ABANT IZZET BAYSAL UNIVERSITY

THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

IDENTIFICATION OF ALTERNATIVE OXIDASE ENCODING GENES IN *CAULERPA CYLINDRACEA*

MASTER OF SCIENCE

ÖMER CAN ÜNÜVAR

BOLU, DECEMBER 2016

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APPROVAL OF THE THESIS

IDENTIFICATION OF ALTERNATIVE OXIDASE ENCODING GENES IN *CAULERPA CYLINDRACEA* submitted by **ÖMER CAN ÜNÜVAR** in partial fulfillment of the requirements for the degree of **Master of Science** in **Department of Chemistry, Abant İzzet Baysal University** by,

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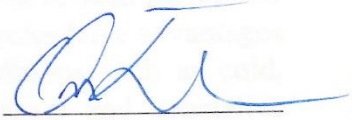
To the seas of the Earth

DECLARATION

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Ömer Can ÜNÜVAR



ABSTRACT

IDENTIFICATION OF ALTERNATIVE OXIDASE ENCODING GENES IN *CAULERPA CYLINDRACEA*
MSC THESIS
ÖMER CAN ÜNÜVAR
ABANT IZZET BAYSAL UNIVERSITY GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF CHEMISTRY
(SUPERVISOR: DR. ERCAN SELÇUK ÜNLÜ)

BOLU, DECEMBER 2016

Alternative oxidases (AOX) are involved in adaptation of organisms to different environmental conditions. Function of mitochondrial AOX proteins have been described for electron flow through electron transport chain and regulation of mitochondrial retrograde signaling pathway. The roles of AOX proteins have been found in sexual reproduction, vegetative reproduction, and resistance against oxidative stress, cold stress, starvation and biotic attacks. Alternative oxidases have been identified in plants, fungi and algae. *Caulerpa cylindracea*, also known as terrorist seaweed, is an invasive marine green algae. Although the natural habitats of the species are Australia coasts, the impact of the invasion have been monitored through Mediterranean and Aegean shores. *C. cylindracea* species have advantages against others by showing higher resistance to stress conditions such as cold, starvation, pathogen attacks and by their capability of sexual and vegetative reproduction. In addition, *C. cylindracea* have degenerative effects on the growth of other species by reducing peripheral oxygen level, changing the chemical structure of the benthos and producing secondary metabolites. Comparing the advantages of *C. cylindracea* over the niche and defined functional roles of mitochondrial alternative oxidase proteins, it is evident that AOX proteins are likely involved developing those advantageous skills in *C. cylindracea*. However, previous research have focused on characterization of the species using phenotypic approaches. There is limited data about biochemical and molecular mechanisms that take part in stress resistance and invasion characteristics. Several characteristics that help *C. cylindracea* to have invasive power individually demonstrated to be under control of AOX proteins in other plant species at molecular level. Thus our general hypothesis is that invasive characteristics of *C. cylindracea* is directly related with AOX proteins. The working hypothesis is that the species express at least one *AOX* gene. To test this hypothesis, the aim of this study is to identify mitochondrial AOX protein encoding gene(s) in *C. cylindracea.* We collected samples from Seferihisar/İzmir and carried out transcriptome analysis. 27737 transcripts were described from assembled RNA-Seq data. Detailed bioinformatic analysis revealed two different genes encoding mitochondrial alternative oxidase proteins. When the Blast analysis performed via DNA sequence, there was no matching. Therefore, characterization studies on the bioinformatics level were

continued based on protein sequences. The domain locations and sequence lengths of the matching sequences show high similarities (Matching percentages of 5 species is greater than 50%) with AOX proteins of the different species after the analysis. The sequence verifications of the identified *AOX* genes were made successfully and they were subcloned into yeast vector for future expression studies. The achieved transcriptome data and identified *AOX* sequences will guide to understand the invasive properties of the species.

KEYWORDS: *Caulerpa cylindracea,* Alternative Oxidases, Algae, Transcriptome Analysis

ÖZET

CAULERPA CYLINDRACEA TÜRÜNDE ALTERNATİF OKSİDAZ KODLAYAN GENLERİN TANIMLANMASI
YÜKSEK LISANS TEZI
ÖMER CAN ÜNÜVAR
ABANT İZZET BAYSAL ÜNİVERSİTESİ FEN BİLİMLERİ ENSTİTÜSÜ
KIMYA ANABILIM DALI
(TEZ DANIŞMANI: DR. ERCAN SELÇUK ÜNLÜ)

BOLU, ARALIK - 2016

Alternatif oksidazlar (AOX) türlerin farklı ortam koşullarına adaptasyon sürecinde yer alırlar. Mitokondrial AOX proteinlerinin elektron transport zinciri boyunca elektron akışında ve mitokondrial retrograde sinyal iletim yolağının düzenlenmesinde işlevleri olduğu açıklanmıştır. Alternatif oksidazların eşeyli ve vejatatif üreme fonksiyonlarında, oksidatif strese, soğuk strese, besin yetersizliğine ve biotik ataklara karşı dirençlerinde rol aldıkları karakterize edilmiştir. Alternatif oksidazlar bitki, mantar ve alglerde tanımlanmışlardır. *Caulerpa cylindracea* terörist yosun olarak da bilinen istilacı bir deniz yosun türüdür. Türün doğal alanları Güneydoğu Avustralya kıyıları olmasına rağmen türün istila etkileri Akdeniz ve Ege Denizi kıyılarında gözlemlenmiştir. Türün diğer türlere göre avantajı, soğuğa, besin yetersizliğine, patojen ataklarına karşı direnç göstermesi ve hem vejetatif hem de eşeyli yollarla üreyebilmesindedir. Ayrıca bu tür istila ettiği bölgelerde oksijen seviyesini düşürüp toprak yapısını değiştirerek diğer bitki türlerin yaşama olanağını yok etmekte ayrıca salgıladığı ikincil metabolitler vasıtasıyla çevresindeki canlılığın da olumsuz etkilenmesine sebep olmaktadır. Bu özellikler, alternatif oksidaz enzimlerinin aktif şekilde kullandığını işaret etmekte olup *C. cylindracea* üzerine alternatif oksidazları kapsayacak şekilde yapılmış herhangi bir çalışma bulunmamaktadır. Tür üzerine yapılan genetik çalışmalar çoğunlukla türün filogenetik konumunun belirlenmesine yönelik olup türün stres direnci ve yayılım karakteristikleri hususunda biyokimyasal veriler yetersizdir. *C. cylindracea*'ya istilacı güç sağlayan özelliklerin birçoğunun moleküler düzeyde AOX proteinlerinin kontrolü altında olduğu farklı bitki türlerinde ayrı ayrı gösterilmiştir. Buradan hareketle genel hipotezimiz *C. cylindracea* türünün istilacı özelliklerinin doğrudan AOX proteinleri ile ilişkili olduğudur. Çalışma hipotezimiz ise türün en az bir *AOX* genini kodladığı yönündedir. Bu hipotezi test etmek için bu çalışmanın amacı *C. cylindracea* türünde mitokondriyal AOX proteini kodlayan gen veya genlerin tanımlanmasıdır. Örnekler Seferihisar/İzmir'den toplanmıştır ve örneklerin transkriptom analizi yapılmıştır. 27737 transkript RNA-Seq verileri kullanılarak tanımlanmıştır. Detaylı biyoenformatik çalışmaları iki farklı mitokondriyal alternatif oksidaz proteini kodlayan gen varlığını açıklamıştır. Blast analizini DNA sekansı üzerinden yapıldığında herhangi bir eşleşme

gözlenmemektedir. Bu sebeple biyoinformatik düzeyindeki karakterizasyon çalışmalarında protein dizileri temel alınarak analizlere devam edilmiştir. Analiz sonrası eşleşen sekanslara bakıldığında gerek domain bölgeleri gerekse sekans uzunlukları olarak farklı türlere ait alternatif oksidaz proteinlerine yüksek oranda (eşleşme yüzdesi 5 tür için %50'den büyük) benzerlik gösterdikleri görülmektedir. Tanımlanan *AOX* genlerinin dizi doğrulamaları başarıyla yapılmış ve gelecek gen ekspresyon çalışmalarında kullanılmak üzere maya vektörü içerisine aktarılmıştır. Çalışma kapsamında elde edilen transkriptom verileri ve *AOX* dizilerinin bilinmesi türün istilacı özelliklerinin anlaşılmasında gelecek çalışmalara yardımcı olacaktır.

ANAHTAR KELİMELER: *Caulerpa cylindracea*, Alternatif Oksidazlar, Transkriptom Analizi

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A : Adenine

aa : Amino acid

ADP : Adenosine diphosphate

AOX : Alternative oxidase protein

AOX : Alternative oxidase gene

ATP : Adenosine triphosphate

BSA : Bis(trimethylsilyl)acetamide

C : Cytosine

cDNA : Complementary deoxyribonucleic acid

CMAS : Confederation Mondiale Des Activites Subaquatiques

CN : Cyanide

COX : Cytochrome c oxidase

DNA : Deoxyribonucleic acid

E : East

E/DNV : The glutamic acid/aspartic acid, asparagine, valine motif

EST : Expressed sequence tag

EtBr : Ethidium bromide

$FADH_2$: 1, 5-dihydroflavin adenine dinucleotide

G : Guanine

g : Gram

g : The Unit of Relative Centrifugal Force, gravity

gDNA **:** Genomic deoxyribonucleic acid

L **:** Litre

LB **:** Lysogeny broth

MLS **:** Mitochondrial localization signal

MLS **:** Mitochondrial Localization Signal

mRNA **:** Messenger Ribonucleic acid

MRS **:** Mitochondrial Retrograde Signalling

N **:** North

n **:** Nano

NADH **:** Nicotinamide adenine dinucleotide

NADPH **:** Dihydronicotinamide-adenine dinucleotide phosphate

NCBI **:** National Center for Biotechnology Information

nt **:** Nucleotide

NGS : Next generation sequencing

^{o}C **:** Centigrade degree

ORF **:** Open reading frame

OXPHOS **:** Oxidative phosphorylation

pAG413 : Yeast expression vector with GAL-promoter element with ccdB killer gene

PCR **:** Polymerase chain reaction

QDC **:** The glutamine, aspartic acid, cysteine motif

qPCR **:** Quantitative polymerase chain reaction

RNA **:** Ribonucleic Acid

RNase **:** Ribonuclease

ROS **:** Reactive Oxygen Species

SA-PMP **:** Streptavidin-Paramagnetic Particles

Seq **:** Sequence

sETC **:** Standard Electron Transport Chain

SNP **:** Single nucleotide polymorphism

SSC **:** Saline-Sodium citrate

T **:** Thymine

UV **:** Ultraviolet

µ **:** Micro

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1. INTRODUCTION

1.1 Algae

Algae are a wide and multifarious group of eukaryotic photosynthetic organisms living in almost every environment. They show a large morphological diversity from unicells to larger forms over 50 meters long. The first algal forms appeared nearly 1 -1.5 billion years ago after the symbiogenesis of a heterotrophic eukaryotic organism with a photosynthetic cyanobacterium (Douzery et al., 2004). This action resulted with the formation of primary plastids that are still found in the Glaucophyta, red algae and green families including embryophtes (land plants) (Reyes-Prieto et al., 2007). These families are generally called Plantae or Archaeplastida (Adl et al., 2005; Cavalier-Smith, 1981). After the secondary endosymbiosis of green and red algae, the photosynthetic protists arose. The chlorarachniophytes and euglenoids achieved the plastids from a green algae in two different secondary endosymbiotic ways, although molecular data proposes that the red algal plastid found in cryptomonads, heterokonts, haptophytes, apicomplexans and dinoflagellates was achieved with an individual secondary endosymbiosis in their accepted ancestor (Archibald, 2005, 2008). The diversity of the photosynthetic eukaryotes are explained by this process which includes cell captures and consequent enslavement. Photosynthesis appears in Archaeplastida (Glaucophyta, red algae, green plants), Chromalveolata (cryptophytes, Stramenopila or heterokonts including diatoms and brown algae, haptophytes and dinoflagallates), Rhizaria (Chlorarachniophyta) and Excavata (euglenoids) (see Figure 1.1).

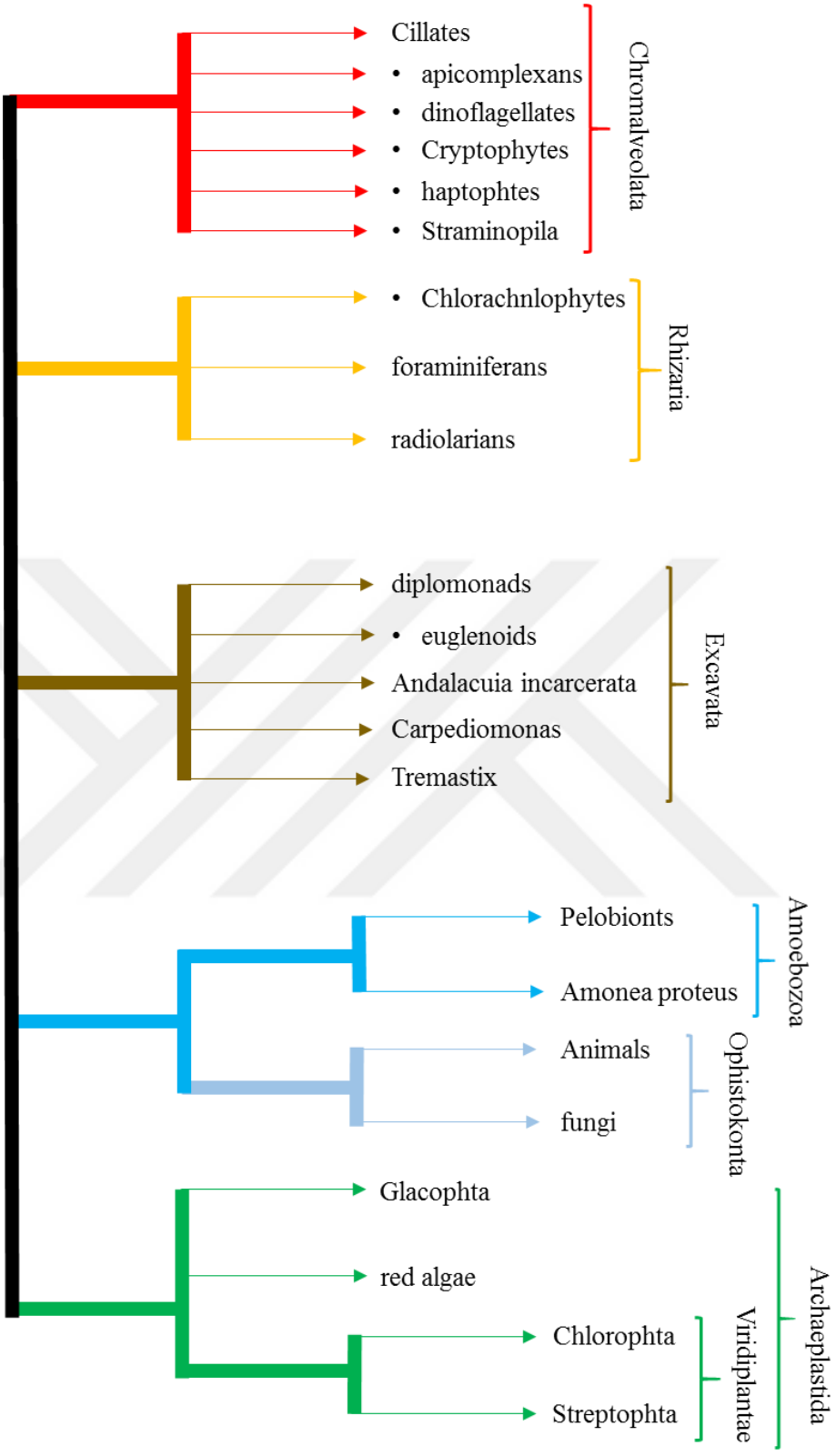

Figure 1.1. Eukaryotic tree of life (modified from Baldauf, 2008; Lane and Archibald, 2008).

The first algae arose after the symbiogenesis of a heterotrophic eukaryotic organism with a photosynthetic cyanobacterium, giving rise to the Archaeplastida. The other photosynthetic protists arose through secondary endosymbiosis of either a green or a red alga and occur in four of six supergroups (marked with respectively green and red circles). The monophyly of the Archaeplastida is well-supported and most recent evidence favours the Glaucophyta as earliest diverging lineage within the Archaeplastida as in the Figure 1.1.

1.2 *Caulerpa* Species

Caulerpa species are multinucleated (coenocyte), modular and colonial algae forms that are common throughout both shallow and deep waters in tropical and subtropical seas. *Caulerpa* species have branched axes and attaching rhizoids and they are capable of vegetative reproduction. 75 species belong the genus *Caulerpa* (Famà et al., 2002). There are 6 described species for Mediterranean Sea. *C. cylindracea* and *C. taxifolia* are known as invasive and have created great environmental issue in Mediterranean countries during recent years (Boudouresque et al., 1995; Verlaque et al., 2000, 2003).

The eastern and western regions of the Mediterranean Sea show different thermal regimes. Temperatures of the eastern part of the Mediterranean Sea ranged from 17^{o}C to 30^{o}C in winter and summer. It is higher than those in western part by about 15^{o}C for these two seasons (Kress and Herut, 2001). Also the eastern part of the Mediterranean Sea has different geographic properties (wide, shallow sandy intertidal platforms made of limestone and biogenic rocks) (Einav and Israel, 2007) and higher salinities than western parts (3.9% - 3.6%) (Berman et al., 1984). The eastern Mediterranean Sea affected to biological invasions which cause from Suez Canal. There are 100 known macrophytes found into the Mediterranean Sea have come from the Indo-Pacific Sea (Boudouresque and Verlaque, 2002, 2005; Galil et al., 1990; Ribera, 2002; Rilov and Galil, 2009; Zenetos et al., 2005). However not all species cross-contaminated from different regions show invasive characteristics in Mediterranean Sea. Because of the invasive properties of *Caulerpa taxifolia* and

Caulerpa cylindracea studies have focused on this two species (Argyrou et al., 1999; Meinesz, 2001; Meinesz and Hesse, 1991; Verlaque et al., 2000, 2003).

1.3 *Caulerpa cylindracea*

Caulerpa cylindracea belongs to Chloropyhta phylum of the order Bryopsidales from the family Calerpaceae and the genus *Caulerpa* have nearly 85 species (Klein and Verlaque, 2008). The species previously was named as *Caulerpa racemosa* var. *cylindracea* however the name has changed to *Caulerpa cylindracea* (Belton et al., 2014). This macroalgae species have a uniaxial siphonous thallus which is mostly divided into a creeping axis (stolon) with rhizoids and erect shoots that are named as ranchis. There are leaf-like or grape-like branchlet attached to the ranchis (Klein and Verlaque, 2008). Figure 1.2 represents the parts of the species. Some studies shows that there are some variations of stolon and erect axis length, cover, growth rate and biomass in the species, and maximum biomass front height, stolon length were observed in September and November (Piazzi et al., 2001; Piazzi and Cinelli, 1999).

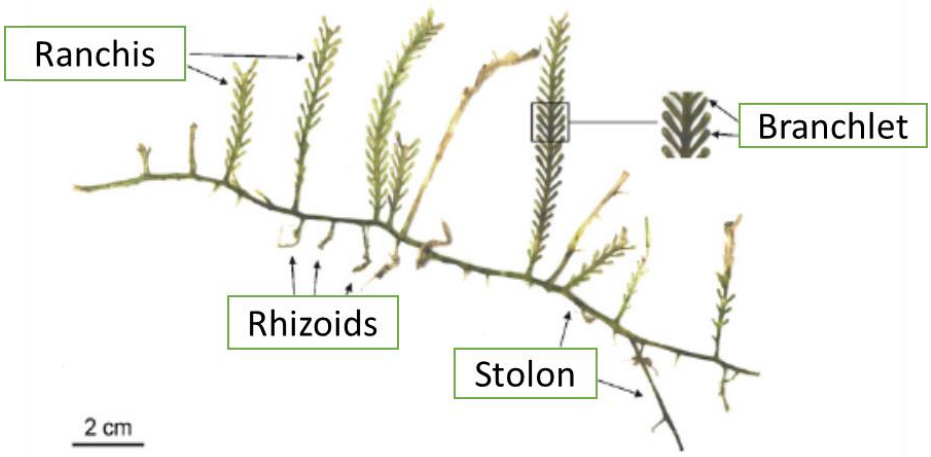

Figure 1.2. The parts of thallus of the invasive *Caulerpa cylindracea* from the Gulf of Marseille (- 30 m). Photograph: Klein J. (Klein and Verlaque, 2008)

The species typically grow up 11 centimeters but exceptionally 19 centimeters in nature. The stolon is attached to the substrate with thin and short rhizoids (Galil, 2006, Klein and Verlaque, 2008). Although it is endemic to South-West Australia,

Caulerpa cylindracea species was recorded in Mediterranean Sea, Libya in 1990 (Nizamuddin, 1991; Verlaque et al., 2003). Genus *Caulerpa* are able to spread over all substrates. They compete with native species and over grove (Piazzi et al., 2005; Ceccherelli and Piazzi, 2001). The reporting data of *Caulerpa cylindracea* in the different regions of the Mediterranean Sea is in given below in Table 1.1 as region, depth and season.

Table 1.1. The reporting data of *Caulerpa cylindracea* in the different regions of the Mediterranean Sea (Modified from Klein and Verlaque, 2008).

Authors	Region	Country	Depth	Year
Nizamuddin (1991)	Tajura, Tripoli	Libya	-	1990
Panayotidis and Montesanto (1994)	Zakynthos, Pylos Bay	Greece	25-35 m	1993
Piazzi et al. (1994)	Leghorn	Italy	4 m	1993-1994
Giaccone and Di Martino (1995b)	Eastern Sicily	Italy	5-10 m	1994
Bussotti et al. (1996)	Genova	Italy	9 m	1995
Buia et al. (1998)	Gulf of Taranto	Italy	6, 9 m	1996
Buia et al. (1998)	Gulf of Taranto	Italy	6, 9 m	1997
Buia et al. (1998)	Gulf of Taranto	Italy	6, 9 m	1997
Gambi and Terlizzi (1998)	Gulf of Salerno	Italy	6, 9 m	1997

Table 1.1. The reporting data of *Caulerpa cylindracea* in the different regions of the Mediterranean Sea (Modified from Klein and Verlaque, 2008) (cont'd)

Gambi and Terlizzi (1998)	Gulf of Salerno	Italy	13-20 m	1997
Panayotidis and Montesanto (1998)	Nissoros Island, Cape Sounion	Greece	Shallow	1995-1996
Piazzi and Cinelli (1999)	Leghorn	Italy	0-3 m	1996
Piazzi and Cinelli (1999)	Leghorn	Italy	0-3 m	1996-1997
Modena et al. (2000)	Varazze, Genova	Italy	5-7 m	1998
Modena et al. (2000)	Varazze, Genova	Italy	5-7 m	1998
Modena et al. (2000)	Varazze, Genova	Italy	5-7 m	1999
Modena et al. (2000)	Sturla, Genova	Italy	5 m	1998
Modena et al. (2000)	Nervi, Genova	Italy	0.1-1 m	1999
Verlaque et al. (2000)	Marseille	France	14-23 m	1997
Verlaque et al. (2000)	Acitreza, Sicily	Italy	-	1998
Verlaque et al. (2000)	Saronikos	Greece	-	1998
Verlaque et al. (2000)	Castellorizo Island	Greece	35-40 m	-

Table 1.1. The reporting data of *Caulerpa cylindracea* in the different regions of the Mediterranean Sea (Modified from Klein and Verlaque, 2008) (cont'd)

Verlaque et al. (2000)	Kalimnos Island	Greece	-	1997
Verlaque et al. (2000)	Samos Island	Greece	-	1998
Verlaque et al. (2000)	Gökova	Turkey	25-50 m	1997
Verlaque et al. (2000)	Famagusta Harbour	Cyprus	8 m	1998
Buia et al. (2001)	Gulf of Salerno	Italy	15 m	1998
Buia et al. (2001)	Gulf of Salerno	Italy	1 m	1998
Buia et al. (2001)	Gulf of Naples	Italy	5 m	1998
Buia et al. (2001)	Gulf of Taranto	Italy	6 m	1998
Cantasano (2001)	Calabria	Italy	1-2 m	1999
Matricardi and Piatti (2001)	Liguria	Italy	-	1995
Matricardi and Piatti (2001)	Liguria	Italy	-	1996
Matricardi and Piatti (2001)	Liguria	Italy	-	1996
Matricardi and Piatti (2001)	Liguria	Italy	-	1996

Table 1.1. The reporting data of *Caulerpa cylindracea* in the different regions of the Mediterranean Sea (Modified from Klein and Verlaque, 2008) (cont'd)

Piazzi et al. (2001)	Leghorn	Italy	2 m	1998
Langar et al. (2002)	Cap Bon	Tunisia	-	-
Pena Martin et al. (2003)	Santa Pola, Alicante	Spain	0-2 m	-
Xhulaj and Kashta (2007)	Vlora Bay	Albania	5 m	2005

The introduction way of the species to the Mediterranean Sea does not known clearly. There are possible ways to introduce as ship traffic (ballast water, ship hull fouling) and aquaria (Klein and Verlaque, 2008).

Caulerpa cylindracea produces caulerpenyne which is a secondary metabolite in order to protect itself from other species in habitats. Also, *C. cylindracea* have an antioxidant system which is used for transformation of the oxygen radicals into water and molecular oxygen (Cavas and Yurdakoc, 2005). Although *C. cylindracea* species has several defence systems (Cavas et al., 2006; Dumay et al., 2002; Jung et al., 2002), there are some species which feed on *C. cylindracea* (Azzurro et al., 2004; Felline et al., 2012; Ruitton et al., 2006).

C. cylindracea shows continuous dense meadows in the Mediterranean Sea, on the other hand specific meadows of the species were not detected in the south-western Australia where is the home range of the *C. cylindracea* (Carruthers et al., 1993; Verlaque et al., 2003). It can penetrate in sparse *Posidonia ocenica* meadows (Ceccherelli and Piazzi, 1999; Ceccherelli and Piazzi, 2001; Ceccherelli et al., 2000, Panayotidis and Montesanto, 1994; Piazzi and Cinelli, 1999; Piazzi et al., 1997a,b; Serio and Pizzuto, 1998; Tsirika and Haritonidis, 2005; Žuljević et al., 2004), although a healthy meadows block the penetration (Ceccherelli et al., 2000). Turf and encrusting species suggest to help *C. cylindracea* although erect algae avoid the spread of the invasive species (Ceccherelli and Campo, 2002). Because of the ecological impact and

the fast spread, *C. cylindracea* is the one of the worst 100 invasive species of the Mediterranean Sea (Streftaris and Zenetos, 2006). Introducing of the invasive seaweeds can affect habitat complexity and sediment biogeochemistry (Lorenti et al., 2011). The penetration of an invasive species may show either direct or indirect source of variation, leading to a diversity of effects (Neira et al., 2006); notably the impact on the fauna depends on some factors of the invaded habitat, also the sediment fraction affected by the invasion after the introduction of the invasive species in a habitat (McKinnon et al., 2009).

1.4 Alternative Oxidases (*AOXs)*

Mitochondria within the eukaryotic cell have many functions as their role in programmed cell death, cellular iron regulation, and calcium signaling. For instance, there is a relation between the cytosolic and mitochondrial calcium molecules in coordinating metabolic homeostasis in calcium signaling (Estaquier et al., 2012; Glancy and Balaban 2012; Lill et al., 2012). In addition, mitochondria are the known as the energy producing organelles since they are responsible for biosynthesis of adenosine triphosphate (ATP) via oxidative phosphorylation (OXPHOS). OXPHOS is the process in which cells produce ATP via the energy released by virtue of the oxidation of nutrients. The center of the process is the standard electron transport chain (sETC). The location of the sETC is the inter membrane of the mitochondria and it consists of five complexes. Four of these complexes (from complex I to IV) are oxidoreductases named as dihydronicotinamide-adenine dinucleotide phosphate (NADPH) dehydrogenase (complex I), the succinate dehydrogenase (complex II), cytochrome c reductase (complex III), cytochrome c oxidase (complex IV); and final complex is an ATP synthase (complex V). In addition to these complexes, there are two mobile components which are cytochrome c and lipid ubiquinone in sETC (Schertl and Braun, 2014).

Electrons are transferred from the coenzymes nicotinamide adenine dinucleotide (NADH) and 1, 5-dihydroflavin adenine dinucleotide ($FADH_2$) into molecular oxygen that is reduced to water during the OXPHOS processing. In depth, complex I, III and IV pair their electron transfer reactions by proton translocation

through the inner mitochondrial membrane. The translocation of protons result in electrochemical gradient that is utilized by the complex V in order to product ATP through phosphorylation of adenosine diphosphate (ADP) (Schertl and Braun, 2014). On the other hand, most of the eukaryotic mitochondria and prokaryotic respiration system have elements in ETC in which electrons can enter or leave like alternative oxidase (AOX) (McDonald and Vanlerberghe, 2004). AOX takes electrons from the ubiquinone pool and catalysis the reduction of oxygen to water, this process is similar to cytochrome c oxidase (COX) (Millenaar and Lambers, 2003). Movement of the electrons via this protein is not coupled to proton translocation and does not contribute to the production of electrochemical gradient. Oxidative potential energy is lost as heat because the electron movement through the AOX avoids the complex III and IV, that are proton pumping complexes in the sETC (Finnegan et al., 2004). AOX is also known as cyanide-resistant terminal oxidase because it shows resistance to the all COX inhibitors as cyanide, nitric oxide and azide.

Cyanide-resistant respiration was discovered at early 20th century as an interest in thermogenic plant during anthesis. The observations were showed that respiration in plants was not completely inhibited by cytochrome oxidase inhibitors, and these discoveries were gradually increased to a wide taxonomic range of organism until 1929. Then, the biochemical and molecular level of AOX dependent (Cyanide (CN)-resistant) respiration has been distinguished (Rychter, 2003; Meeuse, 1975). *AOX* genes have been identified in nearly all of the kingdoms with the exception of Archaebacteria. While AOX is common in nature, loss of the protein has been announced in certain lineages (McDonald and Vanlerberghe, 2006). For instance, AOX protein is common in the fungal groups of Ascomycota and Basidiomycota. In addition, the observations show that more basal groups of Zygomycota and Chytrids have AOX protein (McDonald and Vanlerberghe, 2006). But, there is no AOX protein in Saccharomyces cerevisiae and *Schizosaccharomyces pombe*. Also, *AOX* genes were detected in phyla composed of slow moving or sessile organism among metazoans (McDonald et al., 2009). *AOX* has been studied in a monophyletic group which is called as the Viridiplantae (the green lineage) including green algae and land plants.

AOX is a diiron carboxylase protein which located in the inner mitochondrial membrane as a homodimer facing to matrix. Each monomer is made of six long and

four short α-helices which are organized in an antiparallel manner so coordinates two irons within a structurally preserved four helix-bundle formation. The oxidized state of AOX is inactive. In this inactive AOX from, the two iron within every active site are connected by a hydroxo bridge and ligated by four greatly conserved glutamate residues. There is a key role of the highly conserved tyrosine residue in the catalytic cycle is presented to account for the four electron reduction of O_2 to H_2O. N-terminal of plant AOX is not fully conserved and there is limited information about the structural or functional properties of its N-terminal region in any organism.

Although it is known that N-terminal region is separate from structurally defined 4-helix bundle as specified by the current structure, this region of the each monomer associated with its neighbor, so it is known that functional regulation of the AOX may take place through phosphorylation of the N-terminal addition and this can play a key regulatory role in changing the conformation of four helix bundle (Moore et al., 2013). AOX sequences in plants contain the glutamic acid/aspartic acid, asparagine, valine (E/DNV) or the glutamine, aspartic acid, cysteine (QDC) motif which show to play a role in posttranslational regulation of activity, also E/DNV motif could be correlated to phenotype of thermogenic plant (Moore et al., 1995, 2008, 2013). *AOX*, which is a nuclear-encoded gene in eukaryotes, occurs as a multigene family composing of 3 to 5 members in numerous plants. Two gene subfamilies which show different expression patterns and have different purposes are responsible for encoding of these members in higher plants. First member is named as *AOX1* that is commonly known with its function of induction as stress stimuli in many tissues and exist in both monocot and eudicot plant species. *AOX2* does not exist from the genomes of all monocot species examined that on the other hand it is usually expressed in eudicot species (Clifton et al., 2006).

1.4.1 *AOX* subfamilies

The members of multigene families of *AOX* in plants are regulated differentially (Ito et al., 1997). The gene number of *AOX* in angiosperms is various from 1 to 6 and it consists of variable combinations of different *AOX* subfamilies and kind among the species (Costa et al., 2014). Confirmation for *AOX2* sequences

discovered only in eudicots and not found monocot species examined to date (Clifton et al., 2006). In addition, the sequences of *AOX2* were not found from some eudicot species, but researchers explained this issue as lack of information rather than the gene being absent (Considine et al., 2002). A ten years ago, the common concept about the function of the *AOX* subfamily was that *AOX1* is often induced by the stress stimuli, although *AOX2* constitutively or developmentally expressed (Considine et al., 2002). According to the recent studies *AOX2* gene could be associated with the stress stimuli as *AOX1* (Cavalcanti et al., 2013; Costa et al., 2010). Studies on AOX in angiosperms show that more divergent plants phylogenetically contain similar multigene families although there are more differences in their multigene family composition for closely related species (Costa et al., 2014). Hence, identification of the genes and explanation of their role within the context of parts of multigene families in different species are needed (Borecky et al., 2006). Because, there are specific physiological role of each *AOX* gene and their related protein. Also the evolutionary differences of *AOX* subfamilies in the plant species might have relations for physiological function. In spite of the large data set of sequence information about *AOX* in angiosperm, it is necessary to consider other gene features as intron/exon structure to make a suitable classification. Also, it is necessary to verify that biological activity of the *AOX* sequences which are recovered from bioinformatics technologies. This kind of studies may give information about the functions of general alternative respiratory pathway.

1.4.2 Non-thermogenic activities of AOX proteins

The alternative respiratory pathway plays a role as the source of heating in thermogenic plants because of the powerful relation between heat production and cyanide-resistant respiration in which the AOX act as a terminal electron acceptor (Meeuse and Raskin, 1988; Nagy et al., 1972). The role of AOX in heat production in vivo has been showed for some thermogenic plants (Watling et al., 2006). The main role of the AOX pathway has not explained completely, according to the several hypotheses; its physiological functions are balancing carbon metabolism and electron transport (Simons et al., 1999; Vanlerberghe and McIntosh, 1997), scavenging reactive oxygen species (ROS) in plants (Maxwell et al., 1999), taking a role in the

programmed cell death mechanism (Vanlerberghe et al., 2002) and controlling plant growth rate homeostasis (Hansen et al., 2002; Moore et al., 2002).

AOX gene expression and AOX protein activity changes by biotic and abiotic stresses (Van Aken et al., 2009; Vanlerberghe et al., 2009). ROS are chiefly produced at a low level in organelles like chloroplasts, mitochondria and peroxisomes under optimal growth conditions. It has been proposed that stress-induced AOX prevents the production of ROS (Miller et al., 2010; Pasqualini et al., 2007; Van Aken et al., 2009; Vanlerberghe et al., 2009). Also, the role of alternative oxidase in non-thermogenic plant growth and development was studied but not completely solved. Soybean which contains a *GmAOX2b* antisense gene has decreased vegetative growth and reproductive capacity. This situation shows that AOX is important for fundamental function of plants, as well as being a determinant fertility in the species (Chai et al 2012). But, present information of about these complex functions is incomplete, therefor more studies needs to be done to explain the function of terminal oxidase.

1.4.3 Mitochondrial Retrograde Signaling and AOX proteins

The center of the respiratory pathway is mitochondria, so it is important to identify the effect of abiotic and biotic stress that can cause mitochondrial disturbance in plants, and to see which cell acclimates to conserve mitochondrial function under such conditions. There is limited information about the specific cellular reasons of mitochondrial dysfunction in plants but an evidence show that the reactions of mitochondria to biotic and abiotic stresses contribute significantly to all plant stress responses (Rhoads and Subbaiah, 2007). Mitochondria have an important role in responses to stress by contributing transformed nuclear gene expression. The transformation of nuclear gene expression controlled by organelles via organelle to nucleus signaling is known as retrograde signaling and mitochondria to nucleus signaling (mitochondrial retrograde signaling (MRS)). Answer to mitochondrial disturbance of nuclear gene for *AOX* is the most searched mitochondria-to-nucleus signaling pathway. Many studies about this situation show that the mitochondrial dysfunction, often related with oxidative stress and this disturbance results in the induction of AOX at the both transcript and protein level. Consequently, AOX is

frequently a general sign of mitochondrial dysfunction and/or cellular oxidative stress. There are a lot of abiotic and biotic stresses to increase AOX amount (transcripts, protein or capacity) and it is supported by that when some stresses effect mitochondrial function, AOX might show an important adaptation response (Vanlerberghe, 2013).

1.5 RNA Sequencing

1.5.1 DNA and Gene Expression

Genetic information is stored in deoxyribonucleic acid (DNA) that consist of four nucleotides; Adenine (A), Cytosine (C), Guanine (G), and Thymine (T). DNA is organized as double helix with complementary nucleotides pair which are A-T and G-C as shown in Figure 1.3.

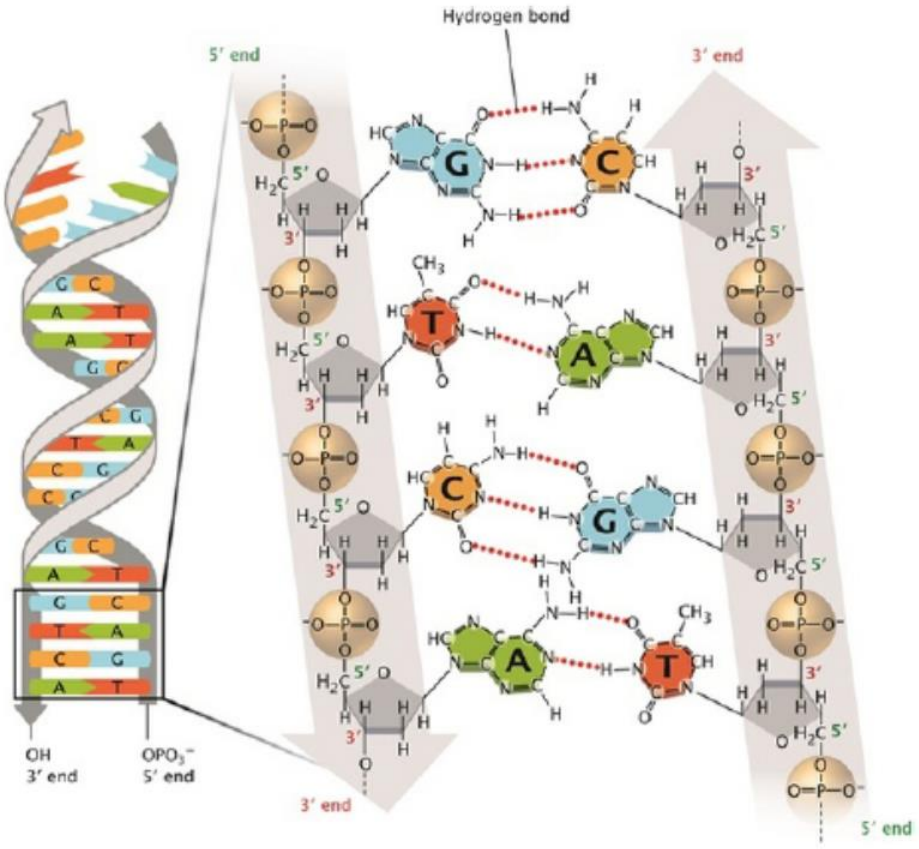

Figure 1.3. The structure of DNA (Pray, 2009)

The whole genome consists of a set of DNA molecules and these sets are called as chromosomes where genes are the important functional units. The central dogma of the molecular biology is described that genes are transcribed to messenger ribonucleic acid (mRNA) which is single stranded and Thymine is replaced by Uracil (U), and then RNA translated to the specific proteins. Exons and introns are the parts of the genes and the exons are the important parts of the mRNA which is the template of the protein, the introns take the regulatory roles mostly. Firstly the whole gene region is transcribed into precursor mRNA (pre-mRNA) which have exons and introns. The intron regions of the pre-mRNA are removed, this process is called splicing and started by proteins in the RNA. These proteins are called as splicing factors. The detection of the intron-exon boundaries is made with detection of short sequences which is called as splice sites. The process of addition of a poly-A tail (a series of Adenine) to an RNA is called as polyadenylation which is started by binding of proteins to a polyadenylation site in an untranslated region of an exon in the pre-mRNA. After these processes the final mRNA is obtained from pre-mRNA (see Figure 1.4). The poly-A tail has an important role in the transportation of the mRNA to the cytoplasm, and regulation of the half-life of the mRNA (Kornblihtt et al., 2004). Translation is the process in which the mRNA is translated into amino acids, performed by ribosomes.

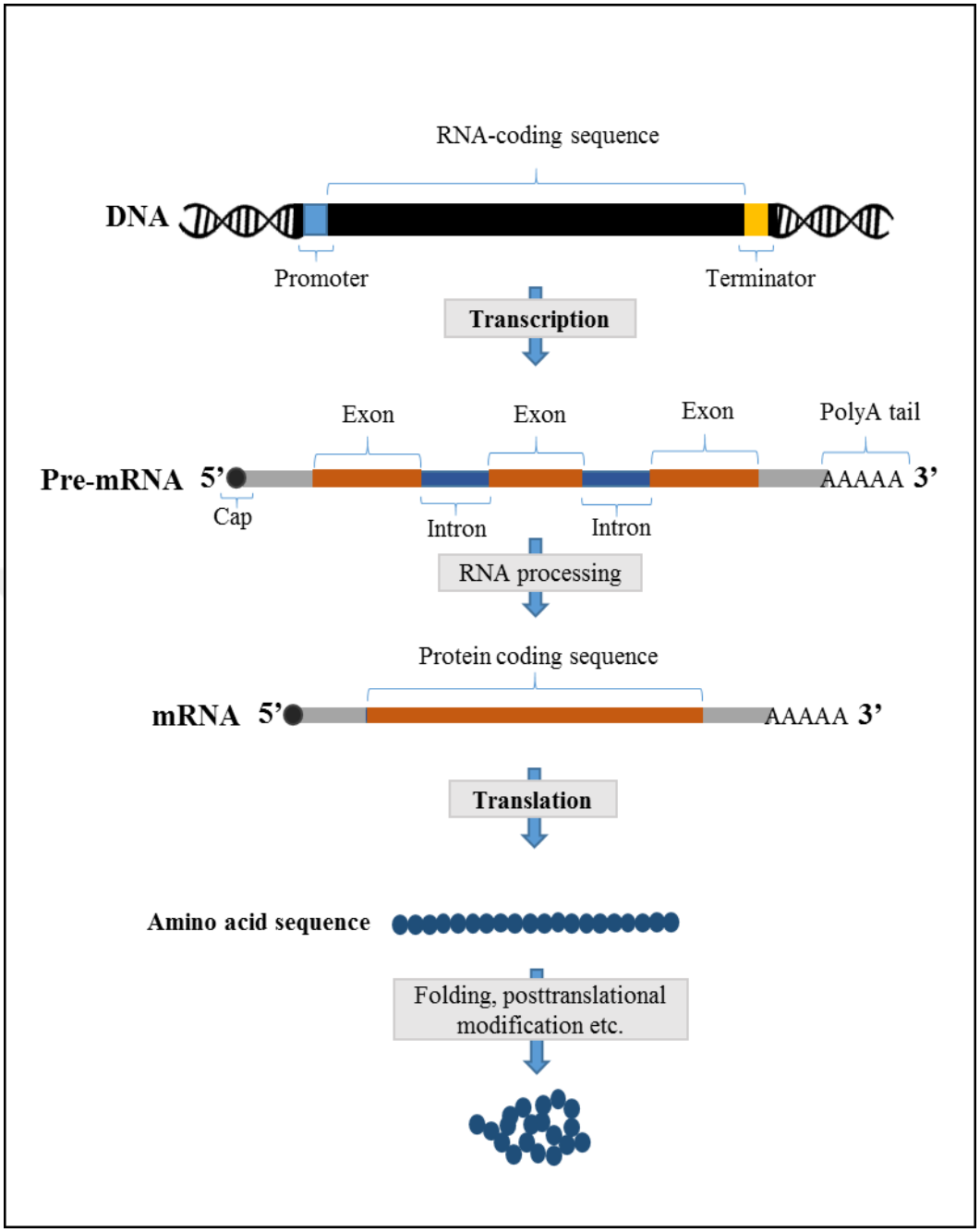

Figure 1.4. The central dogma in molecular biology (Modified from Griffith et al., 2015)

1.5.2 RNA-Seq technology

In the functional genomics studies gene expression is the major focusing area because the genetic information flows from the genomic DNA (gDNA) from the functional proteins (Cheung and Spielman, 2009). The basic protocol of the process

consists of extraction of polyadenylated RNA, conversion into double strand and complementary DNA (cDNA) (more stable) that are shared randomly, selection of the size of the fragments for optimization of the later parts, amplification of the fragments ligation of the adapters to the fragments and sequencing by using an next-generation sequencing (NGS) approach (Griffith et al., 2015). The summary of the basic RNA-Seq study is given in the Figure 1.5.

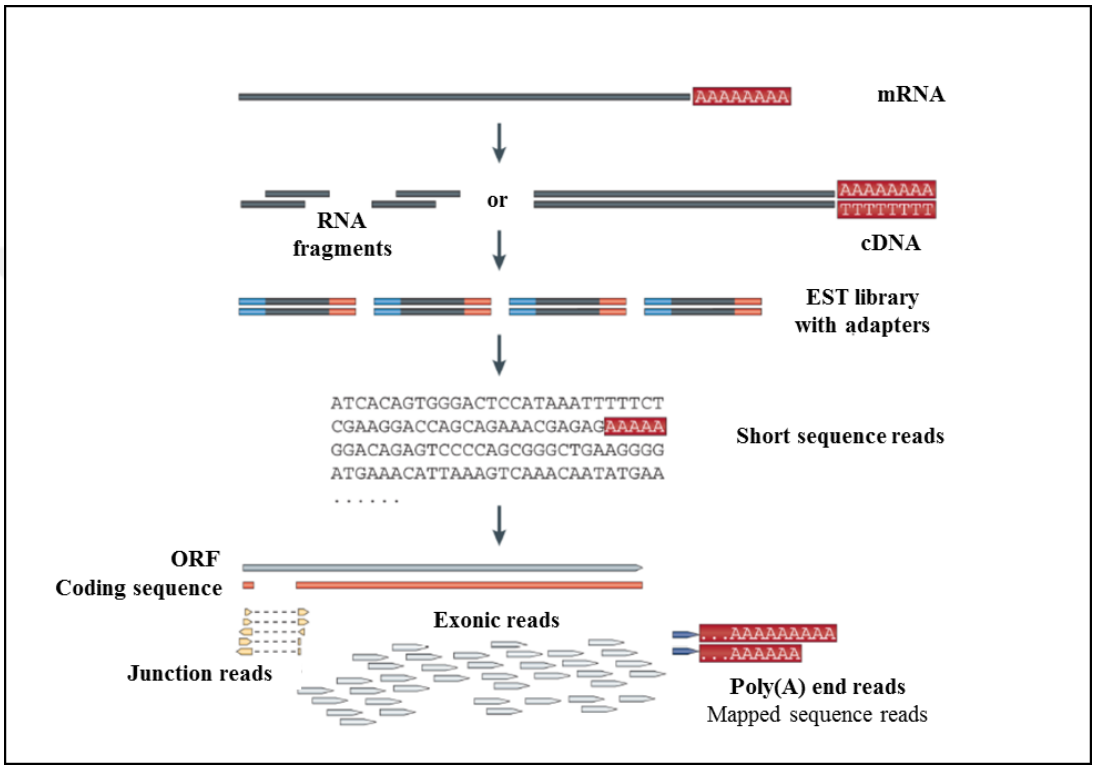

Figure 1.5. The summary of RNA-Seq experiment (Modified from Wang et al., 2009)

Results can be obtained from only one end of a fragment that is called as single-end sequencing or from both ends that is called as paired-end sequencing. Illumina IG (Cloonan et al., 2008; Lister et al., 2008; Morin et al., 2008; Mortazavi et al., 2008; Nagalakshmi et al., 2008; Wilhelm et al., 2008), Applied Biosystems SOLID (Cloonan et al., 2008) and Roche 454 Life Sicence (Barbazuk et al., 2007; Emrich et al., 2007; Vera et al., 2008) systems have already been used for RNA-Sequencing. They are high-throughput platforms.

RNA-Seq technology is still in a fast development and it has several advantages over existing technologies (Table 1.2).

Table 1.2. Advantages of RNA-Seq compared with other methods (Modified from Wang et al., 2009)

	Tiling microarray	**cDNA or EST sequencing**	**RNA-seq**
Technology specifications			
Principle	Hybridization	Sanger sequencing	High-throughput sequencing
Resolution	From several to 100 bp	Single base	Single base
Throughput	High	Low	High
Reliance on genomic sequence	Yes	No	In some cases
Background noise	High	Low	Low
Application			
Simultaneously map transcribed regions and gene expression	Yes	Limited for gene expression	Yes
Dynamic range to quantify gene expression level	Up to a few-hundredfold	No practical	>8,000-fold
Ability to distinguish different isoforms	Limited	Yes	Yes
Ability to distinguish allelic expression	Limited	Yes	Yes
Practical issues			
Required amount of RNA	High	High	Low
Cost for mapping transcriptomes of large genomes	High	High	Relatively Low

RNA-Seq technology is used for undetermined transcriptomes of non-model organisms. Also, the technology can give information about sequence variations (for instance, single nucleotide polymorphisms (SNPs)) (Cloonan et al., 2008; Morin et al., 2008). And, RNA-Seq has very lower background signal than DNA microarrays because DNA sequences can be certainly mapped to unique regions of the transcriptome.

RNA-Seq technology is very accurate for quantifying expression levels just as determined using quantitative polymerase chain reaction (qPCR) (Nagalakshmi et al., 2008) and a spike-in RNA controls with known concentration (Mortazavi et al., 2008).

Also, the results of the RNA-Seq are reproducible highly for technical and biological replicates (Cloonan et al., 2008; Nagalakshmi et al., 2008). In addition to these, procedure of RNA-Seq does not have cloning steps.

2. AIM AND SCOPE OF THE STUDY

Several characteristics that help *C. cylindracea* to have invasive power individually demonstrated to be under control of AOX proteins in other plant species at molecular level. Thus our general hypothesis is that invasive characteristics of *C. cylindracea* is directly related with AOX proteins. The working hypothesis is that the species express at least one *AOX* gene. To test this hypothesis, the main purpose of this study is to identify mitochondrial alternative oxidase encoding gene(s) in *Caulerpa cylindracea* by using next-generation sequencing technology. To reach the main purpose, the special aim was to construct a cDNA library from encoding genes of AOX proteins in the species. Cloning studies of the *AOX* genes into yeast (*Saccharomyces cerevisiae*) vector were conducted for future expression studies. The characterization of those *AOX* genes will help researchers to understand molecular basis of invasion power for *C. cylindracea.*

3. MATERIALS AND METHODS

3.1 Sampling of *Caulerpa cylindracea*

Caulerpa cylindracea was collected by Dr. Ercan Selçuk Ünlü (CMAS 2* diver) and a professional diver from the Sığacık littoral zone (Seferihisar, İzmir, Turkey) at shallow water (12 m) on rocky substrates in September 9th, 2015 (Figure 3.1). The coordinates of the sampling area is 38°10'38.80"N - 26°45'57.24"E. The sampling region and samples of *Caulerpa cylindracea* are given in Figure 3.2.

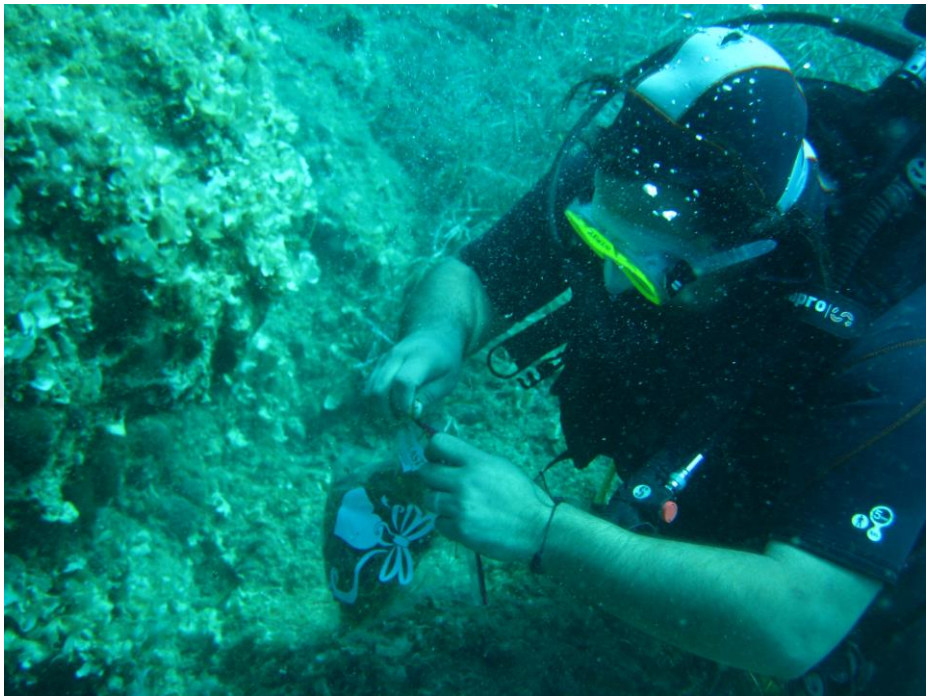

Figure 3.1. Sampling (Dr. Ercan Selçuk Ünlü (CMAS 2* diver))

Figure 3.2. Sampling area and samples (Photographed by Dr. Ünlü)

After collection, the algae washed with sterile water by paying attention to the green stolon tissues and cytoplasm. The samples were separated as 5 grams portions and packaged. Totally 1 kilogram (wet weight) of sample was transported to the laboratory in the 10 L liquid nitrogen transfer tank. During the sampling liquid nitrogen was refilled from Adnan Menderes University to prevent the loss of nitrogen.

3.2 Total RNA Isolation

To prevent the potential contamination from ribonuclease (RNase) during the experiments the following rules were followed in the laboratory.

1. There are two most common sources of RNase that are user's hands and bacteria and the dusts particles. To prevent this type of contamination 70% ethanol (Merck KGaA, 1.00983.2511) was used to sterile the gloves and the all areas that used for experiments.

2. During the experiments, the gloves, the pipettes and the experiment area were cleaned by DNA AWAY™ (Molecular Bioproducts Inc., 7010) and Nuclease Away (BioShop Canada Inc., NUC555.100) solutions.

3. Sterile disposable plasticwares that are RNase-free were used to handle RNA.

Total RNA isolation was carried out by using TRIzol® Reagent (Invitrogen, 15596-026) which is a ready-to-use reagent that was designed for isolation of high quality total RNA and also DNA and proteins from the tissues of plant, animal, human, yeast and bacteria. It contains guanidine isothiocyanate and phenol. The totality of the RNA is protected during the disrupting cells and sample homogenization due to effective inhibition of RNase activity by the reagent with large number of samples and single-step method (Chomczynski and Sacchi, 1987). Precipitation of RNA, DNA and proteins are sequentially available with this method in a single step (Chomczynski, 1993).

At the beginning, 5 grams of Rhizoid tissues and 15 grams of Branchlet, Rachis, Stolon (presented in the Figure 3.3) frozen (under the liquid nitrogen) tissues were prepared for the experiments. The RNA isolation was carried out according to the protocol which is given with TRIzol® Reagent. Firstly, 100 mg of samples were grinded in liquid nitrogen and homogenised in the presence of 1 mL of TRIzol® Reagent. Homogenised sample was incubated at room temperature for 5 minutes to allow complete dissociation of the nucleoprotein complex. 0.2 mL of chloroform (Merck KGaA, 1024451000) was added (0.2 mL of chloroform per 1 mL of TRIzol® Reagent), the solution was shaken vigorously by hand for 15 seconds and incubated at room temperature for 3 minutes, then centrifuged at 12,000 x g at 4°C for 15 minutes (Beckman Coulter, B30135-Microfuge 20R Centrifuge) for separation of the homogenate into a clear upper aqueous layer which contains RNA, and an interphase and a lower organic phase that contain DNA and proteins. Then the aqueous phase was collected into a new tube for isolation procedure.

After the phase separation step, the RNA isolation step was carried out. Firstly, 0.5 mL of 100% isopropanol (Merck KGaA, 1096341000) was added to the tube which contain the aqueous phase, per 1 mL of the reagent used for homogenization, incubated at -20°C and centrifuged 12,000 x g at 4°C for 10 minutes to precipitate the RNA from the aqueous layer. In this step, the pellet of the RNA is often invisible and forms a gel-like pellet on the side and bottom of the tube.

Next step was the RNA washing step. At the beginning of this step, supernatant was removed to the tube carefully for leaving the RNA pellet only in the side and the bottom of the tube. The pellet was washed with 1 mL of 75% ethanol per 1 mL of TRIzol® Reagent used in the initial homogenization. It was vortexed briefly, then centrifuged at 7500 x g t 4°C for 5 minutes and the wash was discarded carefully. After that the RNA pellet was dried completely at room temperature for 5-10 minutes. This part is important that the RNA was not dried completely because the pellet can lose its solubility.

Final step of the total RNA isolation with TRIzol® Reagent is RNA resuspension. For this step, the RNA pellet was resuspended in 50 µL of RNase-free water and incubated in a hot block at 55°C. The resuspended total RNA were store at -80°C.

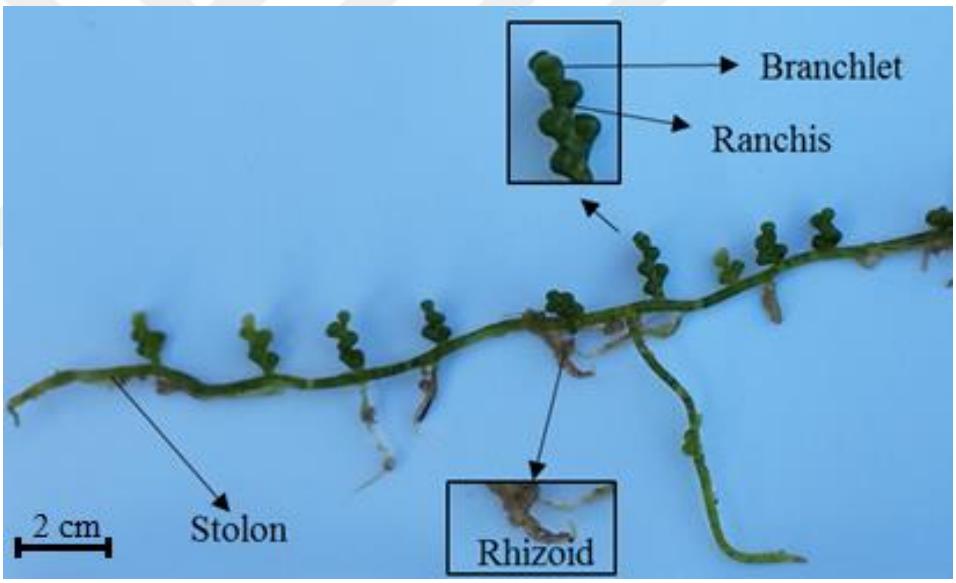

Figure 3.3. The parts of the collected samples (Photographed by Dr. Ünlü)

3.3 Messenger RNA (mRNA) isolation

mRNA isolation was carried out by using PolyATtract® mRNA Isolation Systems (Promega, Z5300). The mRNA isolation was made according to the protocol which is given for PolyATtract® mRNA Isolation Systems kit.

To remove the need for oligo(dT) cellulose and its problems the PolyATtract® mRNA Isolation Systems use the MagneSphere® technology. Isolation of the poly(A) mRNA part can be done free of other contaminations of nucleic acids in nearly 45 minutes. After the process the isolated mRNA can be used for cDNA synthesis and other molecular biology applications. A biotinylated oligo(dT) primer is used for hybridization of the 3' poly(A) region which presents in most eukaryotic mRNA species with a high efficiency. Then the hybrids are captured and then washed and mRNAs separated by using paramagnetic particles with a magnetic stand. The mRNA is eluted by addition of RNase-free water.

There are five steps for the mRNA isolation with PolyATtract mRNA Isolation System kit.

First step is the annealing of probe. For this step 0.1-1.0 mg of total RNA was completed to 500 µL with RNase-free water in a sterile RNase-free 1.5 mL tube and placed in a 65°C hot block for 10 minutes. Then 3 µL of the Biotinylated-Oligo(dT) Probe and 13 µL of 20X the saline-sodium citrate (SSC) buffer were added to the RNA, mixed gently, and incubated at room temperature until completely cooled (nearly 10 minutes).

Second step is the stock solution preparation during the cooling process. To prepare 1.2 mL of sterile 0.5X SSC, 30 µL of 20X SSC was combined with 1,170 mL of RNase-free water in an RNase-free tube. And to prepare 1.4 mL of sterile 0.1X SSC, 7µL of 20X SSC was combined with 1,393 mL of RNase-free water in an RNase-free tube.

The third step is the washing of Streptavidin-Paramagnetic Particles (SA-PMP). Bovine serum albumin (BSA) which is present in the storage buffer is needed for the stabilization of the Streptavidin-Paramagnetic Particles. It is removed by washing the particles. 1 mg/mL The SA-PMPs contains 1 mg/mL BSA and 0.02% sodium azide. The particles were washed three times each with equal volume of 0.5X SSC (300 µL per wash) and they used within 30 minutes after washing process for optimal performance. Then the washed SA-PMPs were resuspended in 100 µL 0.5X SSC completely. (The clumped particles must be discarded, and to prevent clumping the particles should not be frozen and dried out.)

The next step is the capture and washing of annealed Oligo(dT)-mRNA hybrids. The contents prepared in the annealing step were added to the tube containing the washed SA-PMPs, incubated at room temperature for 10 minutes and mixed by inverting every 1 minutes. Then, the SA-PMPs were captured with the magnetic stand, and supernatant was carefully removed. After that, the particles were washed with 0.1X SSC (300 µL per wash) for four times until all of the particles resuspended by flicking the tube gently. After the final washing, supernatant was removed as much as possible without disturbing SA-PMPs.

The final step of the mRNA isolation form total RNA with the kit is the elution of mRNA. The final SA-PMP pellet from the previous step was resuspend in 100 µL of the RNase-free water by flicking gently. The SA-PMPs were magnetically captured and transferred the eluted mRNA to a sterile tube without discarding the particles. And the elution step was repeated by resuspending the SA-PMP pellet in 150 µL of RNase-free water, captured, pooled the eluate with the RNA eluted before. The total volume of the eluted mRNA was 250 µL after the procedure. The transferred particles with the mRNA were removed by centrifuging at 12,000 x g for 1 minute at room temperature and the RNA was transferred to a new RNase-free tube.

After the isolation steps, mRNA samples were stored in -80^{o}C.

3.4 Transcriptome analysis

mRNA samples were isolated from four different tissues (Branchlet, Rachis, Stolon, Rhizoid) pooled as 10 ng/µL and separately to increase due to lack of information about expression quantities of the genes in the species. Then, TruSeq Stranded mRNA Library Prep Kit (Illumina, RS-122-2101) developed for cDNA library preparation with the aim of sequencing was used. Pooled mRNA samples were fragmented in divalent cation solution. After, first strand synthesis was performed by using random primers and reverse transcriptase enzyme. For the purpose of removing RNA chain and synthesizing DNA chain the samples were incubated in a mixture containing DNA polymerase I and RNase H. Adenine molecules were attached to the fragments of the DNA's 3' tails to prevent nonspecific ligation between cDNA fragments. After adapter 1 and adapter 2 (represented in Table 3.1) were attached to

the tails of the fragments by ligation. The samples were amplified by using PCR reaction consisting of 15 replications and concentrations were measured as 2.41 ng/μL.

Table 3.1. Adapters

Adapter 1:	5'-GATCGGAAGAGCACACGTCTGAACTCCAGTCACGCCAATATCTCGTATGCCGTCTTCTGCTTG-3'
Adapter 2:	5'-GATCGGAAGAGCACACGTCTGAACTCCAGTCACCTTGTAATCTCGTATGCCGTCTTCTGCTTG-3'

RNA isolation and sequencing of cDNA process was performed by the laboratory which is working with Ligand Biotechnology Company. The analysis of sequences was done by using HiSeq2000 Sequencing System (Illumina) with 100 nucleotide reads. 83.071.493,00 crude sequence were obtained after sequencing. Firstly, the adapter was separated from sequence fragments then overlapping sequence segments (100 nucleotides) are going to be assembled to generate high coverage quality of the original sequence. 27737 individual gene sequences were recognized after the process.

3.4.1 Identification of *AOX* genes

Blast analysis based on comparative genomic analysis was performed to identify *AOX* genes from 27737 full transcript information. For this purpose, bioinformatics analysis code was written using the Perl scripting language. Performing blast analysis when using a large number of gene sequence according to BIOPERL (Stajich et al., 2002) with the computer is the main principle of the code. All known *AOX* gene sequences were downloaded from the National Center for Biotechnology Information (NCBI) protein database to compare identified *AOX* genes. All of the obtained 27737 transcripts were ran against AOX protein sequence database. Then the sequences were translated into protein sequences and analysis was repeated. There was no results in an analysis based on the gene sequence. Therefore, the sequences were translated into protein sequences and analysis was repeated. Database was created by using 2342 different AOX protein sequences downloaded from NCBI database. Two

protein sequences which are presented below were identified for using high degree of similarity of the known AOX proteins. The gene sequences that belongs to the protein sequences were obtained from the transcriptome analysis data. When the Blast analysis performed via DNA sequence, there was no matching. Therefore, characterization studies on the bioinformatics level were continued based on protein sequences.

Clustel Omega (Sievers et al., 2011) tool was used to obtain similarities between interspecies amino acid sequences. MITOPROT (Claros and Vincens, 1996) tool was used for mitochondrial localization signal of two proteins to verify whether identified proteins are mitochondrial.

3.5 cDNA synthesis

RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., 1621) which is a system for synthesis of first strand cDNA from mRNA or total RNA templates was used. And the following procedure was applied.

The following reagents were added into a sterile, nuclease-free tube on ice in the order as given in Table 3.2.

Table 3.2. Reaction components of cDNA synthesis

Components	Volume
Template RNA (mRNA):	200 ng / µL
Primer:	1 µL
Nuclease-free water:	to 12 µL
5X Reaction buffer:	4 µL
RiboLock RNase inhibitor (20 U/µL):	1 µL
10 mM dNTP mix:	2 µL
RevertAid M-MuLV RT (200 U/µL):	1 µL
Total volume:	20 µL

Then, the mixture was mixed gently, and incubated at 25ºC for 5 minutes followed by 42ºC for 60 minutes. After that, the reaction was set as the Table 3.3.

Table 3.3. Conditions of cDNA synthesis

Step	Temperature	Time
Initial Denaturation	94°C	1 minute
35 cycles	94°C 58°C 72°C	30 seconds 45 seconds 2 minutes
Hold	4°C	-

3.6 Genomic DNA (gDNA) isolation

gDNA samples were obtained by using ZR Plant/Seed DNA MiniPrep kit (Zymo Research, D6020) which has a method for the isolation of inhibitor-free, PCR-quality DNA from of plant and seed species by using chemically inert BashingBeads™.

Before starting the procedure, 2-mercaptoethanol (Sigma-Aldrich, M3148) was added to the Plant/Seed DNA Binding Buffer to a final dilution of 0.5% (v/v) and Zymo-Spin™ IV-HRC Spin Filters which are green topped were prepared for the experiment by centrifuging at 8,000 x g for 3 minutes.

Approximately 150 mg of finely grinded sample was added to a ZR BashingBead™ Lysis Tube and 750 µL of Lysis Solution was added to the tube. The sample was homogenised briefly. After that, the lysate was centrifuged at 10,000 x g for 1 minutes. 400 µL of supernatant was transferred to a Zymo-Spin™ IV Spin Filter which has orange top in a collection tube and centrifuged at 7,000 x g for 1 minute. 1.200 µL of Plant/Seed DNA Binding Buffer was added to the filtrate in the collection tube from previous step. Then, 800 µL of mixture from the step before was transferred to a Zymo-Spin™ IIC Column to a collection tube and centrifuged at 10,000 x g for 1 minute. Supernatant was removed from the collection tube and the previous step was repeated. After, 200 µL of DNA Pre-Wash Buffer was added to the Zymo-Spin™ IIC

Column and centrifuged at 10,000 x g for 1 minute. 500 µL Plant/Seed DNA Wash Buffer was added to the Zymo-Spin™ IIC Column and centrifuged again 10,000 x g at 1 minute. Zymo-Spin™ IIC Column was transferred to a clean 1.5 mL of microcentrifuge tube and 50 µL DNA Elution Buffer was added directly to the column matrix and centrifuged at 10,000 x g for 30 seconds for eluting the DNA. Finally, the eluted DNA was transferred to a prepared Zymo-Spin™ IV-HRC Spin Filter (green top) in a clean 1.5 mL microcentrifuge tube and centrifuged at exactly 8,000 x g for 1 minute. The filtered DNA was suitable for the PCR applications after this processes.

3.7 Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction (PCR) is a technique for DNA amplification and it is powerful and sensitive technique. PCR method was used to amplify *ESU1-AOX* and *ESU2-AOX* genes by using *Taq* DNA polymerase (New England Biolabs, M0320S) which is an enzyme that used commonly in PCR.

The PCR reaction was set up according to the Table 3.4.

Table 3.4. Components of the PCR

Component	25 µL reaction	Final concentration
10X Standard *Taq* Reaction (Mg-free) Buffer	2.5 µL	1X
25 mM MgCl2	1.5 µL	1.5 mM
10 mM dNTPs	0.5 µL	200 µM
10 µM Forward Primer	0.5 µL	0.2 µM
10 µM Reverse Primer	0.5 µL	0.2 µM
Template DNA	1 µL	<1.000 ng
Taq DNA Polymerase	0.125 µL	1.25 units/50 µL PCR
Nuclease-free water	18.375 µL	-

After the preparing of the reaction mixture, the mixture was placed to the thermocycler (Biometra T Professional) under the following conditions (Table 3.5). Designed forward and reverse primers are given in the Table 3.6.

Table 3.5. Conditions of the PCR

Step	Temperature	Time
Inıtial Denaturation	95°C	1 minute
40 cycles	95°C 49°C 68°C	30 seconds 45 seconds 2 minutes
Final Extension	68°C	10 minutes
Hold	4°C	-

Table 3.6. Primers for amplifications of *ESU1-AOX* and *ESU2-AOX*

Name of the primer	Sequence
ESU1-AOX Forward	5'- ACTAGTATGCTGTCTACGAGTCGTCGTTTT -3'
ESU1-AOX Reverse	5'- CTCGAGTTAAGGCACAGTGTGACTGTGCTT -3'
ESU2-AOX Forward	5'-ACTAGTATGTTACAACAAAGATGTCGAAGTTTTACC-3'
ESU2-AOX Reverse	5'- CTCGAGTCAATCCGACGAGGTTTTGGC-3'

3.7.1 Gel electrophoresis

Agarose gel electrophoresis is an effective method to detect and characterize DNA. It is a sensitive method and does not require another applications. With this method, DNA is loaded into the wells of the agarose gel and a current applied. Because of the negatively charged DNA (and RNA) phosphate backbone fragments migrates to the positively charged anode under an electric field. DNA molecules are separated and run different distances because DNA has different mass/charge ratio. The distance run is inversely proportional to the log of its molecular weight (Helling et al., 1974).

For the visualizing the samples, firstly 1% agarose gel was prepared with adding ethidium bromide (EtBr) (Sigma-Aldrich, E1510) which is the common reagent used to mark DNA in agarose gel, under the UV-Light, electrons of the aromatic ring of the EtBr molecule are activated, and when the electrons return to the ground state, releasing of the energy as light occurs (Sharp et al., 1973). 1 µL of PCR products were mixed with Gel Loading Dye Blue (6x) (New England Biolabs, B7021S). Then, 100 volt, current 400 (with Cleaver Scientific NanoPac-300 power supplier) was applied. Finally the results were detected under UV-Light. In addition, Gene Ruler 100 bp Plus DNA Ladder (Thermo Scientific Inc., SMO321) and Lambda DNA-HindIII Digest (New England Laboratories, N3012S) were used as marker to characterize the visualized samples.

3.8 TA Cloning

For the cloning purpose The InsTAclone PCR Cloning Kit (Thermo Scientific Inc., 1214) ,which is a TA system for direct one-step cloning of PCR products with 3’ – dA overhangs, was used. It contains the high quality TA cloning vector pTZ57R/T that is ready to use for efficient ligation providing high quality yields and low background.

Two step cloning protocol that was used in the study is given the below.

3.8.1 Ligation Step

Firstly the Ligation reaction was set up according to the Table 3.7.

Table 3.7. The components of ligation reaction

Reaction components	Volume
Vector pTZ57R/T, (0.17 pmol ends)	3 µL
5X Ligation Buffer	6 µL
PCR product (0.52 pmol ends)	Variable*
Nuclease-free water	To 29 µL
T4 DNA Ligase	1 µL
Total volume of the reaction	**30 µL**

After addition all of the components to the tube, it was vortexed and centrifuged for 5 seconds at 8,000 x g. And the mixture was incubated at room temperature for 2 hours. At the end of the ligation part of the cloning protocol, the ligation mixture was ready to use of bacterial transformation.

3.8.2 Transformation Step

For the transformation *Escherichia coli* (*E. coli*) DH5α competent cells were used. Firstly, 5 µL DNA from the ligation part was added to frozen DH5α competent cells and mixed gently. It was placed on ice for 30 minutes and placed 42°C for 30 seconds in a water bath and after placed on ice for 2 minutes carefully. After that, 250 µL lysogeny broth (LB) media was added to the tube. 1 hour incubation was done at 37°C. Finally, the cells grown in the LB media were plated on LB-ampicillin agar plates and incubated at 37°C for 16 hours. After overnight incubation, colonies grown on plates were picked and analysed with PCR as described above for verification of

ESU1-AOX into pTZ57R/T vector. The vectors verified for *ESU1-AOX* and *ESU2-AOX* fragments were marked as pTZ57R/$T_{ESU1\text{-}AOX}$ and pTZ57R/$T_{ESU2\text{-}AOX}$, respectively.

3.8.2.1 Preparation of Ampicillin stock solution (100 mg/mL)

5.0 grams of ampicillin sodium salt (AppliChem, A0839, 0025) was dissolved in 50 mL of deionized water and filtered and stored at -20°C.

3.8.2.2 Preparation of LB media

10 grams of Peptone from Casein (Tryptone) (Merck KAaG, 1.07213.1000), 5 grams of Yeast Extract (BioShop, YEX401.1) and 5 grams of Sodium chloride (Sigma-Aldrich, 31434-1KG-R) were added to 800 mL of water and the pH of the solution adjusted to 7.0 with sodium hydroxide (Merck KAaG, 1.0642.1000) and the volume was completed to 1 litre. For the plates agar (Lab M Limited, MC002) was added and autoclaved. For the liquid medium, the mixture was autoclaved without adding agar.

3.8.2.3 Preparation of LB-ampicillin media

Before the pouring plates, the media allowed to cool to 55°C after autoclave process. Then, 1 mL of 100 mg/mL ampicillin was added from the stock solution carefully for protection of the bacterial contamination to a final concentration of the ampicillin in the LB of 100 μg/mL and plates were dried and stored at 4°C.

3.9 Ligation *AOX* genes into pAG413 yeast vector

pTZ57R/$T_{ESU1\text{-}AOX}$ and pTZ57R/$T_{ESU2\text{-}AOX}$ was digested with XhoI and SpeI endonuclease enzymes and ligated with pAG413 (Yeast expression vector with GAL-promoter element and ccdB killer gene) plasmid which was also digested with XhoI and SpeI endonuclease enzymes. After digestion process DNA fragments were run on

1% agarose gel and extracted using GeneJET Gel Extraction kit (Thermo Fisher Scientific Inc., K0691). Using a 1:3 (Vector/Insert) ratio, ligation process were carried out using the reaction volumes shown in Table. After the ligation process, the DNA products were transformed into *E. coli* strains by following the protocol described as below (Table 3.8).

Table 3.8. Components of ligation reaction

Component	**Volume (for Vector + Insert)**	**Volume (for only Vector)**
Vector	1.9 µl	1.9 µl
Insert	7.4 µl	-
5X Buffer	4.0 µl	4.0 µl
T4 Ligase	1.0 µl	1.0 µl
dH_2O	5.7 µl	13.1 µl
Total	20.0 µl	20.0 µl

3 µL of ligate was added to the frozen *E. coli* cells and mixed gently. Then, it was placed on ice (for 30 seconds), at 42°C (for 30 minutes) and on ice (for 2 minutes), respectively. After this processes, 250 µL of LB was added to the tube and placed at 37°C (for 1 hour) and plated 150 µL plates. And incubated at 37°C (for 16 hours). After overnight incubation, colonies grown on plates were picked and analysed with PCR as described above for verification of *ESU1-AOX* into pAG413 vector. The vectors verified for *ESU1-AOX* and *ESU2-AOX* fragments were marked as $pAG413_{ESU1\text{-}AOX}$ and $pAG413_{ESU2\text{-}AOX}$, respectively.

4. RESULTS AND DISCUSSIONS

4.1 RNA isolation

The RNA isolation was carried out according to the protocol which is given with TRIzol® Reagent. The resulting RNA concentrations are presented in Table 4.1.

Table 4.1. The concentrations of total RNA and mRNA after isolation

Tissue	**RNA concentration (ng/ul)**	
	Total RNA	***mRNA***
Branchlet	1704	78
Rachis	1366	53
Stolon	1542	59
Rhizoid	631	29

4.2 Transcriptome analysis

The values related to the sequence analyzing are presented in Table 4.2. N50 value which is greater than average fragment length shows that the transcripts were assembled meaningful.

Table 4.2. Values of transcriptome analysis

Number of transcripts	27737
GC content	61,63
N50 value	1032
Average transcripts length	719,14

4.3 Identification of *AOX* genes

There was no result from the PCR because it is clear that the genes responsible for the *AOX* activities of the *Caulerpa cylindracea* have more differences according to the known species. In our analysis, match percentage (> 50%), matching sequence length (> 200 amino acids) and E-value (< 10-5) were taken as a criterion in order to prevent mismatches. The domain locations and sequence lengths of the matching sequences show high similarities with AOX proteins of the different species after looking analysis. The values belong to 5 species that have highly similar according to the protein sequences are shown in Table 4.3. Clustel Omega (Sievers et al., 2011) tool was used to obtain similarities between interspecies amino acid sequences. Similarities between sequences are presented in Figure 4.1 and Figure 4.2.

Table 4.3. Data of the BLAST matching values of AOX proteins of *C. cylindracea*

Sequence ID	**GO matching, Name of the matched species**	**Overlap**	**Matching percentage**	**E-value**	**BIT Score**
c25342_g1_i1_3	Alternative oxidase *Tetraselmis sp, GSL018*	265	58,11	7,00E-92	332
	Alternative oxidase *Ostreococcus tauri*	240	59,17	3,00E-79	289
	Alternative oxidase 2 *Chlamydomonas reinhardtii*	234	58,12	7,00E-79	288
	AOX alternative oxidase *Helicosporidium sp, ATCC50920*	241	56,43	2,00E-80	293
	Alternative oxidase *Volvox carteri f, nagariensis*	208	63,94	1,00E-74	274

Table 4.3. Data of the BLAST matching values of AOX proteins of C. cylindracea (cont'd)

c34342_g1_i1_2	Alternative oxidase *Chlamydomonas reinhardtii*	452	55,31	1E-132	464
	Alternative oxidase 4 *Monoraphidium neglectum*	386	53,37	1E-117	416
	Alternative oxidase *Ostreococcus tauri*	255	59,61	2E-088	320
	Alternative oxidase 4 *Glycine soja*	209	57,42	2E-069	257
	Alternative oxidase 4 *Morus notabilis*	228	50	3E-062	233

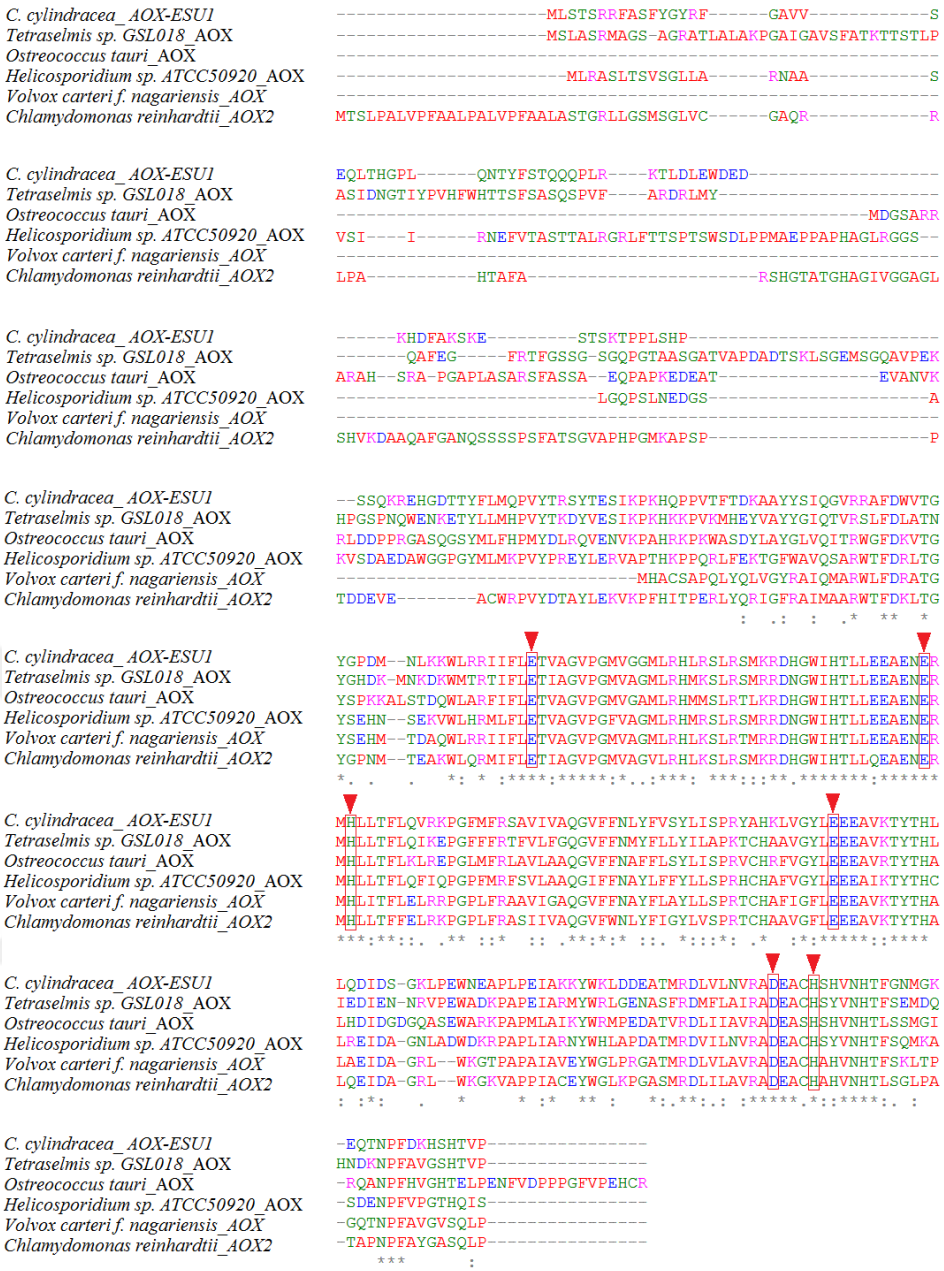

Figure 4.1. Multiple sequence alignment of ESU1-AOX protein

▼: Shows iron binding sites of the identified alternative oxidases.

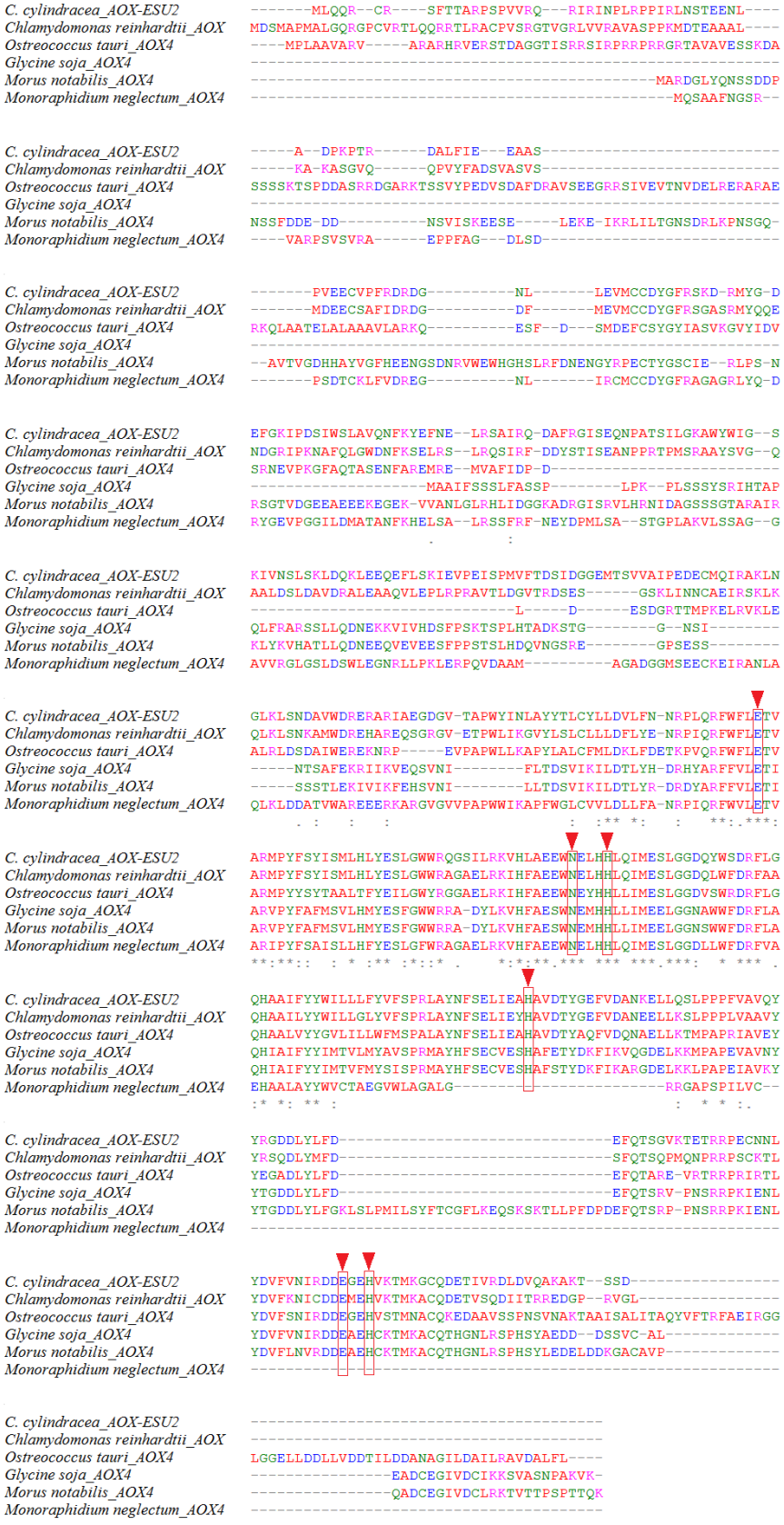

Figure 4.2. Multiple sequence alignment of ESU2-AOX protein

▼ : Shows iron binding sites of the identified alternative oxidases.

The active domain parts of two different AOX sequences that are identified as a result of multiple sequence analysis show a high similarity in terms of amino acids. Detailed nucleotide and protein sequence data of the identified two *AOX* sequences are presented in Table 4.4.

Table 4.4. Sequence properties of *AOX* genes of *C. cylindracea*

Temporary gene name*	*AOX-ESU1*
Sequence ID	ORF_c25342_g1_i1_3
Nucleotide number	966
Nucleotide sequence	ATGCTGTCTACGAGTCGTCGTTTTGCTTCGTTTT ATGGTTACCGATTCGGAGCCGTCGTCAGCGAAC AATTGACGCATGGACCCCTCCAAAACACGTACT TCAGCACTCAGCAACAGCCCCTCCGTAAGACAC TCGACTTGGAGTGGGATGAAGATAAACATGATT TCGCAAAGTCAAAGGAATCCACTTCCAAAACCC CACCATTATCTCATCCTTCGTCTCAGAAGCGCG AACATGGAGATACCACTTATTTCCTAATGCAAC CAGTGTACACTCGCAGCTACACAGAATCCATCA AACCAAAGCATCAACCACCTGTCACCTTCACTG ACAAGGCTGCTTATTACTCCATCCAAGGTGTGC GGAGGGCCTTCGATTGGGTCACTGGCTACGGCC CTGACATGAACTTGAAGAAGTGGCTCAGGAGG ATCATCTTTCTGGAGACTGTTGCAGGGGTGCCT GGGATGGTAGGTGGAATGCTCAGACATCTCAG ATCTTTAAGATCAATGAAGAGAGACCATGGTTG GATCCACACTTTGCTCGAAGAAGCTGAGAATGA ACGAATGCATTTATTGACCTTCCTCCAAGTGCG GAAACCTGGTTTCATGTTCCGATCAGCAGTCAT TGTTGCACAAGGTGTGTTTTTTAATTTGTATTTT GTGTCGTACTTGATATCTCCAAGATACGCACAT AAGCTCGTGGGGTATTTGGAGGAGGAAGCTGT GAAGACTTATACTCATTTATTGCAAGATATCGA TTCTGGAAAGCTTCCAGAATGGAACGAGGCGCC TCTTCCTGAGATCGCGAAGAAGTACTGGAAACT TGACGATGAAGCAACGATGCGAGATTTGGTGTT GAACGTCCGAGCAGATGAAGCATGTCATAGTC ACGTCAATCATACTTTCGGCAACATGGGGAAAG AGCAAACAAACCCATTTGACAAGCACAGTCAC ACTGTGCCTTAA
Amino acid number	321

Table 4.4. Sequence properties of *AOX* genes of *C. cylindracea* (cont'd)

Protein Sequence	**MLSTSRRFASFYGYRF**GAVVSEQLTHGPLQNTY FSTQQQPLRKTLDLEWDEDKHDFAKSKESTSKTP PLSHPSSQKREHGDTTYFLMQPVYTRSYTESIKPK HQPPVTFTDKAAYYSIQGVRRAFDWVTGYGPDM NLKKWLRRIIFLETVAGVPGMVGGMLRHLRSLRS MKRDHGWIHTLLEEAENERMHLLTFLQVRKPGF MFRSAVIVAQGVFFNLYFVSYLISPRYAHKLVGY LEEEAVKTYTHLLQDIDSGKLPEWNEAPLPEIAKK YWKLDDEATMRDLVLNVRADEACHSHVNHTFG NMGKEQTNPFDKHSHTVP

Temporary gene name*	*AOX-ESU2*
Sequence ID	c34342_g1_i1_2
Nucleotide number	1365
Nucleotide sequence	ATGTTACAACAAAGATGTCGAAGTTTTACCACA GCACGTCCTTCCCCGGTGGTCCGACAACGAATC CGGATCAATCCATTGCGTCCTCCAATTCGACTC AACAGTACTGAAGAGAATCTTGCTGATCCCAAA CCGACCCGAGACGCACTCTTCATTGAGGAGGCA GCTTCACCAGTTGAAGAATGTGTTCCATTTCGT GATAGGGATGGAAATCTGTTAGAGGTGATGTGT TGCGATTACGGCTTCCGTTCAAAAGACCGAATG TACGGCGACGAATTCGGCAAGATTCCAGATTCG ATTTGGAGTCTCGCAGTGCAGAATTTCAAATAC GAGTTCAACGAGCTTCGAAGCGCCATTCGACAA GATGCATTTCGCGGAATCTCAGAACAGAATCCT GCCACTTCCATATTAGGCAAAGCTTGGTATTGG ATTGGAAGCAAAATTGTGAACTCGTTGTCTAAA TTGGATCAGAAATTAGAGGAACAGGAGTTTCTG TCCAAGATCGAGGTTCCTGAAATTTCACCTATG GTTTTCACAGATTCAATTGATGGAGGTGAAATG ACGTCTGTTGTGGCTATACCTGAGGATGAATGC ATGCAAATTAGAGCTAAATTGAATGGACTCAAG CTCAGTAACGATGCAGTCTGGGATCGAGAAAG AGCAAGAATTGCAGAAGGAGATGGAGTCACTG CACCATGGTACATCAACTTAGCATACTACACCT TATGTTACCTGCTGGACGTCCTATTCAACAACA GACCTTTGCAGAGATTCTGGTTTTTGGAGACAG TTGCTCGGATGCCTTATTTCAGCTACATTTCGAT GTTGCATTTGTACGAGTCGTTAGGTTGGTGGAG GCAAGGCTCCATTCTGCGTAAAGTGCATTTGGC AGAGGAATGGAACGAATTGCATCATCTCCAAAT CATGGAGTCTTTAGGAGGAGACCAATACTGGA GCGATCGATTCCTCGGACAACATGCAGCCATCT TTTACTATTGGATTTTGCTTTTGTTTTATGTGTTT TCACCAAGATTGGCGTACAATTTCAGTGAGTTG ATTGAGGCTCATGCAGTGGATACTTATGGTGAA

Table 4.4. Sequence properties of *AOX* genes of *C. cylindracea* (cont'd)

	TTTGTTGATGCTAATAAGGAGCTTCTTCAATCG CTTCCTCCTCCCTTCGTCGCGGTCCAGTATTACC GAGGAGATGATTTGTATCTATTCGATGAATTCC AAACTTCAGGAGTCAAGACGGAGACTCGTCGA CCGGAGTGTAATAACCTCTACGACGTCTTCGTG AACATCCGAGATGACGAGGGAGAGCATGTAAA GACGATGAAAGGGTGCCAAGATGAGACCATCG TCCGAGACCTCGACGTCCAAGCCAAAGCCAAA ACCTCGTCGGATTGA
Amino acid number	454
Protein Sequence	**MLQQRCRSFTTARPSPVVRQRIRINPLRPPIRL**N STEENLADPKPTRDALFIEEAASPVEECVPFRDRD GNLLEVMCCDYGFRSKDRMYGDEFGKIPDSIWSL AVQNFKYEFNELRSAIRQDAFRGISEQNPATSILG KAWYWIGSKIVNSLSKLDQKLEEQEFLSKIEVPEIS PMVFTDSIDGGEMTSVVAIPEDECMQIRAKLNGL KLSNDAVWDRERARIAEGDGVTAPWYINLAYYT LCYLLDVLFNNRPLQRFWFLETVARMPYFSYISM LHLYESLGWWRQGSILRKVHLAEEWNELHHLQI MESLGGDQYWSDRFLGQHAAIFYYWILLLFYVFS PRLAYNFSELIEAHAVDTYGEFVDANKELLQSLPP PFVAVQYYRGDDLYLFDEFQTSGVKTETRRPECN NLYDVFVNIRDDEGEHVKTMKGCQDETIVRDLD VQAKAKTSSD

*Temporary gene names are the code names during the study. Gene names will be chosen after the activity determination according to related *AOX* family.

Also MITOPROT (Claros and Vincens, 1996) tool was used for mitochondrial localization signal of two proteins to verify whether identified proteins are mitochondrial. Responsible parts of the mitochondrial localization are shown in Table 4.4 as bold and underlined. The protein sequences which were identified for the analysis of the conservation status of the active domain sites of the protein domains were analyzed in structure analysis module of NCBI database (http://www.ncbi.nlm.nih.gov/Structure). Analysis indicated that identified two proteins were conserved for the amino acid regions which are defined for AOX proteins. The data are presented in the results multiple sequence analysis (Figure 4.1 and 4.2).

As expected, identified proteins conserved for the amino acid sequences providing the activity of alternative oxidase. The parts which are shown with red arrows presents iron binding sites of the identified alternative oxidases in Figure 4.1 and 4.2). These regions are the most important part of the active site of the enzyme that performs the oxidation activity. The results prove that identified two different sequences show alternative oxidase activity.

4.4 Amplification of *ESU1-AOX* and *ESU2-AOX* genes with PCR

Although *ESU1-AOX* was amplified from three different tissues, PCR experiments from the cDNA samples of *ESU2-AOX* did not work (Figure 4.3-A).

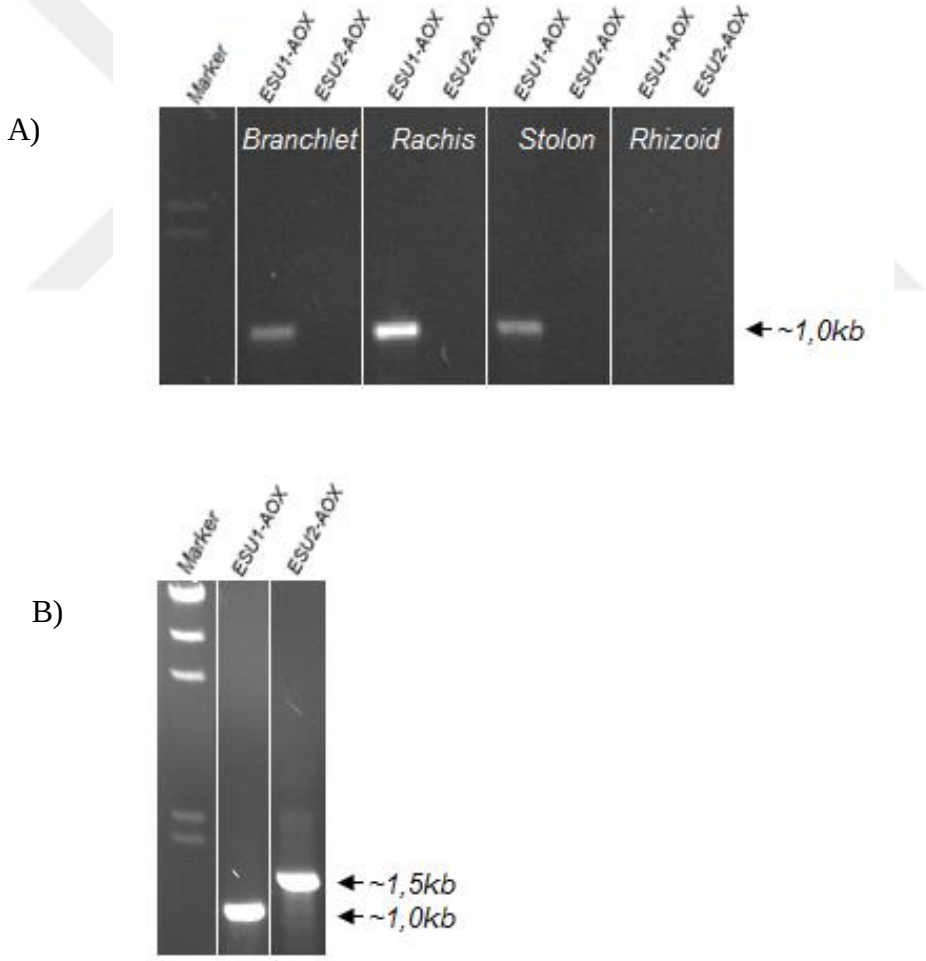

Figure 4.3. PCR results for amplifications of *ESU1-AOX* and *ESU2-AOX*

It was agreed that the problem may be from three sources; wrong PCR conditions, incorrect primers depending on incorrect *AOX* gene sequence and low yield in the cDNA synthesizing. For the testing the first case, experiments were repeated by using different PCR conditions (different temperatures, $MgCl_2$ concentrations and enzyme), but the result was not positive. For the testing of the possibilities of the second and the third reason, different cDNA concentrations from different tissues were used as template, however there was no result again. gDNA samples obtained by using ZR Plant/Seed DNA MiniPrep kit from different tissues were used as template in PCR reactions to verify that the expression levels of the *AOX* genes is lower than the sensitivity levels of the cDNA synthesis kits and for correction of sequences that we have. As shown in Figure 4.3-B *ESU2-AOX* sequence run when gDNA used as a template. This data shows that the expression levels of *ESU2-AOX* genes is lower than the sensitivity of the cDNA kits. Probably, the source of the problem in the amplification of the *ESU1-AOX* genes from Rhizoid tissue is the same. *ESU2-AOX* sequences have been identified by using different tissues with transcriptome analysis. The methods used in this assay is much more sensitive than the standard cDNA protocols. Therefore, it had an advantage in the identification of the genes. According to these data, the expression of the *ESU1-AOX* is continuous and active in the development process of the tissues. Also, different environmental and developmental processes may be predicted in the expression *ESU2-AOX* gene expression. *AOX* genes respond different expression profiles in different developmental processes and stress conditions (abiotic and biotic) (Polidoros et al., 2009). Our prediction is that identified *ESU1-AOX* can be effective under abiotic stress (including cold stress). In the study of Matos et all. (2007), specific *AOX* forms of *A. thaliana* species express in normal conditions (22^{o}C) although the expression levels are higher in cold stress (9^{o}C). Also, different *AOX* forms have limited synthesizing profile either in stress or different developmental levels although expression levels change and specific *AOX* forms are active in only a short cycle of the development (Clifton et al., 2006). Our future targets have interpretations of identified *AOX* genes according to these data. The metabolic effects of identified *AOX* genes in *C. cylindracea* and expression levels according to environmental conditions and state of development of cell with the effects of these genes in vivo are in the future impacts of the study. In addition, the study of Varela-Alvarez et al. (2012), the relations between *Caulerpa* species and the polyploids of the species investigated. According to the analysis, three polyploid levels for *C.*

cylindracea were determined. Certainly, the relations between the activities of the AOX proteins by detect with polyploid properties of the species are important for the future works.

Although the sampling was made sterile and carefully, studies on *Caulerpa* species show that these species are in contact with bacterial colonies in the habitats (Aires et al., 2012). This situation may affect the results of the transcriptome analysis. For the analysis of the effects of the contamination, isolated total RNAs (150 ng) from different tissues were denaturized with formamide firstly, then analysed by running on 1.0 % agarose gel. Only three standard bands (25S, 18S and 5,8S) have detected, it shows that there is no bacterial contaminations or the contamination level is lower than the detection limits (Figure 4.4).

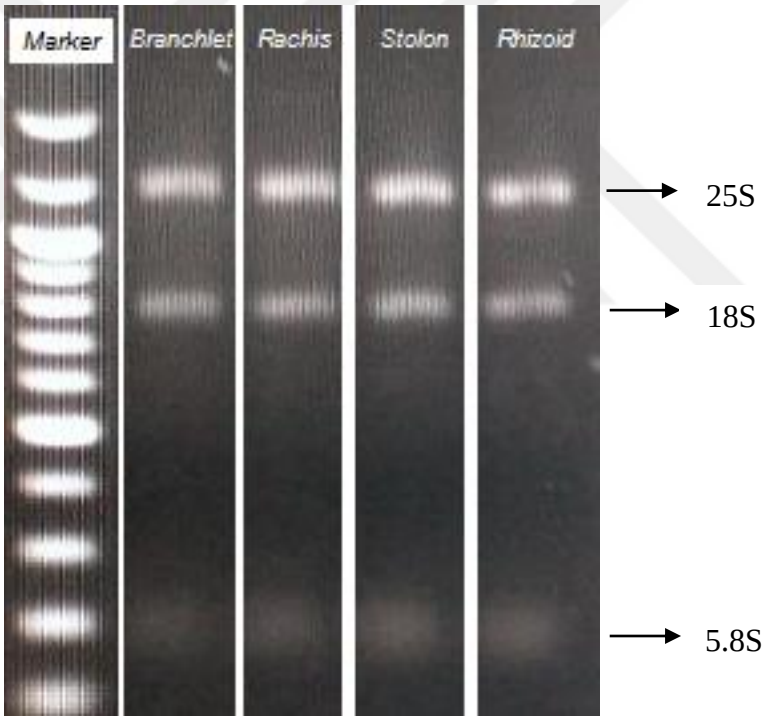

Figure 4.4. *C. cylindracea* total RNA samples

In addition to correction of absence of bacterial AOX protein contamination we used statistical methods to present the differences between bacterial AOX proteins and identified AOX proteins in this study.

For this purpose the peptide lengths of 258 bacterial AOX proteins were compared with the peptide lengths of 311 algal AOX proteins. The bacterial AOX

proteins ranged between 140-240 aa and plant + algal AOX proteins ranged between 167-1049 aa peptide length according to the calculations. The graph of ranges of proteins as peptide length is presented in Figure 4.5.

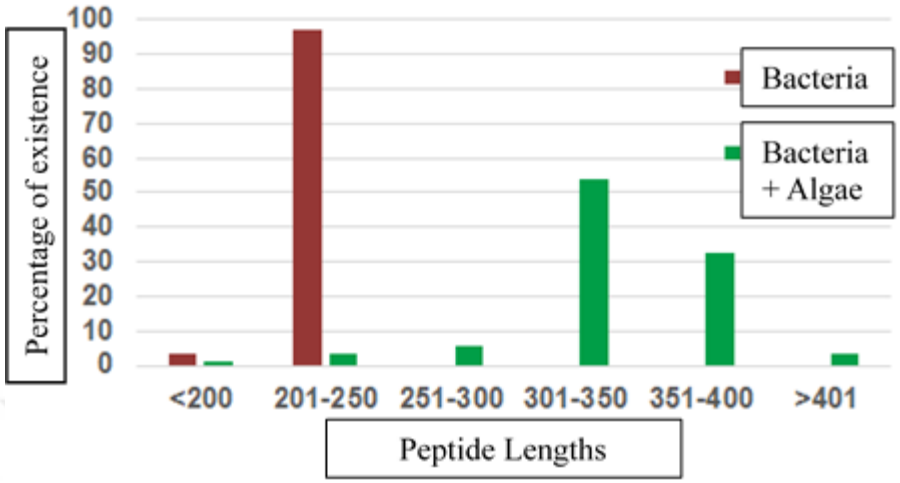

Figure 4.5. Distribution of bacterial and plant + algal amino acid lengths

The results of independent samples T-test show that amino acid lengths of the proteins of bacterial AOX (Avg=216,28; s=10,37) amino acid lengths of the proteins of plant + algal AOX (Avg=340,74; s=66,55) are differ from each other meaningfully (t(328.08) = 32,506; p=0,000). In this regard AOX proteins identified in this study are different from bacterial forms with 321 and 454 amino acid lengths.

Also gDNA samples were isolated from different tissues of the species and performed PCR reaction to understand the possible contaminations from Rhizoid. Figure 4.6 represents the amplifications of *ESU1-AOX* and *ESU2-AOX* genes by PCR from all parts of the species. The results show us that *AOX* sequences that we have are independent from contamination and specific for the species. However this analysis does not mean that there was no sequences data from contamination. Therefore, the data must be verified with similar analysis when we want to use for other genes by using transcriptome data.

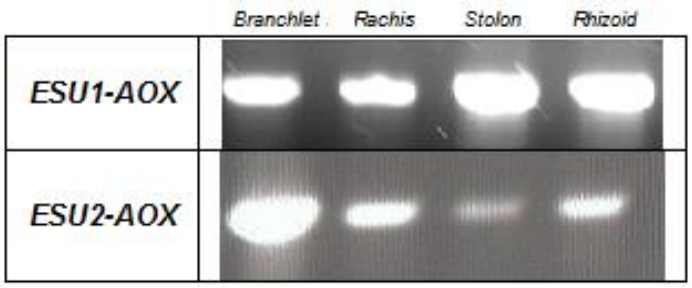

Figure 4.6. The PCR results of *ESU1-AOX* and *ESU2-AOX* genes from different tissues of the species

4.5 Ligation *AOX* genes into pAG413 yeast vector

pTZ57R/$T_{ESU1\text{-}AOX}$, pTZ57R/$T_{ESU2\text{-}AOX}$ and pAG413 plasmid were digested with XhoI and SpeI endonuclease enzymes. Then, pTZ57R/$T_{ESU1\text{-}AOX}$, pTZ57R/$T_{ESU2\text{-}AOX}$ were ligated with pAG413.

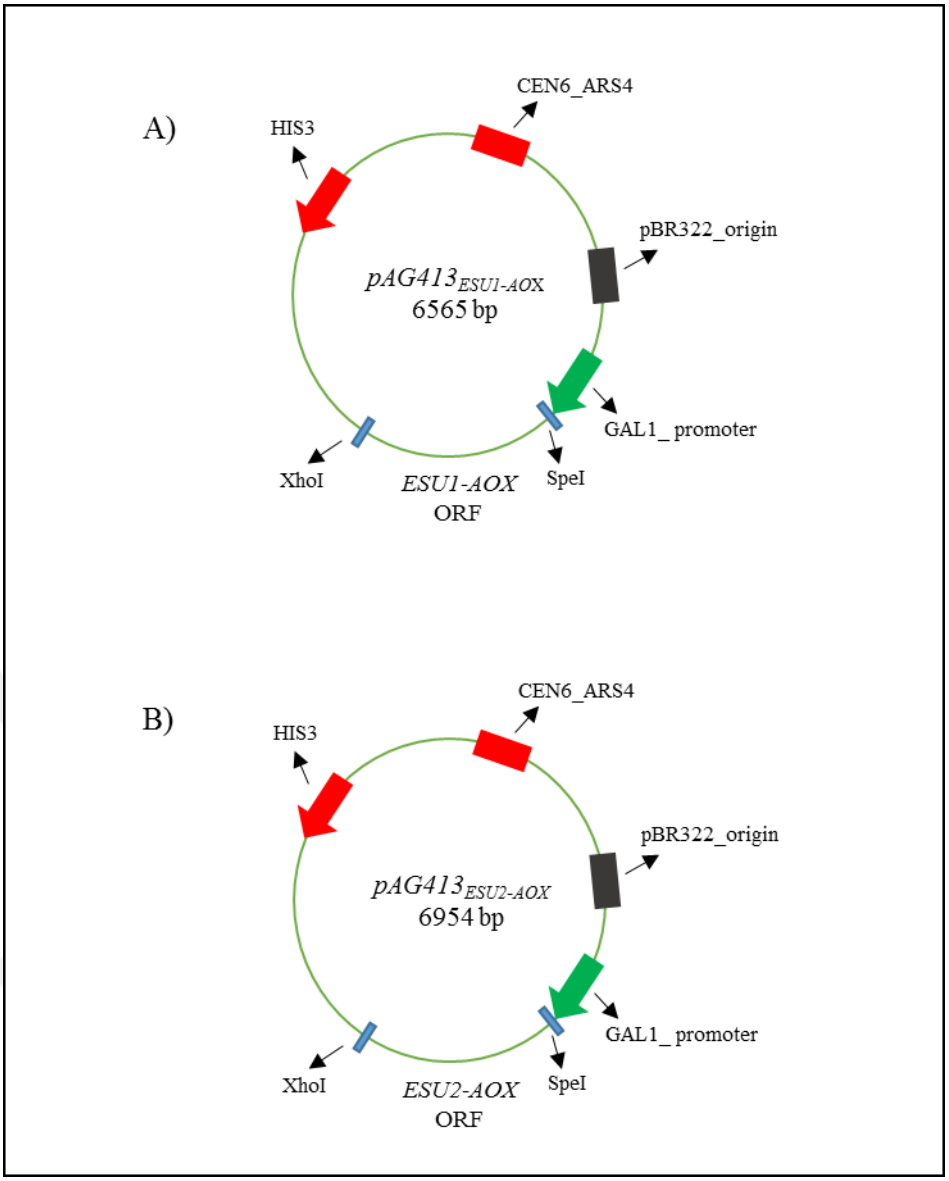

Figure 4.7. Ligated pAG413 vectors with *ESU1-AOX* (A) and *ESU2-AOX* (B) genes

The verification of the ligation process was made and the results were given in Figure 4.8.

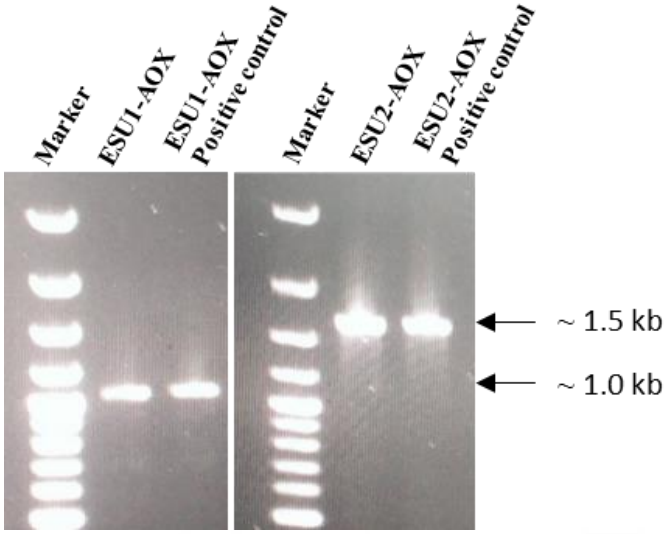

Figure 4.8. Verification of *ESU1-AOX* and *ESU2-AOX* genes in pAG413

The results show that the ligation procedure was made clearly for *ESU1-AOX* and *ESU2-AOX* genes.

4.6 *AOX* gene modifications

The result of NCBI structure analysis module and PFam (Finn et al., 2014) analysis of the amino acid boundaries belong to domain regions of identified AOX protein sequences of *C. cylindracea* species are given in Figure 4.8. Also Figure 4.9 summarizes the mutation parts which are continuing. Accordingly, the synthesizing of 4 different mutants by mitochondrial localization sequence and *AOX* active site and characterizing of the activity are the future works of the study.

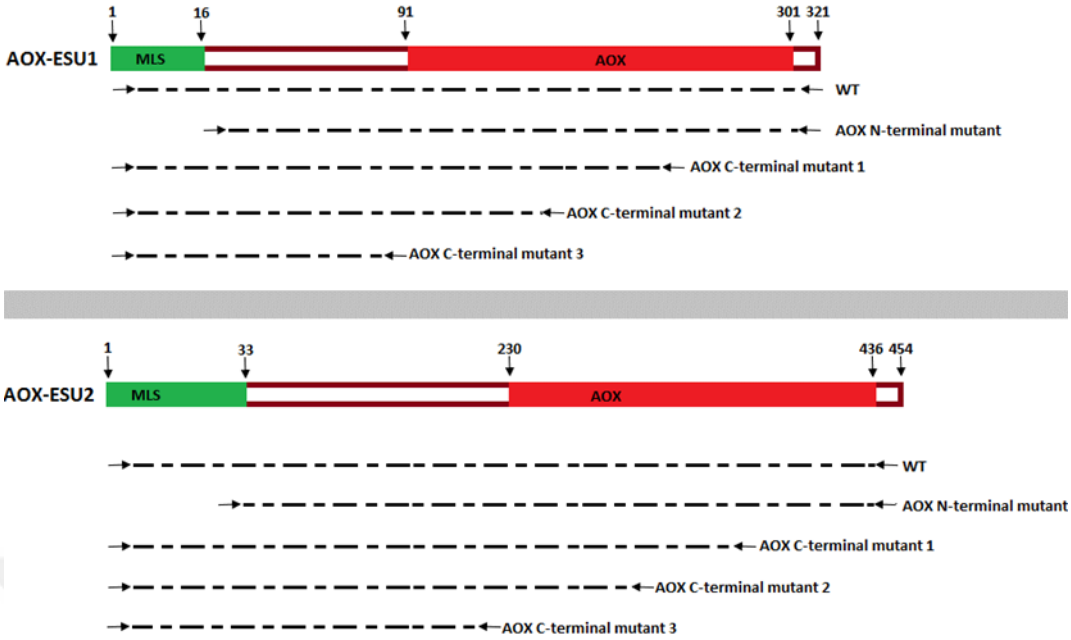

Figure 4.9. Domain properties of AOX proteins and planned mutation sites

MLS: Mitochondrial localization signal, AOX: active site of the Alternative oxidase. Vertical arrows show amino acid positions, horizontal arrows show sites that amplified by PCR by using designed primers.

5. CONCLUSIONS AND RECOMMENDATIONS

In this study it is aimed to identify mitochondrial alternative oxidase encoding genes in *C. cylindracea*. We used advanced next generation sequencing technology to analyze the mRNA sequence information from *C. cylindracea* samples. Comparative genomics approaches revealed two diverse genes showing high similarity to those identified from other plant and algae species. Further characterization of those *AOX* genes will help researchers to understand molecular basis of invasion power for *C. cylindracea.* Since two genes were cloned into yeast expression vector (pAG413), functional characterization of those genes will be carried out in *Saccharomyces cerevisiae* cells without the need of fresh algae samples.

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