exDNA and observed after 1 hour to detect the early spatial distribution of labelled exDNAs.

For transcriptome analysis (Figure 2.2), plants were grown until the appearance of the first true leaves. At this stage, the filter papers were imbibed with the control solution, 1.2 ml of sterile distilled water, or the same volume of 200 ng/µl of sonicated DNA (self or non self). Control and treated plates were harvested at 1, 8 and 16 hrs (two biological replicates per treatment), then immediately frozen with liquid nitrogen. Additional plants that were not harvested for RNA
extraction were maintained for 15 days for final observations on longer terms phenotypic effects evaluated at 10 days post-treatment.

### 2.2.3 Transcriptome and bioinformatics analyses

Total RNA extraction from harvested plant material was performed using RNeasy micro kit from QIAGEN (Cat No./ID: 74004) following the standard extraction protocol and sent to a service provider for the RNA-seq analysis on Illumina Hiseq2500, by single read sequencing 1x15M.

Raw reads per sample were cleaned from adaptors and low quality bases by using the Trim Galore package (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/), applying the default settings with --minimum-length = 100, to remove reads shorter than 100 bp, and --quality cutoff = 10 to trim low quality ends from reads. The cleaned reads were then mapped to the Arabidopsis nuclear and cytoplasmic genomes (version TAIR 10) using the STAR aligner software (version 2.4.2a) (Dobin et al., 2013) allowing a maximum number of 10 mismatches.

Detailed results from the pre-processing are reported in Supplementary Table 2.1. The mapped reads were counted per exon by featureCounts (version 1.4.6-p5) (Liao et al., 2014), using the strand specific count (“--s 2” option) and allowing the counting of read on overlapping features for each feature (“--O” option).

Assessment of replicates correlation in terms of reads per kilobase of transcript (RPKMs) was performed by Pearson correlation and is reported as the correlation matrix and the associated dendrogram (Supplementary Figure 2.1 a,b). The Principal Component Analysis of the same data per treatment per time is also shown (Supplementary Figure 2.1c). Differentially expressed genes (DEGs) call, comparing DNA treatments at each stage with the respective control, were made performing three different statistical approaches (false discovery rate (FDR ) < 0.05): i) DESeq2 (Love et al. 2014); ii) edgeR and iii) edgeR GLM (Robinson et al., 2010). The union of the three approaches was considered for subsequent analyses.

Gene ontology (GO) enrichment analyses on DEGs filtered by $|\log_2(FC)| \geq 1$ were performed using the GOseq package (Young et al., 2010) (FDR ≤ 0.05), and the reference GO annotation for Arabidopsis that was downloaded from Ensembl Plants (http://plants.ensembl.org/index.html).

Lists of genes annotated with the most enriched GOs, showing different expression patterns between self-exDNA and nonself-exDNA treatments at each observation stage (i.e. 1, 8 and 16 hrs) were collected in order to quantitatively assess between-treatment differences and analyze their contribution in detail at single-gene level.
2.2.4 Confocal microscopy experiment

The confocal analyses were performed independently in two different institutes by using three different confocal microscopes (Zeiss LSM-700, Leica TCS and Zeiss LSM-780), in order to confirm and verify the reproducibility of the results.

The analyses were conducted treating roots by a DNA concentration of 100 ng/µL DNA that included labelled DNA by Cy3 dye for an incubation period of 1 hour. Seedlings were then transferred to a new slide, and treated with 2 µM FM4-64 for 5 minutes. Before the confocal observations, seedlings were subjected to two sequential washing steps using tap water. Then they were transferred to the slide for the confocal analyses. As a negative control, Arabidopsis seedlings were incubated for 1 hour with the same amount of Cy3 (without DNA), then exposed to the same treatment with FM4-64. The images were analysed and edited by using the ImageJ free software.

2.2.5 QRT-PCR validation

Seven DEGs in at least one stage/treatment either up or downregulated were selected and their expression was evaluated if concordant with that resulted from the RNAseq analysis and checked for duplication in the TAIR database (Arabidopsis.org). Using the RealTime QPCR online tool from IDT (https://eu.idtdna.com/scitools/Applications/RealTimePCR/). One pair of primers was designed for amplification of the selected genes targeting the exon-exon junction, when possible, to exclude intronic regions arising from genomic DNA or not mature mRNAs. First strand cDNA was synthesized with superscript III (ThermoScientific) starting from 600 ng of the same RNA samples used for the RNA-seq analysis and using manufacturer conditions. Each primer pair was verified in PCRs using first-strand cDNA as template. PCR conditions were as follows: denaturation at 94°C for 4 minutes, cycling at 94°C for 30 seconds, annealing at varying temperatures for 30 seconds, and extension at 72.0°C for 30 seconds. Annealing temperatures ranged from 50°C to 68°C. Reaction products sizes and integrity were visualized by 1.2% agarose gel electrophoresis.

QPCR was performed in 20 µL reaction volumes containing 10 µL Power SYBR™ Green PCR Master Mix (Thermo-Fisher Scientific; catalogue no. 4367659), 500 nM forward and reverse primers and 1:10 dilution of 600 ng stock sample cDNA.

For each gene, we considered two biological replicates with two technical replicates per biological replicate. QPCR amplifications were performed on separate plates, where each plate contained primer pairs for the housekeeping gene GAPDH (AT1G13440.1, Jin et al. 2018). QPCR expression values were calculated for each gene as the difference between the quantification cycle of the gene and the reference gene, averaged over technical replicates (Supplementary Table 2.2).
Concordance between RNAseq and RT-QPCR results was assessed by testing the statistical significance of linear regression of the log2-fold change (log2(FC)) versus the log2(ΔΔCt) results, for those transcripts for which both metrics were available (n = 42, 7 transcripts x 2 DNA treatments x 3 time points). For each metric, the mean of replicated values was used for each combination of transcript, DNA treatment and observation time. Evident outliers were removed before fitting the regression model (Supplementary Table 2.2).

![Schematic representation of the experimental design.](image)

**Figure 2.2.** Schematic representation of the experimental design. Figure published in (Chiusano et al., 2021).

### 2.3 Results

#### 2.3.1 Differential gene expression self and nonself-exDNA exposure

The bioinformatics data processing (Supplementary Table 2.1), revealed that among the 32678 genes reported in the reference Arabidopsis annotation file, filtering by |log2(FC)| ≥ 1 the total number of DEGs was 825 and 1949 in self and nonself treatments, respectively and only 342 genes were in common between the two treatments (Supplementary Table 2.3a), underlining that specific changes were about threefold higher in nonself treatments than in self ones.

Interestingly, the relative number of common DEGs is higher after the first hour while the number of specific DEGs widen in the other two stages post treatment (Supplementary Table 2.3b, Figure 3.3). The Venn diagrams of DEGs at each observation stage (Figure 2.3C) showed that most DEGs were specific per treatment. Moreover, after exposure to self-exDNA the number of DEGs is lower than that in nonself at all the experimental stages if compared with the control.
The complete list of all DEGs and their fold changes, highlighting the filtered DEGs (|log₂(FC)| ≥ 1) and the expression levels (RPKM) at the corresponding hpt are reported for both self and nonself treatments (Supplementary Table 2.4a and b, respectively).

Figure 2.3. Number of differentially expressed genes in treatment with self-exDNA (A) and nonself-exDNA (B) vs control, comparisons and transitions per stage (1, 8 and 16 hpt). In (A) and (B), grey circles represent Arabidopsis thaliana genes not differentially expressed, the red and blue circles represents up and downregulated genes, respectively at 1, 8 and 16 hpt. The circle size is log-proportional to gene numbers. The lack arrows indicate the number of differentially expressed genes (DEGs) that from each stage change their expression becoming up, downregulated regulated of not differentially expressed (line width is log-proportional to the number of transitions displayed on each arrow). In (C) Venn diagram for the self vs. nonself comparisons at each observation stage is reported. The number of up and downregulated DEGs either specific or common in self- and nonself-exDNA, is indicated in red and blue, respectively. Figure published in (Chiusano et al., 2021).
2.3.2 Gene Ontology (GO) enrichment in self and nonself treatments

Results of Gene Ontology (GO) enrichment analysis highlights clear differences across treatments and exposure times for specific classes of GOs. In the Figure 2.4 and in Supplementary Table 2.5, the GO were manually grouped for similar functional processes and cellular functions. Each row represents a single GO whose enrichment has been evaluated for self- and nonself-exDNA treatments at 1, 8 and 16 hpt (columns). The colour of each rectangle in the column shows the pattern of expression of genes enriching the GO represented for by the row (full red: upregulated DEGs; blue: downregulated; light red: both up- and downregulated). Following, the major differences in GOs enriched in nonself- and self-exDNA.

- Group of DNA transcription and RNA translation, the former significantly enriches in downregulated genes exclusively in self-exDNA after 8 hrs of treatment, whereas the group of RNA translation GOs is enriched in upregulated genes at 8 and 16 hrs exclusively in nonself-exDNA treatment.

- GO of Signal transduction is enriched in downregulated genes after 1 hour of treatment with self-exDNA.

- Group of GOs related to hormones: at 16 hpt with self-exDNA, brassinosteroids and cytokinins GOs are enriched by upregulated genes while abscissic acid (ABA) and gibberellin GOs are enriched by downregulated genes. On the other hand, Brassinosteroids homeostasis, Response to ABA, and Auxin efflux GOs are enriched by downregulated genes after 8 hrs with nonself-exDNA (Supplementary Table 2.5). Moreover, the response to salicylic acid is enriched by upregulated genes only in nonself-exDNA.

- Group of GOs related to Biotic stress: they are enriched by upregulated genes after nonself-exDNA treatment in comparison with self-exDNA treatment at 1 and 8 hrs and at 16 hrs the Systemic acquired resistance GO is enriched exclusively in the nonself-exDNA treatment.

- Group of GOs related to Abiotic stress: GOs related to responses to copper and cadmium ions, as well as to ozone and light intensity are enriched by upregulated genes only after 8 hrs in self-exDNA treatment. Furthermore, GOs related to the responses to water deprivation, high light intensity, heat and hyperosmotic salinity as well as to chitin and wounding are enriched by downregulated genes exclusively in self-exDNA treatments (Figure 2.4, Supplementary Table 2.5).

- Group of GOs related to the Oxidative stress: at 8 hrs only after the treatment with self-exDNA, the response to superoxide radical activity is enriched by upregulated genes whereas the response to hydrogen peroxide is enriched by downregulated genes.
- Group of GOs related to Proton and electron transport, ATP related processes, and Oxidoreductase activity are enriched by upregulated genes at 1 and 16 hrs after self-exDNA treatment (Figure 2.4, Supplementary Table 2.5). The GO related to Oxidoreductase activity is instead enriched by downregulated genes after 8 hrs of treatment with nonself-exDNA.

- Group of GO related to Chloroplast: among them, Structure and Photosynthesis are enriched by upregulated genes at 1 and 16 hrs post self-exDNA treatment, while at 8 hrs after nonself-exDNA treatment the GO related to Photosynthesis shows an enrichment in upregulated genes. Moreover, Light harvesting and the Genome and transcription GOs do not show significant enrichment after self-exDNA treatment, while are enriched after nonself-exDNA treatment.

- Group of GOs related to Mitochondrion are enriched by upregulated genes exclusively at 8 hrs after nonself-exDNA treatment.

**Figure 2.4.** Summary of GO enrichment analysis on filtered DEGs. Most enriched GOs (rows) are grouped by functional process or cell compartment. The colour of each rectangle in the column shows the pattern of expression of genes enriching the GO represented for by the row (for each treatment type and stage) (full red: upregulated DEGs; blue: downregulated; light red: both up- and downregulated, with enrichment in upregulated DEGs showing lower p-value compared to the downregulated ones). In white, absence of enrichment is shown. Figure published in (Chiusano et al., 2021).
2.3.2 DEGs associated to enriched GOs

A detailed analysis at gene level of the results of GO enrichment reveals interesting differential features between self and nonself treatment at each time point (Supplementary Tables 2.6a,b; Figure 2.5).

The number of specific DEGs after the treatment with self-exDNA was higher at 1 hour while decreasing at 8 hrs and rising again at 16 hrs. In particular, in this treatment, DEGs are observed enriching groups of DNA, RNA, proton and electron transport, ATP-related processes, NAD/NADP related processes and chloroplast related GOs (i.e., structure and photosynthesis) (Figure 2.5). Moreover, some other GOs (those related to biotic, abiotic stresses and mitochondrion) show a different pattern with a higher number of specific DEGs at the first hour, then progressively decreasing over time at 8 and 16 hpt.

In contrast, after the nonself-exDNA treatment, the number of specific DEGs increased at 8 hrs and persisted also at 16 hpt for all considered GOs, with an evident increase in DEGs numbers for DNA, RNA, abiotic and chloroplast structure GOs.
2.3.3 One hour post treatment

In the group of GOs related to DNA Transcription and translation, DEGs counting show that downregulated genes prevailed and many are also specific for either self- and nonself-exDNA treatment. This highlights different reprogramming of the transcriptional asset in the two treatments. Interestingly, after self-exDNA treatment, we identified the four RNA polymerases encoded by the chloroplast genome (ATCG00170; ATCG00180; ATCG00190; ATCG00740) all expressed over the log$_2$(FC)$>1$ cut-off (Supplementary Table 2.4). In addition, self-specific upregulated DEGs include AT1G66600 (ABO3), a WRKY transcription factor involved in
drought tolerance and in ABA mediated response (Ren et al., 2010); AT5G47220 (ERF2), the ethylene responsive element, and two transcription factors that are involved in ROS response (AT3G46080, AT1G52890). Noticeably, the higher expression of ERF2 is accompanied by the downregulation of specific genes involved in ethylene signal transduction (AT4G34410, AT5G52020) and ethylene responsive transcription factors (AT1G74930, AT1G28370). AT3G01220, exclusively downregulated in self-exDNA treatment, is expressed during seed germination in the micropylar endosperm and in the root cap, and, when mutated, increases seed dormancy and ABA sensitivity (Barrero et al., 2010).

In response to self-exDNA treatment, almost all DEGs enriching the RNA related GOs (Transcription and translation), are encoded by the chloroplast genome (Supplementary Table 2.4).

Moreover, many of the upregulated genes specific of the response to self-exDNA treatment in the GOs related to NAD/NADP processes (in the group of Oxidoreductase activity), are encoded by the chloroplast genome (Supplementary Table 2.4). Similarly, in the group of Proton and electron transport GOs genes exclusively upregulated in response to self-exDNA treatment are mainly involved in the photosystem II reaction center, in photosynthetic electron transfer and in the ATP synthase complex or membrane transporters. Also in the GOs of group of ATP related processes, all the DEGs are self-specific and code for the ATP synthase complex, and all these genes are also contributing to the enrichment of the group of Proton and electron transport related GOs.

Although the lack of enrichment in the GOs related to Mitochondrion in the first hour post treatment with both nonself- and self-exDNAs, there are DEGs associated to these GOs. Interestingly, among these genes AOX1d (AT1G32350) encodes for one of the 4 AOX1 genes of A. thaliana genome (Vanlerberghe & McIntosh, 1994).

When considering the chloroplast related GOs all the 4 groups of GOs are exclusively enriched in upregulated genes in the self-exDNA treatment. Among the DEGs in this group, more than half are specific of the response to self-exDNA treatment and are also encoded by the chloroplast genome.

Considering DEGs within the Systemic resistance group, PAD 4 (AT3G52430: phytoalexin deficient 4), that usually mediates TIR-NB-LRR signaling involved in the pathogen resistance response, as well as in root meristem growth arrest (Kim et al., 2012), is exclusively downregulated in self-exDNA treatment. This is also confirmed by QRT-PCR (Supplementary Table 2.2).

In the Hormone related GOs (indicating processes related to cytokinins, brassinosteroids, ABA, gibberellins, auxins and salicylic acid.), among the upregulated DEGs specific for self-exDNA, it is worthy to mention AT5G15970, encoding for the protein KIN2, that is known to be induced by
ABA and during water deficit stress. Additionally, AT1G15520 (PDR12) encodes for the pleiotropic drug resistance 12, an ABA related ABC transporter localized on the plasma membrane of guard cells and involved in ABA uptake and stomatal closure (Munemasa et al., 2015; Wang et al., 2018b). Overall, the data show a relevant role of ABA and jasmonic acid (JA) in the response to self-exDNA exposure at 1 hour, while, in the case of nonself-exDNA treatment, salicylic acid, ABA and auxin, play a major role at 1 hour (Supplementary Table 2.6b, Figure 2.5).

### 2.3.4 Eight hours post treatment

Among DEGs downregulated in the self-exDNA treatment in the group of DNA enriched GOs, many have the same trend already evident in the 1 hpt (Supplementary Table 2.4). Among these genes, HSFA2A (AT2G26150), a transcription factor that is typically upregulated during stress response (Enomoto et al., 2019), and HSFC1 (AT3G24520), both confirmed by QRT-PCR, together with other AT-HSFA7B (AT3G63350) and 3 heat shock proteins (HSPs), show a significant negative log2(FC) in self-exDNA treatments. The majority of the downregulated genes are ethylene related proteins or ethylene responsive transcription factors.

Considering the group of GOs associated to RNA, the upregulated DEGs, are specific to the nonself-exDNA treatment and, among them, many encode ribosomal proteins, indicating a consistent activation of the translation machinery in the nonself-exDNA treatment at this stage (Supplementary Table 2.6a, Figure 2.4).

Considering signal transduction, the following DEGs are all upregulated specifically in nonself-exDNA: AT3G03530: non-specific phospholipase C4 (NPC4); AT3G08510: phospholipase C 2 (PLC2); AT3G48610: non-specific phospholipase C6 (NPC6); AT3G51460: ROOT HAIR DEFECTIVE4 (RHD4) are all involved in the phosphoinositides signaling pathway, which is recognized as an early response involving membrane reorganization and lipid signaling in defense response (Abd-El-Haliem & Joosten, 2017).

In the group of Oxidative stress, among the 2 genes exclusively upregulated in response to self-exDNA, AT4G25100 codes for Fe superoxide dismutase 1 (FSD1), acting in plastidial, cytoplasmic and nuclear compartments with an anti-oxidative and osmoprotective role (Dvořák et al., 2020).

Counting the DEGs in the GOs Hormone-related it is evident a reduced involvement of ABA and JA associated DEGs in comparison with the first hour, in the response to self-exDNA. In the case of nonself-exDNA treatment, a remarkable specificity characterizes the response in comparison with the self-exDNA treatment, with a clear involvement of salicylic acid, ABA and auxin activity related DEGs (Supplementary Table 2.6b, Figure 2.5).
2.3.5 Sixteen hours post treatment

Interestingly, in the group of DNA related GOs, among DEGs specific for nonself-exDNA treatment and upregulated we find WRKY33 that is involved in both the hypersensitive response and the systemic acquired resistance and WRKY70 is involved in the establishment of the systemic acquired resistance (Cao et al., 2018). This indicates that nonself-exDNA can be sensed as a PAMP by triggering the hypersensitive response and initiating a systemic acquired resistance.

In the group of RNA related GOs, among those exclusively upregulated in response to self-exDNA, most of genes are encoded by the chloroplast genome and many of them are also upregulated at the 1 hour post the same treatment indicating a persistence or a restarting of a similar response. Differently, in the nonself-exDNA treatment, the upregulated genes are more related to rRNA processing, maturation and stabilization, and less to ribosome biogenesis and structure (Supplementary Table 2.6).

The group of Signal transduction GOs, is enriched in nonself-exDNA treatment of specific upregulated genes coding for resistance proteins (Supplementary Table 2.6) and involved in the process of the hypersensitive response (Coll et al., 2011).

In the Systemic resistance group among DEGs exclusively upregulated in response to nonself-exDNA there is AT3G52430 encoding for PAD4 which mediates the TIR-NB-LRR signaling when upregulated and that is involved in the hypersensitive response (Coll et al., 2011).

Concerning the Oxidoreductase activity GOs (NAD/NADP related processes) and Proton and electron transport group, many of the DEGs specifically upregulated after self-exDNA treatment are encoded by the chloroplast genome and all were also upregulated at 1 hour.

For the group of Mitochondrion among the downregulated and specific DEGs in response to self-exDNA AT5G24120 codes for SIGE, a transcriptional factors localized in both chloroplast and mitochondrion, which regulates the chloroplast transcriptional response to light intensity (Belbin et al., 2017).

Considering the Chloroplast group of GOs, the self-exDNA treatment shows a trend similar to that observed at 1 hour for the Structure and Photosynthesis subgroups (Supplementary Table 2.6a, Figure 2.5). Of note, among the specifically upregulated genes, almost all DEGs are encoded by the chloroplast genome and are also upregulated also at 1 hour (Supplementary Table 2.4). Interestingly, among DEGs in nonself-exDNA treatment, none is encoded by the chloroplast genome (Supplementary Table 2.4).

The evaluation of DEGs of Hormone-related GOs, revealed and interesting upregulation in the self-exDNA treatment of two genes: AT4G29740 and AT5G56970 encoding for oxidases/dehydrogenases that catalyse the degradation of cytokinines, that are mainly involved in
cell division processes and cell growth and differentiation (Avalbaev et al., 2012), thus possibly revealing effects related to the growth inhibition caused by self-exDNA exposure (Mazzoleni et al., 2015a).

In the self-exDNA treatment, the genes involved in processes related to cell energy production and balance, oxidoreductases and chloroplast structure and photosynthesis show a recursive upregulation, since the pattern at 16 hpt resembles the one at 1 hour.

2.3.6. DAMP and PAMP Associated Genes

To consider Arabidopsis genes involved in DAMPs or in PAMPs responses, we collected the list of known or putative receptors, mainly considering those responsive to extracellular nucleic acids, described in the literature (Hornung et al., 2009; Greeff et al., 2012; He & Wu, 2016; Bhat & Ryu, 2016; Choi & Klessig, 2016; Gravino et al., 2017; Quintana-Rodriguez et al., 2018; Bacete et al., 2018; De Lorenzo et al., 2018; Hou et al., 2019; Erb & Reymond, 2019; Albert et al., 2020; Li et al., 2020b; Pham et al., 2020; Cheung et al., 2020; Ferrusquía-Jiménez et al., 2020; Bentham et al., 2020). Moreover, we considered the expression patterns in both nonself and self-exDNA treatments comparing the behaviour per stage (Supplementary Table 2.7). The summary of the total number of DEGs from self and nonself-exDNA treatments at different time post exposure is reported in Supplementary Table 2.7. Interestingly, it is evident that in both treatments, there are DEGs in either DAMP or PAMP classes. In particular, in the DAMP class, the number of DEGs increases in both nonself- and self-exDNA treatments during time. In contrast, in the PAMP class, the number of DEGs remains almost stable in self treatments, while it is higher in the first and third stages, in comparison with the second stage post treatment, in nonself-exDNA treatments. Interestingly, the number of DEGs showing a common behaviour in the two treatments increases during time for both classes, although the number of specific DEGs remains higher in nonself treatments, especially in the DAMP class. This may be due to the increase of DAMPs in the later stages of the nonself treatments, due to the cellular disruption revealed by the confocal analysis. Of note, the very low number of specific DEGs in self-exDNA treatments in both classes, particularly at the 16 hpt, may indicate that the differential sensing may be determined in the initial stages post treatment. Nevertheless, from this preliminary analysis, it is evident that the response to self-exDNA is poorly characterized in terms of known receptors of DAMPs or PAMPs (Supplementary Table 2.7). Considering the DEGs that are specific in the self-exDNA treatment, it is worth mentioning AT1G57650, coding for an ATP-binding protein, and AT1G57630, coding for a TIR domain family protein, both upregulated and reported to respond to extracellular nucleic acids (Bhat & Ryu, 2016) and AT1G31540, coding for a TIR-NBS-LRR protein, which is down regulated, all belonging to the DAMP class. AT2G19190, coding for the
Flagellin22-induced receptor-like kinase 1 and AT1G02900, coding for a Rapid alkalinization factor are both down regulated in the DAMP class.

At 8 hpt, specific DEGs from self-exDNA treatments include defensins (three up regulated and one downregulated), and AT1G79680, coding for a cell wall associated kinase (WAK10), which is upregulated and reported to be a calcium receptor, and AT2G33580, in the PAMP class, coding for another membrane kinase, which is downregulated. Interestingly, among the three defensins that are classified as DAMPs and have DEGs in the nonself response, none is in common with the self-exDNA response, and all are downregulated expect the one coded by AT3G24510, that is up regulated. It is of interest to note that the defensin pattern remains almost different in the two treatments also in the third stage. In particular, AT5G33355 remains upregulated also at 16 hpt in self-exDNA treatments while AT3G24510 remains upregulated in nonself-exDNA treatments. AT1G34047 results a DEG at the 16 hpt only in the self-exDNA response. Remarkably, defensins are the major class that is involved in the specific self-exDNA response among the two classes (Supplementary Table 2.7).

### 2.3.7 QRT-PCR results

The Supplementary table 2.2 shows the results from QRT-PCR data of 7 genes compared with the fold change of DEGs. The selected genes were chosen also to confirm some of the marker genes that could depict the behaviour in the two treatments. Worthy to notice the upregulation of the superoxide dismutase in self confirms the oxidative stress which is typical of this treatment. The expected general trend of AOX1d is confirmed in the two treatments per stage, together with the down regulation of HSFA2 (AT2G26150) and HSFC1 (AT3G24520) in the second stage of the self-exDNA treatment, which is even more evident in the nonself one. Expression levels observed by RNAseq and QRT-PCR were well in accordance, as confirmed by the highly significant linear regression between the two series of data emerging from the comparison across 7 gene transcripts, two DNA treatments and three observation time points (Supplementary Figure 2.2, Pearson's $r = 0.814, P = 1.73 \times 10^{-10}$).

### 2.3.8 Differential self- and nonself-exDNA distribution by confocal analysis and phenotypic changes in seedlings

Confocal microscopy of roots exposed to self-exDNA revealed that A. thaliana labelled DNA (with both Cy3 and Alexa Fluor 555 dyes) was mostly visible outside the root (Figure 2.6A-C). At 1 hour, the self-exDNA fluorescence was also visible inside the root, limited to the surface of cells (Figure 2.6D). No fluorescence was seen in the cytoplasm of any of the images.
In contrast, after treatment with nonself-exDNA, labelled-DNA is evident in the root, in the cytoplasm and even at nuclear level (Figure 2.6F-I). The staining with FM4-64 after 1 hpt with either nonself- or self-exDNA highlights a clear difference in the dye uptake and diffusive pattern inside the roots. In fact, the FM4-64 dye remains outside the root exposed to self-exDNA (Figure 2.6E), whereas it enters the cells previously exposed to nonself-exDNA (Figure 2.6L). At the phenotypic levels, the main differential responses to self- and nonself-exDNA are summarized in Figure 2.7. Of note, at macroscopic level, the exposure to self-exDNA produced peculiar phenotypic effects: at 8 hpt with self-exDNA, there is an increase in root hair density and a consistent root browning; at a later stage 10 days post treatment, necrosis of root tips is accompanied by an inhibition of growth and leaf decolouring (Figure 2.7).
Figure 2.6. Fluorescence microphotography (confocal images) of early response (1 h) to self- vs. nonself-exDNA treatments. From the left to the right, the following treatments are shown: Alexa Fluor 555 dye (in white) labelling self-exDNA (A,B) and nonself-exDNA (F,G); Cy3 dye (in blue) labelling self-exDNA (C,D) and nonself-exDNA (H,I); FM4-64 staining (in red) of A. thaliana roots after 1 h exposure to either self-exDNA (E) or nonself-exDNA (L). Scale bars 20 µm in (A–C,E–G,I,L); 10 µm in (D,H). Figure published in (Chiusano et al., 2021).
**Table 2.7.** Summary of the main differences in response to extracellular self- and nonself-exDNA. A differential uptake according to treatments is clear after 1 hour, morphological differences on thin roots and root apices appear after 8 hrs, full inhibition by the exposure to self-exDNA is evident on the whole plants after 10 days. Figure published in (Chiusano et al., 2021).

<table>
<thead>
<tr>
<th>Observation</th>
<th>Self-DNA</th>
<th>Nonself-DNA</th>
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<tbody>
<tr>
<td>1 hour post treatment</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
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<tr>
<td>Labelled DNA (blue) and RMA-64 (red) localization at root and cell level</td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
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<tr>
<td>8 hours post treatment</td>
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<tr>
<td>Root hair density</td>
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<td><img src="image8" alt="Image" /></td>
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<tr>
<td>Root phenotype</td>
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<td>10 days post treatment</td>
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<tr>
<td>Plant phenotype</td>
<td><img src="image13" alt="Image" /></td>
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**Figure 2.7.** Summary of the main differences in response to extracellular self- and nonself-exDNA. A differential uptake according to treatments is clear after 1 hour, morphological differences on thin roots and root apices appear after 8 hrs, full inhibition by the exposure to self-exDNA is evident on the whole plants after 10 days. Figure published in (Chiusano et al., 2021).

### 2.4 Discussion

Mazzoleni and his colleagues, (Mazzoleni et al., 2015a) reported that extracellular self-exDNA, released in the soil during litter decomposition, or made available by experimental exposure, induced an inhibitory effect on root growth and seed germination in several plant species, without affecting heterospecifics. Such findings have been ascribed to different putative mechanisms (Veresoglou et al., 2015), including signalling and self-recognition (Duran-Flores & Heil, 2015;
Bhat & Ryu, 2016), plant root defense (Hawes et al., 2011) and microbe- or damage-associated molecular patterns (Panstruga, 2016; Vega-Muñoz et al., 2018; Heil & Vega-Muñoz, 2019).

These results show a clear-cut pattern in the plant transcriptomic response in the early stages after exposure and before evident phenotypic traits could be detected. Exposure to exDNA resulted in remarkable differences both between exposure to self- vs. nonself-exDNA, and among different stages (1, 8 and 16 hpt) after exposure in each treatment. In parallel, remarkable differences were also highlighted in the early response by confocal microscopy.

In particular, our experiments show that self-exDNA appears to decrease the permeability of plant root cells leading to a reduced entrance in the innermost parts of the root and cells. It accumulates in the outer layers, where it is found in the extracellular space. Conversely, nonself-exDNA diffuses throughout the root, also entering the cytoplasm of cells and the nucleus, and this was never observed in self-exDNA treatments in our experiments.

The uptake of nucleic acid macromolecules in roots was already reported (Paungfoo-Lonhienne et al., 2010). However, to the best of our knowledge, this is the first evidence of exDNA entry in roots showing different distribution patterns between self and nonself-exDNA.

The differences between self and nonself-exDNA treatment are also evident at macroscopic level. After 8 hrs of treatment with self-exDNA an increased root hair density was observed and a consistent root brownish. Of note, only after the exposure to self-exDNA root tips showed a necrotic phenotype, inhibited growth and leaf decolouring at 10 days post treatment.

2.4.1 Contrasting transcriptome dynamics in response to extracellular self vs. nonself-exDNA

The transcriptome analysis of plants treated with self-exDNA showed a limited number of differentially expressed genes compared to the exposure to nonself-exDNA, although remarkable GO enrichments could be revealed.

After one hour, as early response to self-exDNA sensing is the upregulation of chloroplast related genes (Structure and Photosynthesis groups) coded by the chloroplast genome without a differential expression of nuclear genes related to chloroplast activity (Genome and Light harvesting groups). Significant enrichments in upregulated DEGs are evident in self-exDNA treatment also in NAD/NADP related processes, Proton and Electron Transport and ATP related processes. Despite lack of the enrichment in the GOs related to mitochondrion the mitochondrial gene RPS3 (ATMG00090) is upregulated specifically in self-exDNA treatment. It encodes ribosomal protein related to pathogen resistance (Bisgrove et al., 1994). Moreover, the nuclear gene AOX1d (AT1G32350) (Vanlerberghe & McIntosh, 1994) is upregulated in self-exDNA
treatment, as also confirmed by the QRT-PCR, which is typically upregulated in response to stress (Clifton et al., 2006).

In particular, AOX1d contributes to the recovery from the inhibition of Complex III that is involved in the mitochondrial electron transport chain, thus indicating a block of the respiratory chain typical of the self-exDNA treatment. Moreover, the upregulation of AOX1D is coherent with the presence of nitric oxide (NO) which it is supposed to be released after the treatment with self-exDNA because of the upregulation of both NIA2 (Nitrate reductase: AT1G77760) and NIA1 (AT2G15620). NO is an alternative ROS product, determined by a drop of the oxygen concentration, also activating 2 oxoglutarate (Selinski et al., 2018) and determining the inhibition of aconitase (GUPTA 2012), which ends up with the upregulation of AOX1D.

A peculiarity of the exposure to self-exDNA is the hyper-activation of the chloroplast genome activity, in absence of a similar upregulation of chloroplast proteins encoded by the nuclear genome. This discrepancy could be related and also be responsible of the overproduction of chloroplast related ROS whose enhanced production has also been reported to occur as production of \( \text{H}_2\text{O}_2 \) in similar experiments of exposure to self-exDNA (Duran-Flores & Heil, 2018). Moreover, the chloroplast probably fails to remove them as suggested by the lack of the overexpression of genes like ascorbate peroxidases, namely the APX1 gene (AT1G07890), acting as scavengers of \( \text{H}_2\text{O}_2 \) in the chloroplast and as suppressor of the expression of \( \text{H}_2\text{O}_2 \)-responsive genes under photo-oxidative stress (Maruta et al., 2009). This is also known to be accompanied by a downregulation of HSFA2 (Nishizawa et al., 2006), which could explain the evidence here reported of an unexpected pattern of downregulated heat shock related proteins, as revealed for self-exDNA exposure at 8 hrs.

However, further investigations should clarify the discrepancy here highlighted by the QRT-PCR that also shows a downregulation of the HSFA2 at the 8 hpt also in the nonself-exDNA treatment that, however, is not accompanied by the downregulation of heat shock proteins.

Interestingly, the upregulation of chloroplast related genes observed at 1 hpt with self-exDNA completely turned off after 8 hrs.

At 16 hrs, genes encoded by the chloroplast genome showed again upregulation in self-exDNA treatments, indicating a recursive effect and a persisting stressing stimulus. It will be of interest, in future efforts, to monitor the hormonal as well as NO and \( \text{H}_2\text{O}_2 \) waves possibly accompanying the process. Moreover, photoinhibition should also occur, deteriorating the chloroplast machinery for longer exposure.

Considering the classes of genes specifically related to responses to stresses (Biotic and Abiotic Stress, and Systemic resistance) common DEGs are detected in both treatments. Nevertheless, it is of note that the difference in the number of specific genes start to widen between the two
responses after 1 hour (Figure 2.4). Indeed, self and nonself treatments, respectively, show along the three stages after treatment: 15-8-3 versus 22-44-67 DEGs in the biotic stress; 35-58-22 versus 68-199-237 for abiotic stress; 1-2-1 versus 4-8-11 in the systemic resistance. The overall increase in specific DEGs along the three stages in nonself-exDNA treatment, could indicate the establishment of a response against an identified stressor agent. In contrast, the absence of an increasing specificity in DEGs along time after the treatment with self-exDNA, could be related to a not fully deciphered adverse condition, thus leading to the failure of the activation of specific responses.

In particular, genes related to heat, wounding and chitin response were downregulated, while responses to oxidative stress, toxic substances and ions were upregulated in self, involving genes encoding detoxification and anti-oxidation protective enzymes (Kliebenstein et al., 1998; Seki et al., 2002; Bechtold et al., 2004; Simpson et al., 2009). Such results clearly indicate that self-exDNA triggered a response to oxidative stress and detoxification, while downregulating typical stress responsive genes, like HSPs, as it resulted evident at 8 hrs.

After 1 hour of treatment with self-exDNA, the gene PAD4 (AT3G52430: phytoalexin deficient 4) resulted significantly downregulated and this was also confirmed by QRT-PCR. This gene, when upregulated, mediates TIR-NB-LRR signalling involved in the pathogen resistance response thus its downregulation may be associated to the inhibition of TIR-NB-LRR signalling involved in the resistance responses mediated by TIR containing R proteins (Coll et al., 2011). Differently, the response to nonself-exDNA is characterized by the upregulation of the systemic resistance and biotic stress. Indeed, PAD4 (AT3G52430: phytoalexin deficient 4) is upregulated after the treatment with nonself-exDNA. Considering the expression of PAD4, the self-specific downregulation at the first hour and the nonself-specific upregulation at 16 hrs, as also confirmed by QRT-PCR, accompanied by the upregulation of genes involved in the hypersensitive response (AT3G52430, AT2G38470, AT1G91560, AT3G45640, AT5G07390, AT1G01480, AT4G11280 and of several TIR-NBS-LRR proteins), indicates that also the sensing of nonself-exDNA triggers a peculiar phenomenon supported by the upregulation of genes related to systemic acquired resistance in this stage of nonself-exDNA treatments (AT3G45640, AT2G38470, AT1G19250, AT2G13810, AT3G56400, AT5G26920 and AT1G73810). This is consistent with the hypothesis that nonself-exDNA acts as a PAMP triggering plant immune response (Heil & Vega-Muñoz, 2019). Overall, on one hand these findings suggest that the effects of nonself-exDNA recall a PAMP-like response, as it is evolving towards a systemic acquired resistance, that is not revealed from our results from self-exDNA treatments, the latter being possibly more related to a DAMP like response (Duran-Flores & Heil, 2015).

Considering the results of GO enrichment analysis for hormone related responses, after the treatment with self-exDNA there is a consistent upregulation of cytokinin and brassinosteroids
and a downregulation of gibberellins related processes. Differently, in the case of nonself-exDNA, hormonal response trends are limited to an upregulation of genes related to ABA and salicylic acid. The upregulation of most of the genes belonging to the Cytokinin Oxidase/Dehydrogenase (CKX) family in the self-exDNA treatment indicates cytokinins breakdown and suggests that at 16 hrs cytokinin-mediated processes are negatively affected, possibly involving, among others, cell cycle regulation, cell proliferation and shoot and root development (Werner et al., 2003).

Interestingly, self-exDNA treatment induces a downregulation of gibberellins transport. Moreover, two members of SWEET family, SWEET13 and SWEET14 (AT4G25010 and AT5G50800, respectively), known for including a major class of sugar membrane transporters in plants (Chen et al., 2012; Eom et al., 2015) are downregulated exclusively after the self-exDNA treatment at 16 hpt. Recently, they have been identified as transporter of gibberellins at intra- and inter-cellular levels, thus possibly affecting plant development and growth (Kanno et al., 2016). Our finding of a downregulation exclusive of the self-exDNA treatment may be related to the growth inhibition observed at a later stage in our analyses (Figure 2.7) that confirmed what previously highlighted (Mazzoleni et al., 2015a).

### 2.4.2 Hypotheses on the mechanisms of self-exDNA inhibition in plants

The discovery of plant growth inhibition by self-exDNA (Mazzoleni et al., 2015a) could be the result of a mechanism resembling “processes of interference based on sequence-specific recognition of small-sized nucleotide molecules” (Mazzoleni et al., 2015a), that could explain the specificity of the response leading to the inhibition of the cell functionality (Cartenì et al., 2016).

A further hypothesis was suggested to explain the dosage-dependent growth-inhibition by self-exDNA as the phenotypic consequence of a costly immune response (Veresoglou et al., 2015; Duran-Flores & Heil, 2015). In detail, Dalia Duran-Flores and Martin Heil suggested that self-exDNA may function as a DAMP activating a costly resistance mechanisms to signal a hostile environment causing the death of many conspecifics (Duran-Flores and Heil, 2015). This could ultimately causes the delay in seeds germination observed by Mazzoleni in 2015. However, it has been already stated that “the molecular mechanism underlying growth inhibition by eDNA… is uncharacterized” (Bhat & Ryu, 2016).

Self-exDNA fragments that appear in the extracellular space have been suggested to act as DAMPs (Gallucci & Maffei, 2017; Duran-Flores & Heil, 2018) causing the activation of a plant immune response similar to other well characterized DAMP, such as ATP, small signalling peptides and cell wall fragments released following a plant cell damage (Heil et al., 2012; Heil & Land, 2014; Duran-Flores & Heil, 2016; De Lorenzo et al., 2018; Hou et al., 2019). This response consists in a signalling cascade involving membrane depolarization, Ca²⁺ fluxes, ROS production
and MAPK activation and the subsequent induction of a JA dependent immunity against chewing (Wang et al., 2018a). Of note, the JA-dependent immune response causes dosage-dependent metabolic costs which, at the phenotypic level, may become apparent as stunted growth or a transient growth arrest (Heil & Baldwin, 2002; Vos et al., 2013; Guo et al., 2018). Under this scenario, self-exDNA activates a response similar to DAMPs. Indeed, fragmented self-exDNA has been shown to trigger membrane depolarization in maize (Zea mays) and lima bean (Phaseolus lunatus) (Barbero et al., 2016), ROS production, MAPK activation and JA increase in common bean (Duran-Flores & Heil, 2018), and the expression of superoxide dismutase, catalase, and phenylalanine ammonia lyase in lettuce (Lactuca sativa) (Vega-Muñoz et al., 2018).

Considering the response to nonself-exDNA, the upregulation of the response to biotic stress together with the activation of the hypersensitive response in the later stages of this treatment strongly suggest that it could act as a PAMP and not a DAMP. Similarly to the response to DAMPs, PAMPs sensing has been associated with growth arrest/inhibition and immunogenic effects (Heidel & Dong, 2006; Yakushiji et al., 2009; Niehl et al., 2016; Poncini et al., 2017) and although growth reduction is not generally reported for nonself-exDNA treatments few examples exist (Yakushiji et al., 2009).

Starting from the fact that both self and nonself-exDNA can be perceived by cells, it would have been more intuitive to expect a more costly response to foreign DNA, rather than to self-exDNA. This could be also in agreement with the stronger molecular response, in terms of transcriptome changes, here revealed in nonself rather than in self treatments. However, this evidence appears in contrast with the hypothesis of the growth inhibition in self treatments as a consequence of the metabolic cost of the immunity response. As an alternative hypothesis, Mazzoleni et al., 2015 (Mazzoleni et al., 2015a) and later Cartenì et al., 2016 (Cartenì et al., 2016) suggested a different explanation based on a more direct effect, i.e. the possible “interference” of extracellular self- or “similar” DNA (e.g.: homologous, i.e. from phylogenetically related species or even similar, i.e. with convergent structure similarity, although not phylogenetically related) causing inhibition of the whole cell functionality, mediated by sequence-specific recognition of small-sized nucleotide molecules (Ecker & Davis, 1986), which could hamper cell and gene expression functionality (Hannon, 2002) or affect genome stability (Gruenert et al., 2003), inhibiting the growth. This could explain the self-exDNA growth inhibition as a widely conserved property of living beings, and therefore justify its observation over a very wide range of organisms spanning from prokaryotes to metazoan (Mazzoleni et al., 2015b).
2.4.3 EDAP: Extracellular DNA Associated Pathways

DNA fragments may enter the cell, directly interfering with gene expression, as previously shown e.g. for triplex-forming oligonucleotides (e.g. (Chin & Glazer, 2009)) or even interacting at cytoplasmic level with redundant metabolic DNA (Gahan et al., 2008). However, the fluorescence microphotography supports the existence of a perception mechanism discriminating either nonself- and self-exDNA at membrane level resulting in a differential pattern of exDNA localization. In particular, nonself-exDNA (non-similar or phylogenetically distant) enters root tissues and cells, while self-exDNA (conspecific and/or similar or “homologous”) remains outside.

Moreover, the transcriptome analysis reveals that specific and different molecular pathways are triggered by the early response to self- and nonself-exDNA, respectively. With the attempt to indicate these pathways, we proposed the term of Extracellular DNA Associated Pathways, suggesting the new acronym EDAP. This acronym is useful to depict the differential response, since no specific pathway was described before that could explain the difference between the two categories of molecules (self and nonself-exDNA, respectively). Specifically, in the case of nonself-exDNA a remarkable differential gene expression, involving both biotic and abiotic stress related genes, accompanied by the mounting of a hypersensitive response, putatively towards a systemic acquired resistance. Conversely, a minor differential expression is evident in the self-exDNA response, where however is strongly activated the chloroplast genome, evident the oxidative stress with suppression of the genes involved in the scavenging of ROS. Interestingly, from previous works, it is known that the self-recognition triggers an early intracellular Ca$^{2+}$ spike signal and cell membrane depolarization after 30 min of self-exDNA treatment (Barbero et al., 2016). This may be accompanied by the downregulation in signal transduction related genes, as here revealed by the transcriptome analysis. Among these, the CBL-interacting protein kinases, which are known to be active in the Ca$^{2+}$ dependent signalling cascades (Luan, 2009) once bound to Calcineurin B-Like proteins (CBL), also regulates the response to oxygen deficiency and osmotic and salt stress (Lee et al., 2009; Pandey et al., 2015). This could be responsible of a reduced intracellular dynamics ending with a compromised crosstalk between the chloroplast and the nuclear genome activities, after self-exDNA exposure, that can be coupled with the activation of cation cotransporters, such as the vacuolar H$^+$/Ca$^{2+}$ antiporter (Cheng et al., 2004).

From the presented results and from previous efforts, we suggest that the response to self-exDNA exposure can be characterized by the following events:

- The initial sensing triggering a signal, similarly to what happens after DAMPs perception, rapidly propagates throughout the plant (Salvador-Recatalà et al., 2014; Choi et al., 2017; Canales et al., 2018; Toyota et al., 2018).
-The signal arrives to photosynthetic organs: here it can induce the activation of the chloroplast genome (Figure 2.8) that together with the lack of activation of genes acting as chloroplast ROS scavenging, as clearly depicted by our the transcriptome analysis and presumably due to the inhibition of the chloroplast-nuclear cross-talk, induces a positive feedback on ROS production which is associated to the MAPK activation and JA production that was reported in other experiments of exposure to self-exDNA (Duran-Flores & Heil, 2018).

From previous work, the mentioned inhibition is also reported to induce ROS production (Duran-Flores & Heil, 2018). This is linked to O₂ drop down that may contribute to the activation of the NO pathway determining the downregulation of ethylene response after exposure to self-exDNA.

We hypothesize that the inhibition of the intracellular dynamics and crosstalk can be a typical initial cell response after the sensing of external self-damages, the length of the exposure to the stressor agent strongly affecting cell fate and the extent of the damage to the whole organism.

### 2.4.4 Future perspectives

These preliminary evidences surely need more investigations and further assessments to better characterize what we here propose to describe as Extracellular DNA-Associated Pathways, introducing the acronym EDAP, that need to explain overlap and discrepancies in the sensing of self and nonself-exDNA, highlighting novel perspectives on this subject.

In particular, further analysis will be performed to define the location and the nature of the involved receptors or sensing mechanisms. Since fundamental questions concerning the response to nonself- and self-exDNA have been well addressed in A. thaliana, it could be interesting to explore in detail master regulator genes involved in the differential response in the attempt to depict the molecular pathway activated by the exposure to both exDNAs.
Figure 2.8. Model of Extracellular DNA Associated Pathways (EDAP) in plants. The exposure to either self- or nonself-exDNA produces differential cellular responses. The self-exDNA treatment triggers an electric response, starting with a sensing at membrane level, with calcium spikes followed by a reduced permeability of the roots, and a cascade of events involving the chloroplasts and inducing ROS production. On the other hand, nonself-exDNA enters the cells where it is metabolized activating a cascade of events inducing a hypersensitive response. Figure published in (Chiusano et al., 2021).
Chapter 3: exDNA AND UNICELLULAR EUKARYOTIC ORGANISMS: EFFECTS ON MICROALGAE

The exDNA in aquatic systems has been found as free molecules in the water column, or in sediments, if complexed with colloids and particles (Deflaun et al., 1986; Karl & Bailiff, 1989; Dell’Anno & Danovaro, 2005; Herndl & Reinthaler, 2013; Torti et al., 2015) or as component of the EPS of marine bacteria biofilms (Decho & Gutierrez, 2017; Passerini et al., 2019). In these contexts, exDNA has been mainly discussed for its nutritional role, i.e. it is an important phosphate (Dell’Anno & Danovaro, 2005) and carbon (Lennon, 2007) source for many marine microbial communities (Dell’Anno & Corinaldesi, 2004; Dell’Anno & Danovaro, 2005). Moreover, due to its longer persistence, it is also an important pool for genetic recombination and genetic modification via HGT (Corinaldesi et al., 2005; Mao et al., 2014) which has been particularly discussed in the context of marine biofilms (Corinaldesi et al., 2005; Mao et al., 2014; Decho & Gutierrez, 2017).

In aquatic environments, the inhibitory effects of self-exDNA is almost unexplored as well as the effects of the exposure to nonself-exDNA. This is the first study in which this effect is deeper investigated on two photosynthetic microorganisms: *Chlamydomonas reinhardtii* and *Nannochloropsis gaditana*, representative of freshwater and marine environments, respectively.

*C. reinhardtii* was selected because of its well-characterized cellular and molecular biology that makes it an advantageous model organisms for both unicellular photosynthetic organisms but also for studying many processes of multicellular autotrophs. Despite less characterized than *C. reinhardtii*, *N. gaditana* is a promising model for marine microalgae giving the possibility to perform both physiological and molecular analysis upon treatment with the compounds of interest. Moreover, being characterized by a more resistant cell wall, it would be interesting to explore its susceptibility to either nonself- and self-exDNA.

### 3.1. Microalgae: a model system for photosynthetic organisms

Microalgae are unicellular photosynthetic microorganisms, able to grow alone or in symbiosis with other organisms in a wide range of environmental conditions in either marine or freshwater and able to live in the water column as well as onto sediment (Khan et al., 2018).

Microalgae (including diatoms and green algae together with cyanobacteria) have been studied for over a century (Mann & Myers, 1968; Bowler et al., 2008) in that they offer several advantages over higher plants such as a faster growth rate, higher yield in term of biomass production and sustainable growth requiring only marginal resources (Ben-Amotz & Avron, 1983; Levering et al., 2016). The recent advances in genome sequencing and in genome editing technologies as well as the availability of several wild-type and mutant strains increased the interest of the research on
microalgae species (Fu et al., 2019). Moreover, they have recently attracted considerable interest worldwide, due to the application of the production of many bioactive compounds useful as nutritional supplement, pharmaceutical products and biofuels production (Hamed, 2016).

3.1.2 *Chlamydomonas reinhardtii*: a model for freshwater microalgae

*Chlamydomonas reinhardtii* (Figure 3.1a) is a freshwater single-celled green alga easily cultured in laboratory conditions and growing as a haploid with vegetative life cycle but able to form zygotes if environmental conditions become harsh. Indeed, sexual reproduction can increase the rate of adaptation of *C. reinhardtii* to new or changing environmental conditions (Sasso et al., 2018).

It is considered a model organism for study many plant physiological processes such as the basis of photosynthesis and light perception. Moreover, the study of the structure, function and biogenesis of motile *C. reinhardtii* cilia that share similarities with those of mammals, promoted our understanding of cilium dysfunctions in humans (Brown & Witman, 2014).

Other characteristics make *C. reinhardtii* an excellent model organism (Sasso et al., 2018):

- Rapid growth rate (doubling time in optimal condition: 8 hrs (Harris, 2001);
- Availability of light-sensitive photosynthesis mutants (Levine, 1969; Spreitzer & Mets, 1980);
- Sequenced and annotated nuclear (Merchant et al., 2007), mitochondrial (Vahrenholz et al., 1993) and chloroplast genome (Maul et al., 2002) capable of genetic transformation (Boynton et al., 1988; Remacle et al., 2006) and genome editing (Ferenczi et al., 2017);
- Construction of genome-wide library of mapped, indexed insertional mutants (Li et al., 2016).

3.1.3 *Nannochloropsis gaditana*: a model for marine microalgae

*Nannochloropsis gaditana* (Figure 3.1b) is a unicellular eukaryotic and photosynthetic marine microalga considered a promising tool for many industrial applications, particularly for the biofuel production in microalgae (mainly for the production of triacylglycerols) (Corteggiani Carpinelli et al., 2014; Cecchin et al., 2020) and for food additives production (being source of poly-unsaturated fatty acids (PUFAs), polyphenols, carotenoids and vitamins) (Cecchin et al., 2020; Zanella & Vianello, 2020).
The main aspects that would encourage the use of *Nannochloropsis gaditana* as model organisms are the following:

- Rapid growth rate;
- The ability to survive in a wide range of conditions (i.e. pH, temperature and salinity);
- The capacity to produce and accumulate a large amounts of lipids also in standard cultural conditions but particularly under nitrogen deficiency (Cecchin *et al.*, 2020);

**Figure 3.1.** Structure of *Chlamydomonas reinhardtii* (a) and *Nannochloropsis gaditana* (b) cell. In *C. reinhardtii* cell (a) most of the cell volume is occupied by a single cup-shaped chloroplast housing the machinery for the photosynthesis. It contains the pyrenoid and the eyespot. The former concentrates the inorganic carbon in the cell against a concentration gradient while the latter, orients cells swimming toward or away from the light (phototaxis). The two cilia at the top of the cell are motile and are fundamental for the mating processes. Other features of the cell are a centrally located nucleus, a cell wall mainly composed of proteins, Golgi bodies, vacuoles and mitochondria. Cell diameter: 5-10 µm. This image is adapted from (Sasso *et al.*, 2018) Schematic presentation of the main features of the *N. gaditana* cell (b). Cell diameter of 2–5 µm This image is adapted from (Zhang *et al.*, 2019).

### 3.2 Materials and methods

#### 3.2.1 DNA extraction and fragmentation

The genomic DNA from *C. reinhardtii* and *N. gaditana* (self-DNA) was extracted adapting the protocols of Jagielski, 2017 (Jagielski *et al.*, 2017) and Jeffrey Doyle, 1991 (Doyle, 1991). The algal cells were collected and ground with pestle in a pre-chilled mortar in presence of Polyvinylpyrrolidone (PVP) powder and re-suspended in pre-warmed CTAB isolation buffer (2%...
CTAB, 1.4 M NaCl, 100 mM Tris pH 8.0, 20 mM EDTA) for 1 hr in a waterbath set at 60°C. The DNA was extracted within an equal volume of chloroform-isoamyl alcohol (24:1) and centrifuged 15 minutes at max speed. The supernatant was collected and the DNA was precipitated over-night at -20°C with two volumes of ethanol 100% v/v and 1/10 of NaOAc 3 M pH 5.2. The ethanol was removed after 15 minutes of centrifugation at max speed (4°C), the pellet was washed with 70% ethanol v/v and centrifuged for 10 minutes at max speed. The pellet was air-dried and then dissolved in sterile milliQ water. The no-self DNA was from the phylogenetically distant Sardina pilchardus (nonself-DNA). It was extracted by the abdominal muscle following the same procedure here reported from the incubation in the CTAB buffer.

After DNA extraction, the re-suspended DNA was treated with RNase A (Thermo fisher) for 1 hr at the final concentration of 0.25 mg/ml at 37°C. The RNase A was then inactivated at 70°C for 20 minutes.

Final DNA concentration has been quantified using Qubit fluorimeter, kit dsDNA HS Assay (Thermo Fisher). The quality and integrity of the DNA were evaluated by 1% agarose gel electrophoresis. The DNA preparation was sheared using a Bioruptor Plus (Diagenode) sonicator, set for 12 cycles at high power setting with 60 seconds ON and 10 seconds OFF to get fragmented DNA according to (Mazzoleni et al., 2015a).

### 3.2.2 Algal growth and growth inhibition assay

Cultures of *Chlamydomonas reinhardtii* (strain CCAP 11/32b) and *Nannochloropsis gaditana* (strain 639) obtained from the Algal Collection University Federico II were grown in Bold’s Basal Medium (BBM) and Artificial Seawater Medium (ASW) both supplemented with vitamins, respectively at 24 ± 1°C, on a rotatory shaker at 100 rpm with a photoperiod of 16:8 hours light/dark at 100 E m⁻² s⁻¹ and subculturing were weekly maintained in fresh medium (Bischoff, 1963; Chiaiese et al., 2011). Growth inhibition assays were assessed with the modified protocol reported in Nunes et al., 2014 (Nunes et al., 2014). Specifically, the assay was performed in 24 well microplates and incubated for 168 hrs. The cell density was measured every 24 hrs. Each microalgae species was treated with fragmented self and non-self DNA at 0; 3; 10 and 30 ng/µL in triplicates. A schematic overview of the experimental design is represented in Figure 3.2.

The specific growth rate (µ) was calculated for each species according to the following equation:

\[
\mu = \frac{\ln N_2 - \ln N_1}{t_2 - t_1}
\]

where \(N_2\) and \(N_1\) represent the cell density (cell ml⁻¹) at time \(t_2\) and \(t_1\) (expressed in hrs).

The generation time (Tg) was calculated based on the following equation:
The percentage of inhibition (%I) for both species was calculated with respect to control at 168 hpt, using the following equation:

\[ \% I = \frac{\mu_c - \mu_t}{\mu_c} \]

with \( \mu_c \) representing the specific growth rate in the control group and \( \mu_t \) the specific growth rate in the DNA treatment, respectively.

Statistical analyses were performed by Shapiro Wilk’s and Bartlett tests using the SPSS 27.0 software.

**Figure 3.2.** Schematic overview of the experimental design. DNA PREPARATION: extraction, RNase treatment and sonication of DNA from C. reinhardtii or N. gaditana (self-DNA) and from Sardina pilchardus (nonself-DNA), according to Mazzoleni et al., 2015a. TREATMENTS: the exposure of both microalgae to three different concentrations (3, 10 and 30 ng/µL) was performed in triplicate. ANALYTICAL APPROACHES: Growth curves of microalgae and microscopy were performed to evaluate the effects of both exDNA. BB medium stays for Bold-Basal medium.

**3.2.3 Microscopic analysis of cell structure and chlorophyll distribution**

Morphological analyses were performed with two independent microscopes by using a 63x oil immersion objective. The confocal microscope Zeiss LSM 700 was used to observe the cellular chlorophyll distribution (Ex/Em: 405/300-800 nm) and the microscope Leica DM6000B was used.
to observe cells using fluorescein isothiocyanate (FITC) filter, whose excitation wavelengths includes the excitation maxima of all known types of algal chlorophylls and of many photosynthetic accessory pigments, and 4',6-diamidino-2-phenylindole (DAPI) that binds strongly to adenine–thymine-rich regions in DNA (Ex/Em: 488/525 nm and 358/460 nm, respectively).

3.2.4 DNase treatment and cell vitality assays

*C. reinhardtii* cell aggregates were analysed at 168 hpt with self DNA at 30 ng/µL. 13.5U of RNase-Free DNase (Qiagen) were added to the microalgae cell culture and a phenotypic analysis was carried using the microscope Leica DM6000B, 1 hour post DNase treatment.

3.3. Results

3.3.1 Self-DNA inhibits growth of freshwater and marine algae

*C. reinhardtii* growth curves (Figure 3.3a,b) show that the cell density is not affected at all concentrations of nonself-exDNA (Figure 3.3a) and at lower concentrations of self-exDNA (Figure 3.3b) following over time the same sigmoidal trend of the control. Conversely, the treatment with self-exDNA at 30 ng/µL significantly affects the culture cell density along the time course. In particular, the differences between the self-exDNA and the other treatments start to widen among 48 and 72 hrs reaching a cell density remaining almost stable until 168 hpt.

Moreover, as the concentration of nonself-exDNA increases, cells grow more than the control (Figure 3.3a) and also for self-exDNA at 3 and 10 ng/µL the cell density reaches a value higher than the control at 168 hrs.

In *N. gaditana*, the treatment with nonself-exDNA at 3, 10 and 30 ng/µL only slightly affects algal growth in a concentration dependent manner (Figure 3.3c) while the treatment with self-exDNA strongly affects the growth in a concentration dependent manner showing the highest effect at 30 ng/µL at 168 hrs (Figure 3.3d).

The evaluation of the growth rate inhibition to control (GRI (%)) at 168 hrs for *C. reinhardtii* and *N. gaditana* (Table 3.1) shows that for both microalgae the treatment with self-exDNA at 30 ng/µL strongly affects the growth (Table 3.1). The generation time (Tg) of *C. reinhardtii* at 168 hrs is around 3-4 days after all treatments and only after the treatment with self-exDNA at 30 ng/µL it shows a delay reaching the value of 22 days if compared with the control (Table 3.1). In *N. gaditana*, after all nonself-exDNA treatments the Tg only slightly changes when compared with the control. Differently, after the treatment with self-exDNA the Tg increases in a concentration
dependent manner reaching in self-exDNA at 30 ng/µL a value at least 6 times higher than the control (Table 3.1).

![Graph showing cell growth analysis for Chlamydomonas reinhardtii and Nannochloropsis gaditana](image)

**Figure 3.3.** Cell growth analysis. Cell growth of *Chlamydomonas reinhardtii* (a,b) and *Nannochloropsis gaditana* (c,d) after the exposure to different concentration of nonself- and self-exDNA. * indicates statistically significant difference among treatments within a specific time (post Hoc ANOVA, Dunnet test with p < 0.05).
### C. reihnardtii

<table>
<thead>
<tr>
<th>Treatment (exDNA ng/µl)</th>
<th>Average cell density (x $10^4$ cells*ml$^{-1}$ ± sd)</th>
<th>Tg (days ± sd)</th>
<th>GRI (% of control ± sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (BB-m)</td>
<td>42.2 ± 3.6</td>
<td>3.6 ± 0.2</td>
<td>-</td>
</tr>
<tr>
<td>Nonself 3</td>
<td>42.7 ± 3.3</td>
<td>3.8 ± 0.4</td>
<td>6.3 ± 9.2</td>
</tr>
<tr>
<td>Nonself 10</td>
<td>45.7 ± 2.8</td>
<td>3.4 ± 0.1</td>
<td>-4.8 ± 3.8</td>
</tr>
<tr>
<td>Nonself 30</td>
<td>52.9 ± 7.7</td>
<td>3.2 ± 0.4</td>
<td>-13.1 ± 12.3</td>
</tr>
<tr>
<td>Self 3</td>
<td>51.8 ± 8.6</td>
<td>3.6 ± 0.7</td>
<td>-1.6 ± 19.2</td>
</tr>
<tr>
<td>Self 10</td>
<td>57.1 ± 8.7</td>
<td>3.0 ± 0.3</td>
<td>-17.8 ± 9.7</td>
</tr>
<tr>
<td>Self 30</td>
<td>13.7 ± 0.6</td>
<td>22.4 ± 3.3</td>
<td>83.7 ± 2.7</td>
</tr>
</tbody>
</table>

### N. gaditana

<table>
<thead>
<tr>
<th>Treatment (exDNA ng/µl)</th>
<th>Average cell density (x $10^4$ cells*ml$^{-1}$ ± sd)</th>
<th>Tg (days ± sd)</th>
<th>GRI (% of control ± sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (BB-m)</td>
<td>65.3 ± 9.8</td>
<td>6.7 ± 0.2</td>
<td>-</td>
</tr>
<tr>
<td>Nonself 3</td>
<td>52.8 ± 9.8</td>
<td>10.2 ± 0.2</td>
<td>11 ± 1.5</td>
</tr>
<tr>
<td>Nonself 10</td>
<td>57.6 ± 9.8</td>
<td>8.8 ± 0.3</td>
<td>28 ± 2.8</td>
</tr>
<tr>
<td>Nonself 30</td>
<td>59.7 ± 19.6</td>
<td>8.5 ± 1.3</td>
<td>14 ± 10.2</td>
</tr>
<tr>
<td>Self 3</td>
<td>58.0 ± 13</td>
<td>10.3 ± 0.3</td>
<td>2.0 ± 1.7</td>
</tr>
<tr>
<td>Self 10</td>
<td>51.2 ± 11</td>
<td>11.7 ± 0.3</td>
<td>28 ± 1.6</td>
</tr>
<tr>
<td>Self 30</td>
<td>42.2 ± 13.2</td>
<td>36.6 ± 11.2</td>
<td>81.6 ± 9</td>
</tr>
</tbody>
</table>

**Table 3.1.** Average cell density (x $10^4$ cells*ml$^{-1}$), generation time (Tg) and growth rate inhibition (% of control) (GRI (%)) of *C. reihnardtii* (A) and *N. gaditana* (B) at 168 hpt at different concentrations of nonself- and self-exDNA. sd: standard deviation.
3.3.2 Morphological changes after treatment with exDNA

*C. reinhardtii* cells treated at the three different concentrations of nonself-exDNA show a morphology and a chlorophyll autofluorescence similar to the control (Figure 3.4 a,b and Supplementary Figure 3.1a). This appears also for the self-exDNA treatments at 3 and 10 ng/µL (Supplementary Figure 3.1a), while at 30 ng/µL the cells show an altered morphology, appearing in a palmelloid phenotype and forming aggregates (Figure 3.4 a,b and Figure 3.5a).

Also *N. gaditana* cells treated with the three different concentrations of nonself-exDNA show a morphology and a chlorophyll autofluorescence similar to the control. At lower concentrations of self-exDNA treatments (3 and 10 ng/µL) the cells appear with an altered organization of the chlorophyll (Supplementary Figure 3.1b) that becomes remarkable at 30 ng/µL, a concentration in which the formation of aggregates is evident too (Figure 3.4b, Figure 3.5b and Supplementary figure 3.1b). In both species, the aggregates appear as heteromorphous organizations with cells surrounded by a dense substance appearing like an extracellular matrix.

**Figure 3.4.** Microscopy analysis of *C. reinhardtii* (a) and *N. gaditana* (b) samples in controls and after treatments with nonself- and self-exDNA at 30 ng/µL. In red, the chlorophyll autofluorescence (Ex/Em\(^{1}\): 405/300-800). The composite is also shown (Merge). Microscope: Zeiss LSM 700. Excitation at 405, detection in the range 300-800, Objective: 63x 1.40 Oil Dic. \(^{1}\)Ex/Em=Excitation/Emission.
3.3.3 Microscopic evaluation of aggregates formation after treatment with self-exDNA

In order to evaluate the formation and the nature of cellular aggregates and extracellular matrices observed after the treatment with self-exDNA at 30 ng/µL, we stained the aggregates formed in both species with DAPI (Figure 3.6). The dye remarks the localization of DNA in the cell nucleus and in the organelles (plastids and mitochondria) in both controls and treatments. Moreover, in the self-exDNA treatments, it is also staining the entire heteromorphous aggregates including the extracellular matrices.

For further investigations, we monitored the cell aggregate formation over time in the treatment of *C. reinhardtii*. An aliquot of the cellular suspension was analysed under microscope starting from 48 hrs, to 96 and 168 hpt by using DAPI staining and FITC filtering (Figure 3.7). The presence of palmelloid cells is evident already at 48 hrs, and, interestingly, the formation of aggregates surrounded by an extracellular matrix appears since this stage. At 96 hpt the cellular aggregates become clearer and the DAPI diffusely stains also the extracellular formations. At 168 hpt, the heteromorphous formations acquire a more evident and structurally defined organization (Figure 3.7, 168 hpt and Figure 3.8a). Interestingly, 1 hpt with RNase-Free DNase of aggregates formed at 168 hpt with self-exDNA 30 ng/µL resulted in disaggregation of the extracellular matrices embedding the *C. reinhardtii* cells (Figure 3.8 b).
Figure 3.6. Fluorescence analysis of C. reinhardtii (a) and N. gaditana (b) samples in the controls and after treatments with self-exDNA at 30 ng/µL. Cellular aggregates were analysed using DAPI staining. Microscope: Leica DM6000B, Ex/Em: 358/460 nm, Objective: HC PL APO 63x 1.40 Oil, scale bar = 10 µm.
Figure 3.7. Fluorescence analysis of C. reinhardtii after treatment with self-exDNA at 30 ng/µL: focus on cellular aggregates formation. Cellular aggregates were analysed using DAPI staining. Microscope: Leica DM6000B, Ex/Em: 358/460 nm, Objective: HC PL APO 63x 1.40 Oil, scale bar = 10 µm.
Microscopy analysis of C. reinhardtii aggregates after treatment with DNase. An aliquot of C. reinhardtii cells treated with self-exDNA at 30 ng/µL was observed before and after the treatment with 6 µL of 2,25 U/µL RNase-Free DNase (Qiagen) for 40’. After treatment with DNase, aggregates break up. In red, the chlorophyll autofluorescence (Ex/Em: 405/300-800). Microscope: Zeiss LSM 700, Excitation at 405 and detection in the range 300-800, Objective: 63x 1.40 Oil Dic, scale bar = 10 µm.
3.4. Discussion

ExDNA has been found in nearly all terrestrial and aquatic habits (Nagler et al., 2018). In marine and freshwater environments it is reported to be freely present in the water column as well as in sediments (Deflaun et al., 1986; Karl & Bailiff, 1989; Dell’Anno & Danovaro, 2005; Nielsen et al., 2007; Torti et al., 2015; de Aldecoa et al., 2017) and its persistence in the environment is influenced by surrounding conditions, for example, the adsorption to particles (Lorenz & Wackernagel, 1987; Agnelli et al., 2007), the presence of bacterial nucleases (Demanèche et al., 2001; Bylemans et al., 2018), temperature and salinity of the water (Okabe & Shimazu, 2007; Borin et al., 2008). Most of recently published efforts describe the exDNA as a mineral source of phosphate (Dell’Anno & Danovaro, 2005) and carbon (Lennon, 2007). In the context of marine microbial communities or, when located in the extracellular polymeric substance of marine biofilms, exDNA is also considered a source for genetic recombination and horizontal transfer genes (Corinaldesi et al., 2005; Mao et al., 2014).

Recently, Mazzoleni et al. 2015 demonstrated that the exposure to fragmented self-exDNA triggers inhibitory effects on conspecifics in several species, while the treatment with nonself-exDNA did not show similar effects (Mazzoleni et al., 2015b). The inhibitory effect was firstly observed in plants (Mazzoleni et al., 2015a) and subsequently reported in species of different taxonomic groups, showing a general biological phenomenon revealing ecological and evolutionary implications (Mazzoleni et al., 2015a).

In aquatic environments, the inhibitory effects of self-DNA is almost unexplored. This is the first study in which this effect is deeper investigated on two photosynthetic microorganisms, representative of freshwater and marine environments, respectively.

Our results show that the presence of self-exDNA in culture growth medium affects the life cycle of both freshwater and marine microalgae. Indeed, it reduces cell growth, increases the generation time of almost 5 times, and determine a growth inhibition rate of almost the 80% for both microalgae species when compared with the control at 30 ng/µL. Interestingly, this inhibition appears to have a concentration dependent effect in N. gaditana. In C. reinhardtii, the self-exDNA at 10 ng/µL seems to exert the highest inhibitory effect at 96 hpt, while the cell growth overcomes the control at 168 hpt, appearing to recover from the inhibition. Finally, the cell growth seems to be favoured by the presence of a very low concentration of self-exDNA (3 ng/µL) in the medium. This could be interpreted as a nutritive advantage at very low concentration that is opposite to the evident inhibitory effects at higher concentrations. These results, while confirming the remarkable inhibitory effect in both species at higher self-exDNA concentrations, also reveal the positive effects of lower concentrations due to the well-known nutritive role of exDNA, as extensively discussed in the literature (Dell’Anno & Danovaro, 2005; Levy-Booth et al., 2007; Nagler et al.,
This can also explain the positive recovery from the self-DNA inhibition revealed in *N. gaditana* at 10 ng/µL considering 96 and 168 hpts.

It is to note that the cell growth at 168 hpt in all the tested concentrations of nonself-exDNA seems to slightly promote *C. reihardtii* cell growth, while reducing the growth in *N. gaditana*. This highlights that species can have different sensitivity to DNA exposure and that the nature of the DNA, as well as the environmental context in which this exposure takes place, can affect species growth and generation time, paving the way to the need for further investigations in the field.

Interestingly, the microscopy analysis revealed the formation of a heteroamorous organization after treatment with self-exDNA at 30 ng/µL. These organizations appear to be formed by both morphological altered cells with palmelloids phenotype and extracellular aggregates similar to extracellular matrices.

Considering palmelloid cells within extracellular aggregates, it is well-known that *C. reihardtii* cells when exposed to stress agents, such as organic acids (Iwasa & Murakami, 1968), chloroplatinic acid (Nakamura *et al.*, 1975), herbicide paraquat (Metaxatos *et al.*, 2003) and sodium chloride (NaCl) salt stress (Khona *et al.*, 2016) or also when grown in a phosphate limited medium (Olsen *et al.*, 1983), form multicellular aggregates known as palmelloids. These cells growing in form of multilayers are thought to retain the ability to proceed into cell division in the mother cell but to be unable to completely separate after division and thus remaining within a common cell wall and membrane (Khona *et al.*, 2016). Accordingly, the treatment with self-exDNA at 30 ng/µL gradually arrests cell growth determining the formation of these cells unable to complete cell division. Nevertheless, other molecular and morphological analysis are required to better characterize the nature of palmelloids, for example, to investigate if they are effectively more resistant to environmental stresses and also to what extent the cell cycle progression resulted affected by the exposure to higher concentrations of self-exDNA.

Concerning the extracellular aggregates formed only after treatment with self-exDNA at 30 ng/µL, our time laps analysis revealed that palmelloids and other materials forming the extracellular matrix start to accumulate from 48 hpt and becoming progressively evident and structurally defined up to 168 hpt. These organizations resemble extracellular structures such as plant root extracellular traps (Wen *et al.*, 2009), mammalian neutrophil extracellular traps (Brinkmann *et al.*, 2004) and biofilms (Whitchurch *et al.*, 2002) where it is widely reported in literature that the exDNA has both a structural and functional role. The presence of exDNA within this aggregates was confirmed by both DAPI staining and by their dissolution after the treatment with DNase.
Recently, on the basis of the observation of an inhibitory effects of self-exDNA, a novel role has been proposed for these extracellular structures, i.e. sites where the exDNA can be organized and trapped. Indeed, within this structures exDNA can play its well-known role of protection against foreign attacks, and, as suggested in (Monticolo et al., 2020) be limited in its bio-availability within the environment as a free molecule, limiting the extracellular self-exDNA self-inhibitory effects. The formation of these aggregates composed of also exDNA only after the treatment with self-exDNA at 30 ng/µL could be indicative of a mechanism involving a specific recognition of the nature of the exDNA (i.e. conspecific or heterospecific) also recently described in plants (Chiusano et al., 2021). Nevertheless, future microscopy and molecular efforts are required to clarify if a mechanism of differential reception is involved in the sensing of self- and nonself-exDNA. Furthermore, further analysis should be performed to better characterize the nature of these aggregates and their formation and composition also in *N. gaditana*.

### 3.5 Future perspectives and impact

Further molecular and morphological analysis should be performed to confirm and to better characterize both the status of *C. reihardtii* cells within aggregates (i.e. *palmelloids* cells) as well as the aggregates composition. Moreover, the same issues should be addressed for the marine microalgae *N. gaditana*. Then, further microscopy and molecular efforts are required to clarify if a mechanism of differential perception is involved in the sensing of self- and nonself-exDNA, thus justifying the formation of aggregates only after treatment with higher concentration of self-exDNA. These results supported the great potential in the use of self-exDNA for industrial application, also deserving important ecological implication. For instance, the development of strategies based on the application of exDNA specific for microbial organisms affecting microalgae growth, may be useful to increase the biomass yield of microalgae cultivated in bioreactors. The scaling up phase required for making the proposed strategies suitable for industrial purposes, would take advantages from the powerful tool represented by microbial libraries where different fragments of the target species DNA are inserted in the microbial genome allowing specific amplification under highly quality conditions. On the other hand, the application of the self-exDNA could be of ecological interest for the biocontrol in aquatic ecosystem of microalgae overgrowth, i.e. to control the harmful phenomenon of harmful algal blooms.
4.1 ExDNA in multicellular organisms

In multicellular organisms and in particular in human body, the presence of exDNA has been widely discussed from different points of view (Aucamp et al., 2018). Considering its origin, exDNA can derive from endogenous as well as of exogenous sources, of both mitochondrial (Zhang et al., 2010; Rykova et al., 2017) and nuclear (Barrat et al., 2005; Bhagirath et al., 2015) origin. It is mainly produced following apoptotic cell death (e.g. in patients with gastric (Sai et al., 2007) or prostate cancer (Delgado et al., 2013)) or necrotic processes (Grabuschnig et al., 2020) (e.g. following physical injury or trauma (Gögenur et al., 2017)). Of note, it has been also described that it can be actively released by highly proliferating cells (Stroun et al., 1977) such as stimulated or not human lymphocytes (Anker et al., 1975) and activated neutrophils during bacterial infection (Brinkmann et al., 2004). The exDNA of exogenous origin instead, can enter the body via many processes such as the digestion of food sources, transplantation or transfusion and microbial infection (Aucamp et al., 2018). Focusing on its location, exDNA has been found freely in bodily fluids such as plasma, urine and saliva (O’Driscoll, 2007) or bound to other plasma constituents (e.g. RNA, lipids and proteins) (Gahan & Stroun, 2010) and released through extracellular vesicles (Thierry et al., 2016; Pathan et al., 2019). Moreover, there are evidences of its presence on the outer leaflet of the plasma membrane (Meinke et al., 1973; Bennett et al., 1986) and as component of defensive structures known as neutrophil extracellular traps (NETs) (Brinkmann et al., 2004; Tamkovich & Laktionov, 2019; Sofoluwe et al., 2019).

Following the advance in next-generation sequencing technologies, it is nowadays possible to detect the exDNA naturally present in healthy humans (Lo et al., 1997) or occurring during pathological conditions (Fuchs et al., 2010; Hawes et al., 2015; McCarthy et al., 2015; Vakrakou et al., 2018; Lowes et al., 2020b). This paved the way for the non-invasive diagnosis of pathological conditions, therapy monitoring, follow-up tests (Thierry et al., 2016) and for non-invasive detection of fetal genetic disorders (Larrabee et al., 2004). Concerning its functions, a fundamental protective role of exDNA has been discussed in the context of NETs where it chemotactically attracts and immobilises pathogens promoting the disruption of microbial membrane integrity, leading to cell lyses (Brinkmann et al., 2004; Hawes et al., 2015; Monticolo et al., 2020). When abnormally released or present in in an anomalous compartment it may also act as a "Damage-associated molecular pattern" (DAMP) (Duran-Flores & Heil, 2018; Monticolo et al., 2020). In this case, it may trigger the activation of a non-infectious host inflammatory immune response (Seong & Matzinger, 2004; Roh & Sohn, 2018). Interestingly, exDNA can putatively function as an endocrine signalling molecule. Indeed, alone or enclosed within RNA/lipoprotein complexes, it is able to modify the biology of the recipient cells, as in the...
transformation of normal cells into cancer cells (Bendich et al., 1965; Gahan & Chayen, 1965; García-Olmo et al., 2000, 2010; Gahan, 2003; Hawes et al., 2015). According to that, García-Olmo and colleagues introduced the concept of “genometastasis” referring to the occurrence of metastasis via the in vitro transfection of cells with DNA carrying dominant oncogenes derived from a primary tumour and circulating in the plasma (García-Olmo et al., 2000; Gahan & Stroun, 2010). This was also demonstrated in vivo to promote cell transformation, tumorigenesis and also it drives tumour progression (García-Olmo et al., 2010; Trejo-Becerril et al., 2012).

Overall, the presence of either exogenous DNA that can act as a “pathogen associated molecular pattern (PAMP) or endogenous DNA in aberrant compartments triggers a response following the activation of receptors that sense the DNA in the cytoplasm or in the endosomal compartment called pattern recognition receptors (PRRs) (Paludan & Bowie, 2013; Gallucci & Maffei, 2017). Whether cytoplasmic or endosomal system, of exogenous or endogenous origin, the sensing of exDNA activates the host immune system and interferon mediated pro-inflammatory responses (Desmet & Ishii, 2012; Man et al., 2016; Tao et al., 2016; Gasser et al., 2017; Zhou et al., 2017; Szczesny, 2018; Wang et al., 2019b; Motwani et al., 2019).

Despite the recognition of nucleic acids is well-described in mammals, it is still poorly characterized either the differential response or the sensing of nonself-exDNA and self-exDNA.

To explore the effect of the treatment with exDNAs on human cells we used the line PNT2 (human prostatic epithelial cell lines). This cell line has been immortalized through the transfection with SV40 virus expressing both large T and small t oncogenes. They have a well-differentiated morphology, are serum dependent and non-tumorigenic in mice, grow as colonies until full confluency and show an epithelioid shape (Berthon et al., 1995).

4.1.2 Human cell line as model system for heterotroph organisms

In vitro cell culture offers the possibility to obtain relevant information of cellular and molecular responses to external stimuli in higher multicellular and organisms (i.e. humans) otherwise impossible to simply evaluate. In particular, the immortalized cell lines are generally adopted for in vitro studies for their ability to indefinitely proliferate (following laboratory manipulation) and for being cultivable for long periods. Among the main advantages of using immortalized cell lines the most relevant are their low cost, easily manipulation in lab, their being usually available and the presence of many standardized and well characterized cell lines deriving from cells of different tissues having mutations or chromosomal abnormalities generated either naturally or by genetic manipulation. Moreover, they are, at least theoretically, homogeneous, genetically identical populations, and thus providing consistent sample for obtaining reproducible results (Carter & Shieh, 2010; Kaur & Dufour, 2012).
For these features, cell lines provide an excellent tool for studying the physiology and biochemistry (e.g., metabolic status, developmental processes) of either normal or diseased cells. The basic structure of a human cell is represented in Figure 4.1. Furthermore, they provide an excellent system for testing the cytotoxic effect of drugs or natural and synthetized compounds but also for producing biological compounds (e.g., vaccines, therapeutic proteins) (Vanlerberghe & McIntosh, 1994).

![Figure 4.1 Schematic representation of human cell.](image)

### 4.2 Materials and methods

#### 4.2.1 DNA extraction and fragmentation

The genomic DNA from PNT2 cells (self-exDNA) was extracted by using the following procedure. Cells were resuspended in pre-warmed CTAB extraction buffer (2% CTAB, 100 mM Tris HCl pH=8, 20 mM EDTA, 1.4 M NaCl 0.2% β-mercaptoethanol and 0.1 mg/mL proteinase K (added just before use)) and incubated at 60°C for 1 hour.

Then, an equal volume of chloroform-isooamy alcohol (24:1) was added to the supernatant and centrifuged 10 minutes at max speed. The aqueous phase was collected and the DNA was precipitated at -20°C within one volume of isopropanol for 30 minutes. The isopropanol was removed after 15 minutes of centrifugation at max speed (4°C) and the pellet was washed with ethanol 75% v/v and centrifuged for 10 minutes at max speed. The ethanol was discarded and the pellet was dried and dissolved in sterile milliQ water.

The DNA from the tomato Ailsa Craig young leaves (nonself-exDNA, phylogenetically distant species) was extracted by adapting the procedure described in (Sahu et al., 2012).
Briefly, Ailsa Craig young leaves were collected and ground in a pre-chilled mortar in presence of Polyvinylpyrrolidone (PVP) powder. Then, it was suspended in 2 volume of pre-warmed suspension buffer (50 mM EDTA, 120 mM Tris-HCl, 1 M NaCl, 0.5 M sucrose, 2% Triton-X 100, and 0.2% b-mercaptoethanol (added just before use) and placed in a waterbath set at 60°C for 40 minutes. The suspension was centrifuged at 10,000 rpm for 15 minutes at room temperature and it was added an equal volume of extraction buffer (20 mM EDTA, 100 mM Tris-HCl, 1.5 M NaCl, 2% CTAB, 1% b-mercaptoethanol (added just before use) to the supernatant that was incubated at 60°C for 30 minutes.

The DNA was extracted within an equal volume of chloroform-isoamyl alcohol (24:1) and centrifuged 15 minutes at max speed. The DNA was precipitated in one volume of chilled isopropanol and keep at −20°C overnight. The isopropanol was removed after 15 minutes of centrifugation at max speed (4°C) and the pellet was washed with was washed with 70% ethanol v/v and centrifuged for 10 minutes at max speed. The pellet was air-dried and then dissolved in sterile milliQ water.

After DNA extraction, the re-suspended DNA was treated with RNase A (Thermo fisher) for 1 hour at the final concentration of 0.25 mg/ml at 37°C. The RNase A was then inactivated at 70°C for 20 minutes.

Final DNA concentration has been quantified using Qubit fluorimeter, kit dsDNA HS Assay (Thermo Fisher). The quality and integrity of the DNA were evaluated by 1% agarose gel electrophoresis. The DNA preparation was sheared using a Bioruptor Plus (Diagenode) sonicator, set for 12 cycles at high power setting with 60 seconds ON and 10 seconds OFF to get fragmented DNA according to (Mazzoleni et al., 2015a).

4.2.2 Evaluation of cellular viability

PNT2 cells were grown in RPMI 1640 medium supplemented with 2mM Glutamine with 10% Foetal Bovine Serum (FBS). Cells were maintained in incubator at 37 °C in atmosphere made of 95% air and 5% CO₂. For propagation, cells were detached with trypsin (0.25% p/v) and collected with complete culture medium. After centrifugation at 180 g for 10 minutes, pellets were suspended in fresh medium, properly diluted and plated again. For the experiment, 5000 cells were seeded in 96-well plate (Burker chamber has been used to count cells). After 20 hrs cells completely adhere to the well surface and the medium was removed and 90 µL of fresh medium was added to each well.

The experimental design (Figure 4.2) included the following treatments: (1) milliQ water for control; (2) self-exDNA treatment: addition of fragmented PNT2 DNA and (3) nonself-exDNA
treatment: addition of fragmented Ailsa Craig DNA. The final concentrations of DNA were of 0.03 ng/µL, 0.3 ng/µL, 3 ng/µL and 30 ng/µL. Each treatment was performed in triplicates.

The cell viability was assessed using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay (Applichem A2231) according to Gerlier Denis and Nicole Thomasset (Gerlier & Thomasset, 1986), 1986 and every 24 hrs and up to 96 hpt, cells were treated with 10 µL (5 mg ml\(^{-1}\)) of MTT and incubated for 3 hrs. The absorbance at 570 nm was recorded using a HTS platform freedom EVO 200 - TECAN). Cell viability for each replicate was calculated as following:

\[
\% \text{ cell viability} = \left( \frac{A_{570 \text{ treatment}}}{A_{570 \text{ control}}} \right) \times 100.
\]

Statistical analyses were performed by using post hoc Tukey test with ANOVA using the SPSS 27.0 software.

### 4.2.3 Video microscopy time-lapse

For time-lapse video microscopy, PNT2 cells were seeded in 96-well plate at a density of 5000 cells/well following the same experimental designed for the evaluation of cellular viability described above. In order to monitor the cell trajectories as well as the morphological features after DNA treatments, Carl Zeiss Axio Observer z1 inverted epifluorescence microscope, x,y,z fully motorized, equipped with Hamamatsu Orca Flash 4 camera and chamber incubator XL (Pecon, Germany) for CO\(_2\), temperature and humidity control and controlled by Zen 3.3 software was used. The images were acquired with a 20X air objective and for each well 4 different fields were selected. The acquisition time was set as follow: each 15 min for the first hour (n° of scenes 5), each 60 min for the next 24 hrs (n° of scenes 25). Tile mode was used for defining multiwell positions, then, all data were collected as AVI files. The AVI files were organized with the AVI splitter 2.30 software (Website: [https://www.brizsoft.com/avisplit/](https://www.brizsoft.com/avisplit/)). Cell count and cell tracking of the control and nonself- and self-exDNA treatments at 0.3 ng/µL were analysed by using two plugins of the software ImageJ. For the cell count of the number of total cell, dead cells and mitotic events was used the ImageJ plugin Cell counter. For the cell tracking was used the plugin Manual tracking. The cell tracking was performed by selecting from each replicate of the control, nonself- and self-exDNA treatments a sample of almost 20 cells including 10 isolated cells (indicated as Isolated), i.e. cells that at the beginning of the time lapse were not located yet in an organized group (cluster) and 10 cells already located into clusters (indicated with Clustered) (Supplementary Figure 4.1). Cells have been followed for the entire time of acquisition and the resulting table, containing the coordinates of each cell for each time, was then collected and analysed. An example of the graphical results of manual tracking is reported in Figure 4.3. The results of the manual track were analysed by using tidyverse and ggplot R packages. For the
evaluation of cell migration, speed and distance, the mean square displacement (MSD) was calculated as following according to Gorelik and Gautreau, 2014 (Gorelik & Gautreau, 2014):

$$MSD(\tau) = \text{mean}([r(t + \tau) - r(t)]^2); \quad r = \sqrt{x^2 + y^2}$$

Where x and y denote positions of the cell in a specific frame, t denotes time and τ indicates the time amount separating the 2 positions occupied by the cell.

![Figure 4.2. Schematic overview of the experimental design. DNA PREPARATION: extraction, RNase treatment and sonication of DNA from human cell line PNT2 (self-DNA) and from Ailsa Craig (tomato) (nonself-DNA), according to Mazzoleni et al., 2015a. TREATMENTS: the exposure of PNT2 to different concentrations of both exDNAs (0.03, 0.3, 3 and 30 ng/µL) was performed in triplicate. ANALYTICAL APPROACHES: PNT2 cell viability and video time lapse microscopy were performed to evaluate the effects of both exDNA. MTT stays for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide.](image-url)
Figure 4.3. Tracking analysis of a subset of cells after the treatment with nonself- and self-exDNA and in the control. Coloured points represent the selected cells (left panel) and coloured lines (right panel) represent their trajectories. Inverted epifluorescence microscope: Carl Zeiss Axio Observer z1 with 20x air objective.

4.3 Results

4.3.1 ExDNA treatment decreases human cell viability

The treatment with both nonself- and self-exDNA affects the viability of PNT2 cells when compared with the control in a concentration and time dependent manner (Figure 4.4). The differential effect between exDNAs is more evident at lowest concentration at 24 and 48 hrs. Indeed, at 24 PNT2 cell viability is affected by the treatment with self-exDNA at 0.03 and 0.3 ng/µL of almost 10% more than nonself-exDNA. In detail, at 24 hrs nonself- and self-exDNA at 0.03 ng/µL decrease cell viability of 12% ± 1.31 and 23% ± 1.78, respectively and at 0.3 ng/µL they decrease cell viability of 11% ± 4.16 and 19% ± 2.22, respectively. At 48 hrs, the difference between nonself- and self-exDNA is particularly evident at 3 ng/µL where the viability resulted reduced of 10% ± 0.74 and 22% ± 1.76, respectively. At 72 hrs and at 96 hrs, the cell viability is affected with no significant differences between nonself- and self-exDNA (Figure 4.4).
**Figure 4.4.** PNT2 cell viability after the exposure nonself- and self-exDNA. Cell vitality (expressed as % of the control) was evaluated after the treatment with nonself- and self-exDNA at 0.03, 0.2, 3 and 30 ng/µL at 24, 48, 72 and 96 hpts. * and ** indicates statistically significant differences between treatments and control with p < 0.05 and p < 0.01, respectively (post Hoc ANOVA, Tuckey test).

4.3.2 The treatment with exDNAs affects cellular physiology

The treatment with both nonself- and self-exDNA at 0.3 ng/µL observed for 24 hrs in video time increases the number of dead cells when compared with the control (Table 4.1a and b and Figure 4.5). However, the increase in dead cells is more evident after the treatment with self-exDNA. In detail, the number of death cells in the control, nonself- and self-exDNA after 24 hrs is 7 ± 3, 12 ± 4 and 20 ± 2 respectively.

Considering the occurrence of mitotic events within 24 hrs, in the control the 24% ± 3.84 of cells successfully completed cell division while after the treatment with nonself-exDNA and in particular with self-exDNA, mitotic events represent only the to 17.09% ± 1.72 and 8.7% ± 2.58, respectively (Table 4.1b). The figure 4.6 shows the cumulative sum of mitotic events (i.e. the sum of mitosis events of a frame and the previous ones) and highlights the differences in the occurrence of mitotic events among treatments in comparison with the control. Interestingly, cells treated
with self-exDNA starting the mitosis process, proceed through cell cycle more slowly than the control and the last step of cell division (i.e. the cytokinesis) seems to be particularly delayed (Figure 4.7).

The analysis of manual cell tracking results of either isolated cells or cells within clusters highlights additional features related to cellular migration. The analysis of mean squared displacement MSD (r) shows a differential trend between the isolated and clustered cells in the treatment with self-exDNA when compared with nonself-exDNA and the control. Indeed, after the treatment with self-exDNA clustered cells appears to move less than the clustered cells in nonself-exDNA treatment and in the control (Figure 4.8 and Supplementary Figure 4.2 and 4.3).

A

<table>
<thead>
<tr>
<th></th>
<th>Cell count (1 hour)</th>
<th>Cell count (24 hrs)</th>
<th>Number of dead cells</th>
<th>Number of mitotic events</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>190 ± 15</td>
<td>210 ± 21</td>
<td>7 ± 3</td>
<td>46 ± 9</td>
</tr>
<tr>
<td>Nonself-exDNA</td>
<td>156 ± 28</td>
<td>154 ± 28</td>
<td>12 ± 4</td>
<td>37 ± 2</td>
</tr>
<tr>
<td>Self-exDNA</td>
<td>145 ± 9</td>
<td>124 ± 12</td>
<td>20 ± 2</td>
<td>12 ± 3</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th></th>
<th>Dead cells (%)</th>
<th>Mitotic events (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.6 ± 1.63</td>
<td>24.1 ± 3.84</td>
</tr>
<tr>
<td>Nonself-exDNA</td>
<td>7.5 ± 2.30</td>
<td>17.09 ± 1.72</td>
</tr>
<tr>
<td>Self-exDNA</td>
<td>14 ± 1.74</td>
<td>8.7 ± 2.58</td>
</tr>
</tbody>
</table>

*Table 4.1. Cell count of the total number of cells, dead cells and mitotic events. (a) Total cell count at 1 and 24 hrs in the control and after the treatment with nonself- and self-exDNA. In (b) is reported the percentage of dead cells and mitotic events. The cell death increases while mitotic events decreases after treatment with both exDNAs. These effects are particularly evident after self-exDNA treatment.*
Figure 4.5. Microscopy analysis of cell death events after the treatment with non-self- and self-exDNA at 0.3 ng/µL at 24 hrs compared with control. Cell death increases after treatment with both types exDNAs. This effect is particularly evident after self-exDNA treatment. Inverted epifluorescence microscope: Carl Zeiss Axio Observer z1 with 20x air objective.

Figure 4.6. Number of mitotic events during the 24 hrs after the treatment with non-self- and self-exDNA at 0.3 ng/µL. For each time point, mitotic events are indicated as cumulative sum (i.e. mitotic events occurred in each frame are summed with those occurred in previous ones). Mitotic events decreases after treatment with both exDNAs. This effect is particularly evident after the treatment with self-exDNA.
Timing of mitosis after the treatment with self-exDNA and in the control. Mitosis appears delayed after the treatment with self-exDNA. Inverted epifluorescence microscope: Carl Zeiss Axio Observer z1 with 20x air objective.

Mean squared displacement of isolated and clustered cells after the treatment with nonself- or self-exDNA at 0.3 ng/µL. The mean value of MSD (τ) for both isolated and clustered cells is indicated as mMSD (µm²/s). After the treatment with self-exDNA, clustered cells show a reduced movement when compare with clustered cells in nonself-exDNA treatment and in the control.

4.4 Discussion

The presence of exDNA in human body derived from either exogenous and endogenous sources (Aucamp et al., 2018) and thus acting as PAMPs or DAMPs, respectively, is recognized by pathogen related receptors (PRRs) (Paludan & Bowie, 2013; Gallucci & Maffei, 2017) that upon
sensing activate the host immune system and particularly interferon mediated pro-inflammatory responses (Desmet & Ishii, 2012; Man et al., 2016; Tao et al., 2016; Gasser et al., 2017; Zhou et al., 2017; Szczesny, 2018; Wang et al., 2019b; Motwani et al., 2019). The activation of the immune response following the recognition of exDNA is known to increase the probability to develop an autoimmune disorder (e.g. systemic lupus erythematosus (Barrat et al., 2005) and rheumatoid arthritis (Rykova et al., 2017)), but also to drive a local auto-amplification loop that leads to exaggerated cell death (Linkermann et al., 2014).

Interestingly, both self and nonself DNA determine a decrease in cell viability, although self DNA appears almost more effective. This effect is more evident at higher concentrations and at later stages after exposure (at 72 and 96 hpt) (Figure 4.4).

Nevertheless, future analyses are needed to confirm that the cell death index increases proportionally with DNA concentration and to evaluate if the self- and nonself-exDNA activate different pathways of cell death among the many recognized and characterized for mammals (Galluzzi et al., 2018).

After the treatment with both exDNAs, the number of mitotic events is reduced, with a major effect after the treatment with self-exDNA (Figure 4.6 and Table 4.1). In addition, after the treatment with self-exDNA it seems that cells going through mitosis encountered problems in completing cytokinesis (Figure 4.7). One of the mechanisms that is associated with both the reduction of mitotic events and the increased cell death is the mitotic catastrophe (Castedo et al., 2004) described as a mechanism of cell death activated by the sensing of mitotic failure (Vitale et al., 2011). Nevertheless, further analysis, either molecular or physio-morphological evaluations are fundamental to understand which are the molecular effects occurring during cell division, and if the reduced mitotic index depends on different mechanisms following the two independent treatments with nonself- and self-exDNA.

Interestingly, our results show a clear trend in clustered cells migration after treatment with self-exDNA. Indeed these clusters seem to move less than clustered cells in the control and in nonself-exDNA treatment (Figure 4.8 and Supplementary Figure 4.2 and Supplementary Figure 4.3). This highlights that cells in clusters under self-exDNA treatments are less prone to movement. Presumably, this could be ascribed to cell inhibition or cell suffering as shown by the accompanying results on viability and cell status.

### 4.5 Future perspectives

Further studies are necessary to evaluate the molecular mechanisms involved in human cells response and, in case, if a differential sensing of self- and nonself-exDNA occurs. The characterization of cell death types, the analysis of the cell cycle arrest and the evaluation of
associated markers to specific stress responses should be performed to better depict the phenomena here highlighted.
Chapter 5: exDNA AND MULTICELLULAR HETEROOTROPHS: EFFECTS ON CIONA ROBUSTA

The ascidian chordate *Ciona robusta* (previously known as *Ciona intestinalis*), is a filter-feeding marine sessile invertebrate belonging to the Subphylum of Urochordates, considered as the closest living relatives of vertebrates (Delsuc *et al.*, 2006). It is a widely distributed organism particularly able to survive in a wide range of environmental conditions (i.e. contaminated as well as clean seawater) (Lambert & Lambert, 1998). Indeed, due to its adaptive behaviour, *Ciona* developed the ability to settle and grow on manmade structures such us nets, floats and ropes, aquaculture gear and also on living organisms (i.e. shells). This process, known as biofouling, negatively impact on the economy of shellfish industries (Fitridge *et al.*, 2012).

The ascidians are planktonic swimming larvae in the first part of their life (Figure 5.1a). After substrate settlement, larvae undergo a process of metamorphosis, becoming adult sessile filter feeder animals (Figure 5.1b). Like the majority of tunicates, *Ciona* is a hermaphrodite oviparous; eggs and sperm are released through the atrial siphon and fertilization occurs externally. Ascidians develop in a relatively short time considering that a swimming tadpole hatches within 24 hrs post fertilization (hpf) at 18°C (Figure 5c-i) (Satoh, 2003). Despite none of the distinctive features of the chordate body plan is clearly present in the adult form of ascidians, at larval stage the basic landmarks of the phylum, such as the notochord, the dorsal hollow nerve cord and segmented muscles are recognizable, justifying their close phylogenetic relationship with vertebrates (Figure 5.1l) (Holland *et al.*, 2004).

5.1 Advantages of *Ciona robusta* as model system

Because of their relative simplicity and phylogenetic position, ascidians have recently emerged as model system used to investigate the molecular mechanisms underlying cell-fate specification during chordate development. They offers many advantages (Satoh, 2001):

- Fast embryonic development (completed within 24 hpf at 18°C);
- Transparent embryos and larvae allowing a direct tissue observations;
- Invariant cleavage pattern (i.e. the positioning and timing of the cell divisions is identical between different individuals of the same species) and availability of accurate fate maps tracing the embryonic development of different ascidian species (Conklin, 1905; Nishida, 1987; Hotta *et al.*, 2007);
- Sequenced and well-annotated genome (Dehal *et al.*, 2002) together with a large amount of cDNA and EST (Expressed Sequence Tag) information freely available in the tunicate community. The *Ciona* genome is quite small and compact, it is composed of about 16000 protein coding sequences, with very few duplicated genes (Dehal *et al.*, 2002).
Of note, most of the promoters are relatively short and usually located in close proximity to the transcription start site of the genes (Corbo et al., 1997; Fanelli et al., 2003; Squarzoni et al., 2011). *Ciona* embryos, indeed, permit a simple and detailed visualization of gene expression by whole-mount *in situ* hybridization (Satoh, 2001).

Thus, the genome sequence and the simplified chordate body plan that contains rudiments of most vertebrate tissues, make *Ciona* a recognized model species for studying molecular mechanisms underlying the chordates and vertebrates developmental program (Satoh et al., 2003; Satou et al., 2019).

Moreover, due to its ability to adapt to changing condition and harsh environment in coastal ecosystems, *C. robusta* could be an excellent model for studying species response to the occurrence of environmental stress (Li et al., 2020a) and thus a useful tool for ecological and toxicological analyses (Eliso et al., 2020; Hotta et al., 2020).

In this work, we used the model *C. robusta* to evaluate if the exDNA of both non-self- and self-origin displays any effects on a marine multicellular organism and on its development. In particular, we evaluated its effect on the main *C. robusta* developmental stages: 110-cell stage (at the beginning of gastrulation stage), neural plate formation and neurula stages tailbud formation stage and larval phase.
Figure 5.1. The ascidian Ciona intestinalis. (a) C. intestinalis tadpole larva and (b) C. intestinalis adult. In b, the arrow head and the asterisk evidence the incurrent and the outcurrent siphon respectively. The sperm duct (SD) and the egg duct (ED) are also shown. (c-i) Embriogenesis; (c) Fertilized egg, (d) 2-cell embryo, (e) 4-cell embryo, (f) 16-cell embryo, (g) gastrula, (h) early tailbud, (i) mid-tailbud. (l) Transverse section of the tail with the phenotypic features of chordates is shown (l). Ep, epidermis; ES, endodermal strand; Mu, muscle; NC, nerve (this image is adapted from Satoh et al., The ascidian tadpole larva: comparative molecular development and genomics). Embryos dimension = 200 µm, larvae dimension = 1 mm, adult dimension = 100-200 mm.

5.2 Materials and methods

5.2.1 DNA extraction and fragmentation

The DNA from C. robusta (self-exDNA) was extracted from sperms and oocytes by using the following procedure: sperms and oocytes were suspended in the sperm isolation buffer (SIB) (200 mM Tris pH 8.0, 100 mM EDTA). Proteinase K (final concentration: 50 µg/ml) and sodium
dodecyl sulfate (SDS) (final concentration: 1%) were added. The mixture was incubated overnight in a waterbath set at 37°C. The DNA was extracted within an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and centrifuged 2 minutes in an Eppendorf Centrifuge at max speed. Then, it was added to the supernatant an equal volume of chloroform-isoamyl alcohol (24:1) and centrifuged 2 minutes at max speed. This step was repeated twice in order to completely remove phenol contaminants. The aqueous phase was collected and the DNA was precipitated at -20°C within two volumes of ethanol 100% v/v for 30 minutes. The ethanol was removed after 15 minutes of centrifugation at max speed (4°C), the pellet was washed with 70% ethanol v/v and centrifuged for 10 minutes at max speed. The pellet was air-dried and then dissolved in sterile milliQ water. The DNA from the phylogenetically distant Zea mays (Maize) young leaves (nonself-exDNA) was extracted by adapting the procedure described in (Sahu et al., 2012). Zea mays (Maize) young leaves were collected and ground in a pre-chilled mortar in presence of Polyvinylpyrrolidone (PVP) powder and re-suspended in pre-warmed CTAB isolation buffer (2% CTAB, 1.4 M NaCl, 100 mM Tris pH 8.0, 20 mM EDTA) for 1 hour in a waterbath set at 60°C. The DNA was extracted within an equal volume of chloroform-isoamyl alcohol (24:1) and centrifuged 15 minutes at max speed. The supernatant was collected and the DNA was precipitated over-night at -20° C with two volumes of ethanol 100% v/v and 1/10 of NaOAc 3 M pH 5.2. The ethanol was removed after 15 minutes of centrifugation at max speed (4°C), the pellet was washed with 70% ethanol v/v and centrifuged for 10 minutes at max speed. The pellet was air-dried and then dissolved in sterile milliQ water.

After DNA extraction, the re-suspended DNA was treated with RNase A (Thermo fisher) for 1 hour at the final concentration of 0.25 mg/ml at 37°C. The RNase A was then inactivated at 70°C for 20 minutes. Final DNA concentration has been quantified using Qubit fluorimeter, kit dsDNA HS Assay (Thermo Fisher). The quality and integrity of the DNA were evaluated by 1% agarose gel electrophoresis. The DNA preparation was sheared using a Bioruptor Plus (Diagenode) sonicator, set for 12 cycles at high power setting with 60 seconds ON and 10 seconds OFF to get fragmented DNA according to (Mazzoleni et al., 2015a).

5.2.2 Animals and embryos

Adult specimens of Ciona were collected in the Gulf of Taranto, Italy, and acclimatized at ~20°C for 2–3 days in open system tanks and fed every day with a solution of marine into the facilities of Stazione Zoologica Anton Dohrn and fed every day with a solution of marine microalgae concentrates (Shellfish Diet 1800 Instant Algae). Then, they were exposed to continuous lighting for a few days in order to accumulate mature gametes and to prevent gamete spawning. Gametes were recovered from the gonducts of several animals to perform the in vitro fecundation. The
eggs were washed and fertilized in filtered seawater into agarose-coated plate. After two washing steps, the fertilized eggs were used for the experiment.

5.2.3 Sample preparation and treatments

Two experiments were performed: the first one, to observe the effect of nonself- and self-exDNA at 24 hpt and the second one to follow the entire development. The experimental design (Figure 5.2) included the following treatments: (1) control (2) treatment with self-exDNA: addition of fragmented C. robusta DNA and (3) treatment with nonself-exDNA: addition of fragmented Zea Mays (Maize) DNA at different concentration (50, 100, 150, 200 and 250 ng/µL). The experiments were performed within a 24 well plate in a final volume of 500 µL (first experiment) or 1 ml (second experiment).

From the agarose-coted plate 5 or 10 fertilized eggs for the first and the second experiment, respectively, were selected and seeded into each well by using a glass Pasteur pipette.

In the second experiment, the development was evaluated by observing the following stages: 110 cells, gastrula period, neural plate formation, neurula and tailbud period and at 24 hpf, in a final volume of 1 ml 10 embryos were seeded. For each stage, two embryos of each treatment were isolated, blocked into 3-(N-morpholino) propanesulfonic acid (PARA-MOPS) 8% and sea water (1:1), manually dechorionated and observed under Apotome.2 (ZEISS) Microscope. Morphological anomalies were assessed by observing the larvae after 24 hpf under the stereoscope and motility defects were evaluated before the addition of PARA-MOPS at a final concentration of 4%.
5.3 Results

The evaluation of the development at 24 hpt reveals that both exDNAs induce a concentration dependent effect. Embryos treated with the lowest concentration of both nonself- and self-exDNA (50 ng/µL) complete their development until the larval stage similarly to the control (Table 5.1 and Figure 5.3). After the treatment with both nonself- and self-exDNA at 100 ng/µL, embryos complete the development similarly to the control. Indeed, the control as well as treatments with both exDNAs show that only one embryo fails to reach the larval stage. The treatment with nonself-exDNA at 150 ng/µL allows all embryos to complete their development but 40% showed structural and motility defects (Figure 5.3). Differently, self-exDNA at 150 ng/µL blocks the development of 40% of embryos with also one adult larvae showing both structural and motility defects (Figure 5.3). The major differences between the two treatments occurs at 200 and 250 ng/µL. After treatment with nonself-exDNA at 200 ng/µL only 1 embryo fails to reach the larval
stage, and, at the highest concentration (250 ng/µL), nonself-exDNA arrests the development of almost half of the population while the other properly completes the development without structural anomalies (Supplementary figure 5.2). Conversely, the treatment with self-exDNA at both 200 and 250 ng/µL concentrated arrests the development in 100% of embryos (Supplementary Figure 5.1).

Having being assessed that both exDNAs induced interesting effects visible at 24 hpt, we decided to perform a second experiment to observe the embryos treated with both nonself- and self-exDNA at 200 ng/µL during the main developmental stages (i.e. 110-cell stage, neural plate formation and late neurula, tailbud period and larval stage).

From the microscopy observations, samples treated with nonself-exDNA proceeded during the development up to the tailbud stage similarly to the control, embryos treated with self-exDNA show a delay in the development particularly evident during the neural plate formation and neurula stage (Figure 5.4). Furthermore, after 24 hpt, the treatment with self-exDNA blocked the embryos in an undefined stage while the treatment with nonself-exDNA allows embryos to reach the larval stage (Figure 5.4).
<table>
<thead>
<tr>
<th>n° of embryos</th>
<th>Larvae normally develop.</th>
<th>Arrested develop.</th>
<th>Larvae with anomalies (ng/µL)</th>
<th>n° of embryos</th>
<th>Larvae normally develop.</th>
<th>Arrested develop.</th>
<th>Larvae with anomalies</th>
<th>n° of embryos</th>
<th>Larvae normally develop.</th>
<th>Arrested develop.</th>
<th>Larvae with anomalies</th>
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</table>

N/A indicates not applicable.
Table 5.1. Evaluation of Ciona robusta embryos development after the treatment with different concentrations of nonself- and self-exDNA at 24 hrs. The total number of embryos, of those completed or arrested (arrested develop) during the development and the number of larvae with anomalies are indicated in A. The percentage of normally developed larvae, of the embryos arrested during the development and of larvae with anomalies are reported in B. Anomalies were classified as structural observed under stereomicroscope) or functional (assessed before adding the PARA-PBS 4%). The images were taken using the Apotome.2 (ZEISS), scale bar = 100 µm.

<table>
<thead>
<tr>
<th>exDNA (ng/µL)</th>
<th>Control</th>
<th>Nonself</th>
<th>Self</th>
<th>Nonself</th>
<th>Self</th>
<th>Nonself</th>
<th>Self</th>
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<td>0</td>
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<tr>
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<td>0</td>
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<td>100</td>
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</table>
Figure 5.4. *C. robusta* embryos development (early gastrula period at 110 cells, neural plate, tailbud formation and larval stage) after treatment with nonself- and self-exDNA at 200 ng/µL. Embryos were manually dechorionated. The images were taken using the Apotome.2 (ZEISS), scale bar = 100 µm.
5.4 Discussion

For its phylogenetic position the marine invertebrate *C. robusta* is a suitable model system to investigate the effect of nonself- and self-exDNA treatments on the embryonic development in a chordates. Moreover, due to its adaptive behaviour and its ability to survive in changing conditions and harsh environments, it is an excellent model for studying how species respond to the occurrence of environmental stresses (Li *et al.*, 2020a) and thus a useful tool for ecological and toxicological analyses (Eliso *et al.*, 2020; Hotta *et al.*, 2020).

Our results show that both exDNA types affected *C. robusta* development in a concentration dependent manner (Table 5.1). In particular, at the highest concentration tested (250 ng/µL), the percentage of embryos reaching the larval stage after 24 hrs is reduced if compared with the control but the effect was striking after the treatment with self-exDNA (developmental block of 100% of embryos) (Table 5.1, Figure 5.2, Supplementary Figure 5.1 and 5.1).

Interestingly, after the treatment with self-exDNA at 200 and 250 ng/µL in almost half of arrested embryos, the pigmented cells are anyway visible (Supplementary Figure 5.1). Pigmented cells are normally located in *C. robusta* central nervous system and precisely in the sensory vesicle. This structure contains different sensory receptor cells, which are mostly organized in two pigmented organs: the anterior otolith cells (Dilly, 1962) which are gravity receptors guiding the swimming behaviour of the tadpole larvae (Tsuda *et al.*, 2003) and the more posterior ocellus involved in light perception (Dilly, 1964). The processes involved in their histological specification start during the late gastrula stage and ends at neurula stage (Nishida & Satoh, 1989). In our results, the presence of pigmented cells in embryos blocked after the treatment with highest concentrations of self-exDNA could indicate that processes involved in cellular specification still occurred. However, these processes are probably not followed by those activated to coordinate the distribution of cells in proper tissues. Of note, it has been shown that the exposure to toxic compounds such as bisphenol A (at high concentrations: 20-30 µM) (Gomes *et al.*, 2019), fluconazole and ethanol (Battistoni *et al.*, 2018) induces neurodevelopmental toxicity in ascidians by impairing the movement of pigmented cells that anyway are still morphologically detectable. Similarly, it has been demonstrated that in embryos deformed for high temperature different cell types such as the notochord and pigmented ocellus cells are still present and histochemically recognizable. Nevertheless, they fail to find their proper positions during embryogenesis during the post-gastrula architectural arrangements (Irvine *et al.*, 2019). The authors point out that since the differentiation of the ocellus and otolith occurs long after gastrulation just before the larval stage, at least to the tailbud cell-cell relationships are intact enough. This means that during the neural plate formation and tailbud stages the main molecular pathways acting in these cell type specification (i.e. bone morphogenetic protein (BMP)/Chordin, FGF and wingless-related integration site (Wnt) signalling (Esposito *et al.*, 2015) or alternatively, compensative regulative
processes occur. Overall, to verify if the treatment with self-exDNA produces cellular and molecular changes and to clarify if it affects the pigmented cell migration and the pathways acting during neurula and tailbud stages, either immunohistochemical assays or molecular analysis must be planned as future efforts.

The effects of self-exDNA on the main developmental stages revealed that the whole process resulted delayed. This was particularly evident during the neural plate formation with a final block in an undefined stage (Figure 5.4). Of note, it has been demonstrated that one of the main processes involved in the neural plate formation fundamental for the proper embryos development from fertilization to organ formation (Webb & Miller, 2003; Deguchi et al., 2015; Chen et al., 2017) in both vertebrate and invertebrate is the generation and propagation of transient Ca\(^{2+}\) waves. In particular, it seems conserved the function of transient-type Ca\(^{2+}\) channels during neural tube closure (Abdul-Wajid et al., 2015) and in promoting the differentiation of anterior neural plate (Hackley et al., 2013; Akahoshi et al., 2017). Similarly to what has been previously demonstrated in plant (Barbero et al., 2016), the self-exDNA may exert its effect by impairing the plasma membrane potential and Ca\(^{2+}\) homeostasis of embryos and thus causing the arrest of the processes required for the proper neural plate formation. This effect can depend on a specific mechanism of sensing activating a differential cellular response thus triggering peculiar signal pathways. Nevertheless, further histological, morphological and molecular analysis will be necessary to deepen these preliminary observations and thus clarifying processes and pathways involved in the arrested or impaired development.

Conversely, the treatment with nonself-exDNA does not completely arrest the development of embryos, neither at the highest concentrations (Supplementary Figure 5.2). Indeed when observed during the main developmental stages, embryos treated with nonself-exDNA at 200 ng/µL appeared similar to the control. At 200 and 250 ng/µL embryos that failed in complete the development after 24 hrs appears arrested in an undetermined stage and just in one case (at 200 ng/µL) the pigmented cells were evident (Supplementary Figure 5.1). Peculiar of the nonself-exDNA treatment is the occurrence of structural anomalies and motility defects in some larvae after 24 hrs.

Mutagens like the bisphenol A and also the N-ethyl-N-nitrosourea have been demonstrated to affect the complete development and motility of ascidians Ciona intestinalis (Matsushima et al., 2013) and Ciona savignyi (Nakatani et al., 1999), respectively. For instance, the bisphenol A at low concentration (from 1 to 3µM) decreases the number and induces abnormal tail-beat movements of hatched larvae suggesting an action on the developing larval central nervous system through probably binding of the estrogen-related receptor. The N-ethyl-N-nitrosourea instead impairs the notochord formations, and thus its role in coordinating the proper development and patterning of numerous tissues (in both vertebrates and invertebrates (Fleming et al., 2004))
leading to a truncated tail, although the development of the tail muscles and caudal nerve tracts appears largely normal.

Also in the case of nonself-exDNA, other experiments are necessary to clarify if mechanisms involved in nonself-exDNA response are similar to those activated by the toxic compound above mentioned.

Overall, this study offers an initial insight on the role of exDNAs in influencing the development of a marine invertebrate after fertilization. Moreover, being the ascidian development extensively studied from both a morphological and a molecular point of view, i.e. proteins and genes involved in the main signalling pathways are mostly well-characterized, future analysis aiming at investigating a single factor involved in a specific process or the whole pathway, are possible and also required to define physiochemical and molecular responses of *C. robusta* to both self- and nonself-exDNA and to further define the observed phenotypes.

### 5.5 Future perspectives

The future work on *C. robusta* should take into account the identification of the stage at which the treatment with self-exDNA blocks the development using both immuno-histochemical assays to further characterize cellular populations and molecular analysis and depict mechanisms involved in the arrested or impaired development. Moreover, it would be of interest to better characterize the phenotypes observed after nonself-exDNA treatment (i.e. motility defects and morphological anomalies observed in larvae). To depict the whole phenomenon, the clarification of mechanisms through which embryos sense both exDNAs would be fundamental.
Chapter 6: Final discussion

The inhibitory function of self-exDNA, highlighted for the first time by Mazzoleni and his colleagues (Mazzoleni et al., 2015a,b), reveals unexpected functional roles of exDNA that appear to be crucial for determining species interactions at community (Zhang et al., 2016) and ecosystem (Cartení et al., 2016) levels. Experimental evidences from different research groups fuelled these results supporting the idea of a differential perception of exDNAs from different sources (i.e. self- and nonself-exDNA). The response of plant systems to self-exDNA, differently from nonself-exDNA, consists in a cascade of events involving the plasma membrane depolarization and the intracellular Ca$^{2+}$ release (Barbero et al., 2016), generation of ROS, specific MAPK activation and the involvement of particular immune system factors (Duran-Flores & Heil, 2018).

Following these results, many hypotheses on the perception and the mechanisms activated by self- and nonself-exDNA in cells and organisms of different clades have been made (Mazzoleni et al., 2015a,b; Duran-Flores & Heil, 2015; Cartení et al., 2016; Vega-Muñoz et al., 2018) although mostly remaining at speculative level.

The results shown in this thesis revealed that the treatment of organisms from different species with self-exDNA inhibits cell division and decreases cell growth causing a delay in the overall development plan. In A. thaliana, the treatment with self-exDNA induces the overproduction of ROS, of putatively chloroplast origin, which is accompanied by the lack of their scavenging. This produces a phenomenon of internal toxicity that ends in the necrosis of root apex and presumably of the whole root tissues, if the stimulus continues to persist over time (Figure 2.7). In algae (both freshwater and marine ones), the higher concentration of self-exDNA tested (30 ng/µL) strongly reduced the cell growth when compared with the control (Figure 3.3). Considering the response in human cells at 24 hpt, self-exDNA at 0.3 ng/µL decreases cell viability (Figure 4.4), increases cell death (Table 4.1 and Figure 4.5) and reduces mitotic events (Table 4.1 and Figure 4.6), where it seems to affect particularly the phase of cytokinesis. Moreover, the observed trend of a reduced MSD ($\tau$) and thus a decreased cell movement in human clustered cells after treatment with self-exDNA (Figure 4.8 and Supplementary Figure 4.2 and Supplementary Figure 4.3), suggests that upon a specific recognition of self-exDNA, cells in clusters are less prone to movement. Presumably, this could be ascribed to cell inhibition or cell suffering as shown by the results on viability and cell status. In C. robusta, the self-exDNA at 200 and 250 ng/µL strongly delays embryos development (Figure 5.4) probably affecting the signals organizing the cellular positioning in the adult tissues. Interestingly, despite the concentrations at which the observed phenotypic effects where particularly evident are far from those detected in various aquatic environment (Torti et al., 2015), considering that Ciona sp are typically found in areas of low flow or minimal wave exposure (Carver et al., 2007), where the exDNA concentrations are
expected to be higher than in flowing water, these organisms may putatively have evolved a mechanism to cope with these concentrations contributing to their ability to survive in extreme environmental conditions.

Interestingly, also the exposure to nonself-exDNA produces characteristic responses. For instance, in A. thaliana the transcriptome analysis revealed that the treatment with nonself-exDNA at 200 ng/µL elicits a remarkable differential gene expression, involving both biotic and abiotic stress related genes, accompanied by the mounting of a hypersensitive response, putatively triggering a systemic acquired resistance (Figure 2.8) particularly evident in the later stages of this treatment. These results strongly suggest that it could act as a PAMP, which, similarly to the response to DAMPs, can lead to growth arrest/inhibition and immunogenic effects (Heidel & Dong, 2006; Yakushiji et al., 2009; Niehl et al., 2016; Poncini et al., 2017). In human cells, the higher concentrations of nonself-exDNA (3 and 30 ng/µL) decrease cellular viability when compared with the control (Figure 4.4). This is particularly evident at 72 hpt. Moreover, the treatment with nonself-exDNA at 0.3 ng/µL, increases cell death (Table 4.1 and Figure 4.5) and decreases mitotic events (Table 4.1 and Figure 4.6) when compared with control, even if to a lower extent when compared with self-exDNA. Future analysis could also in this case confirm that nonself-exDNA may be perceived as a PAMP, thus activating a different type of programmed cell death related to immunological responses. A peculiar response to nonself-exDNA is also visible in C. robusta where, as the concentrations increase, there are evident morphological anomalies, i.e. larvae complete the development but their movement are altered and their structure show evident alteration (Figure 5.3).

As mentioned before, it is clear that all species are able to recognize and discriminate between exDNAs of different origin. Of note, the fluorescence microphotography experiments performed on A. thaliana suggest that the differential recognition may occurs at cell membrane level, possibly on the extracellular surface of the plasma membrane or on its immediate surroundings. This could explain the accumulation of extracellular self-DNA on cell surfaces in contrast with the entrance and the active endocytosis stimulated by the nonself-DNA, although further investigation should address possible explanations. Interestingly, in microalgae the formation of extracellular aggregates containing exDNA, only after the treatment with self-exDNA at 30 ng/µL (Figure 3.4), suggests a mechanism of specific recognition occurring at membrane level. Furthermore, being these aggregates absent after the treatment with lower concentrations of self-exDNA, we speculate that the recognition may occur only once a threshold concentration is perceived.

Despite these evidences, additional microscopic and morphological analysis are required to further investigate the exDNAs interaction with cellular systems. In addition, also the nature of the putative membrane receptor (e.g. composed of aminoacids, nucleic acids, glycans or a
combination of these categories) remain to be elucidated. Of note, in animals has been found the presence of DNA molecules (Meinke et al., 1973; Bennett et al., 1986) and glycosylated RNAs (Flynn et al., 2021) on the plasma membrane of cells. On the basis of our results suggesting a specific recognition at membrane level or in its surrounding and the finding of either DNA or RNA molecules on plasma membrane, it can be speculated that nucleic acids themselves, known to be present in the extracellular environment could be involved in the self/nonself-exDNA discrimination in plants and presumably in all living beings. This is an interesting point to be addressed also in plants and other organisms.

In A. thaliana, from the results of the transcriptome analysis and the microphotography observations of the interaction of exDNAs with root apex, we modelled the response of plants to extracellular nucleic acids for which we propose the new acronym of EDAP stating for extracellular DNA associated pathways (Chiusano et al., 2021). According to our results and together with previous observations reported by other groups, upon self-exDNA sensing an early intracellular Ca\(^{2+}\) spike signal and cell membrane depolarization rapidly propagates throughout the plant reaching photosynthetic organs, as reported by Barbero et al. (Barbero et al., 2016), inducing the activation of the chloroplast genome. The hyperactivation of the chloroplast leads to an overproduction of ROS which, in absence of activation of their scavenging, presumably due to the inhibition of the chloroplast-nuclear cross-talk, induces a positive feedback on ROS production which is associated with the MAPK activation and JA production. We suggested that the inhibition of the intracellular dynamics and crosstalk can be a typical early cell response after the sensing of self-exDNA. Moreover, if the stressor agent, i.e. the self-exDNA, is not timely removed, the whole organism would be finally affected by DNA damage, determining cell cycle arrest and growth inhibition, that at the macroscopic level are also evident in our observation of root apex necrosis after 10 days from the exposure to self-exDNA. In the case of nonself-exDNA we suggested that following its uptake, it is metabolized activating a cascade of events involving both biotic and abiotic stress related genes, promoting the establishment of a hypersensitive response, putatively triggering a systemic acquired resistance.

Despite further analyses are necessary to evaluate if the molecular response in other organisms can be ascribed to similar processes, we can speculate on the basis of our results that similarly to plants, part of the response is activated also in other organisms, like the growth inhibition. For instance, in A. thaliana, the exposure to self-exDNA induced the overproduction of ROS as a consequence of the chloroplast activation. This could affect the whole plant metabolic processes, eventually causing also DNA damage and cell cycle arrest. Interestingly, the findings in the freshwater microalgae C. reinhardtii showing palmeloids cells (Khona et al., 2016) (i.e. stress induced cells failing in completing cellular division), after the treatment with self-exDNA at 30 ng/µL, is in agreement with the proposed cell cycle block upon DNA damage in A. thaliana.
Moreover, in *C. reinhardtii*, it has been demonstrated that cell cycle progression is influenced by light intensity and in particular, it is arrested by high light intensity exposure (Voigt & Münzner, 1987). Such condition comes with an increased production of ATP (Michaels & Herrin, 1990), the activation of the chloroplast Photosystem II and the photosynthetic electron transport chain (Voigt & Münzner, 1987) and consequently it leads to an increased generation of ROS (Aksmann et al., 2016), accompanied by a reduction of their scavenging (mainly relying on the ascorbate peroxidase (Kuo et al., 2020)). The overproduction of ROS has been demonstrated to affect the *C. reinhardtii* cell cycle (Pokora et al., 2018). However, further molecular and analyses and biochemical assays are required to clarify the role of the chloroplast, the occurrence of ROS production, in the differential response to self- and nonself-exDNA.

In *C. robusta* the treatment with self-exDNA at higher concentrations (200 and 250 ng/μL) affects the development, particularly delaying the neural plate formation and neurula stage. Embryos are ultimately arrested in an undefined stage showing cells that are typical of the adult larvae. Similarly to what has been demonstrated in plants (Barbero et al., 2016), where one of the main processes occurring in the early sensing of self-exDNAs is the release of intracellular Ca²⁺, embryos of both vertebrate and invertebrate in order to proceed properly during the developmental stages from fertilization to organ formation, require transient Ca²⁺ waves propagation, particularly occurring during the neural plate formation (Chen et al., 2017; Deguchi et al., 2015; Webb and Miller, 2003). Interestingly, the transient-type Ca²⁺ channels for neural tube closure (Abdul-Wajid et al., 2015) and also in *C. robusta*, seem to be conserved over the evolutionary scale in invertebrate and vertebrate. Ca²⁺ propagation promotes the differentiation of anterior neural plate (Akahoshi et al., 2017; Hackley et al., 2013). If the self-exDNA perception could interfere with the physiological Ca²⁺ waves and, consequently, delays the process of neural plate formation leading to the embryo development arrest, remains to be elucidated. Future histological, morphological and molecular analysis will be necessary to deepen these preliminary observation and thus clarifying processes and pathways leading to arrested or impaired development.

In human cells PNT2, an interesting difference between type of exDNA response at 0.3 ng/μL is the reduction of mitotic events (Table 4.1 and Figure 4.6) in self-exDNA, that can be caused by a delayed cell cycle. Interestingly, it seems that the cytokinesis phase is affected in self treatments (Figure 4.7). Cell cycle arrest has been extensively discussed for human cells and can be caused by many factors (Calcinotto et al., 2019) among which the DNA damage, as also suggested in *A. thaliana*, is considered one of the main causes (Pack et al., 2019). In addition to the reduced mitosis, the increased cell death is more evident in self- than in nonself-exDNA treatment (Table 4.1 and Figure 4.5). The reduction of mitotic events together with an increased cell death suggest a mitotic catastrophe (Castedo et al., 2004). The fact that also nonself-exDNA induces similar responses even if to a lower extant, could also foster the hypothesis that nonself-exDNA, acting
similarly to plants as a PAMP, induces PNT2 cell death (that can be of different nature than self-exDNA) or an inflammatory response and consequently the release of also self-DNA. Following, the response to self- and nonself-exDNA may overlap and the effects as time proceed becomes indistinguishable. Again, further analyses will be required to better address such mechanisms and the differences of cellular response to exDNAs.

The results of this PhD thesis confirm that organisms of different taxonomic groups (both unicellular and multicellular ones) are able to perceive and discriminate both self- and nonself-exDNA in a concentration dependent manner, supporting the hypothesis of a general biological phenomenon (Mazzoleni et al., 2015b), i.e. the general occurrence of an inhibitory effect of self-exDNA. In particular, we showed here, for the first time, that both terrestrial and aquatic organisms, either living in freshwater or in marine context, are influenced by the treatment with self-exDNA and that upon the exposure to a certain concentration of self-exDNA, cell growth, morphology and putatively physiology resulted affected.

Overall, the presented results confirm the existence of a general biological phenomenon where the presence of self-exDNA negatively affects the growth and the development of cells and organisms. The self-exDNA inhibitory effects also bears significant implications at ecosystem level. For instance, soil biodiversity may be related to either removal or accumulation of DNA, also contributing to the phenomena described as ‘soil sickness’ and ‘replant diseases’ that in agriculture are examples of species-specific inhibition unrelated to either nutrient availability or soil-borne pathogens (Carteni et al., 2016). Moreover, the inhibitory effect of self-exDNA suggests additional functional roles of exDNA at community level in regulating, for example, the interactions between competing/coexisting species, thus contributing to the establishment of species interactions.
Chapter 7: Future perspectives and applications

The finding of a specific inhibitory effect of self-exDNA sets the basis for the development of applicative strategies for the growth control of many organisms thus deserving also practical applications. In 2014 Mazzoleni and his colleagues proposed for the first time an interesting pharmacological and biotechnological application of fragmented self-exDNA in the context of biological control of the same species through the production of selective compounds with pharmacological activity also improving the performance of antibiotic applications (Mazzoleni et al., 2014).

Our results also supported the great potential in the use of self-exDNA for industrial and medical application. For instance, after being characterized, invading contaminant microorganisms that can reduce microalgae growth in industrial bioreactors, can be controlled using compounds based on sequence similarities without affecting microalgae growth. Moreover, the sensitivity of exDNA to the DNase, paves the way for the setting up of protocols for the algal growth promotion in industrial bioreactors based on DNase applications thus avoiding the accumulation of the exDNA that is involved in the decrease of growth rate. The scaling up phase required for making the proposed strategies suitable for industrial purposes would take advantages from the powerful tool represented by microbial libraries where different fragments of the target species DNA are incorporated in the microbial genome allowing specific amplification under highly quality conditions. On the other hand, it could be of ecological interest the biocontrol in aquatic ecosystem of microalgae overgrowth, i.e. to control the harmful phenomenon of algal blooms. Similar strategies can be applied for the control of the biofouling problem due to Ciona sp. whose growth on manmade structures (such us nets, floats and aquaculture) negatively impact on the economy of shellfish industries.
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**Supplementary Figure 2.1.** Correlation analysis (A) of gene expression (in RPKM) in all replicates Control (C01, C08, C16), Self (S01, S08, S16), and Nonself (N01, N08, N16) indicate each stage per treatment. The associate cluster dendogram (B) and the principal component analysis (PCA) (C) are also shown. Figure from (Chiusano et al., 2021)

**Supplementary Figure 2.2.** Correlation analysis between log2FC RNA-seq and log2 ΔΔCt of QRT-PCR. The correlation was done using Person correlation, the R2 is also reported. Figure from (Chiusano et al., 2021)
**Supplementary Figure 3.1.** Microscopy analysis of *C. reinhardtii* (a) and *N. gaditana* (b) samples in controls and after treatment with nonself- and self-exDNA at 3, 10 and 30 ng/µL. In red, the chlorophyll autofluorescence (Ex/Em: 405/300-800). Microscope: Zeiss LSM 700, Excitation at 405, detection in the range 300-800, Objective: 63x 1.40 Oil Dic.
Supplementary Figure 4.1. PNT2 cells observed by microscope analyses. Green square highlights an example of cell cluster while dashed green squares identify many examples of isolated cells. Inverted epifluorescence microscope: Carl Zeiss Axio Observer z1 with 20x air objective.
**Supplementary figure 4.2.** Isolated (blue) and clustered (yellow) cells MSD ($\tau=1$) after the treatment with nonself- and self-exDNA compared with the control.
Supplementary Figure 4.3. Clustered and isolated cells distribution in bidimensional space (a) and velocity (μm*s⁻¹) vs time (h) (b). Different coloured lines and dots are representative of each cell.
**Supplementary Figure 5.1.** The effect of nonself-exDNA (a) and self-exDNA (b) at 200 and 250 ng/µL on C. robusta embryos development. Apotome.2 (ZEISS), scale bar= 100 µm.
List of publications with contributions


Contribution: Microscopy observation, bioinformatics analysed, contribution in writing paper and during the whole revision process


Contribution: results analysis, paper organization, writing and revision


Contribution: Revision process


Contribution: Paper organization, writing and revision


Contribution: Data acquisition


Contribution: Paper organization, writing and revision


It is ready to be submitted a review paper to summarize the “state of art” on the presence and putative roles of exDNA in terrestrial and aquatic environment.

Contribution: Revision process

Papers in preparation

-Emanuela Palomba et al., The effect of extracellular self and nonself-DNA on the freshwater microalgae Chlamydomonas reinhardtii and on the marine microalgae Nannochloropsis gaditana

-Emanuela Palomba et al., Multifunctional roles of the extracellular DNA
Expected submission period for both: November

Conference attendance and presentations


In November 2018, I was selected to hold a short presentation of an abstract submitted for the congress “Exoflometry: the first international workshop on exotic cytometry”. I also received the prize “Menzione GIC” for the best abstract.

List of non-book component

**Supplementary Table 2.1.** Summary of the RNA-seq pre-processing results showing, for each replicate, the total number of raw reads and the percentage of reads discarded after the cleaning steps and uniquely mapped on the *Arabidopsis thaliana* genome.

**Supplementary Table 2.2:** List of Genes that were significantly differentially expressed (DEGs) in at least one treatment/stage selected for validation by RT-qPCR.

**Supplementary Table 2.3.** Number of total genes and number of DEGs with assigned GO per treatment.

**Supplementary Table 2.4.** List of statistically significant differentially expressed genes (DEGs) in self and nonself-DNA treatments and average RPKM values. Log2FC are shown for all DEGs. DEGs with a Log2FC ≥|1| are indicated in color cells: in red if upregulated and in blue if downregulated.

**Supplementary Table 2.5.** Enriched Gene Ontologies (GOs) in self and nonself-DNA treatments organized in classes and groups. In red (UP) or in blue (DOWN) the GOs enriched by up or downregulate filtered DEGs (expression pattern) are shown.

**Supplementary Table 2.6:** Number of filtered DEGs per class of GOs in self and nonself-DNA treatments at 1, 8 and 16 hours post treatment (hpt).

**Supplementary Table 2.7:** List of DAMPs and PAMPs in *A. thaliana*. 
