Effects of *Ascophyllum nodosum* Extract on Giant Reed (*Arundo donax* L.) Gene Expression and Cold Stress Tolerance

by

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A Thesis Submitted to

Saint Mary's University, Halifax, Nova Scotia

In Partial Fulfillment of the Requirements for

the Degree of Master of Science in Applied Science

August, 2016, Halifax, Nova Scotia

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Table of Contents

Abstract

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Abstract: A new promising energy crop, giant reed (*Arundo donax*. L), has the potential for high biomass production with low agricultural input. However, two field experiments completed in Nova Scotia showed that giant reed cannot survive the winter without protection. The present study focused on applying a biostimulant, *Ascophyllum nodosum* extract (ANE), to enhance cold resistance of giant reed. The gene expression of giant reed was analyzed by transcriptome analysis; the chlorophyll content and foliar electrolyte leakage were measured. The results showed that application of ANE up-regulated many genes including those related to light-harvesting and cellular stress. Low concentration of ANE (1 ml/L) increased chlorophyll content and decreased the leaf electrolyte leakage after freezing. Thus, the preliminary data of the present study suggests that the ANE treatment has significant effects on giant reed gene expression, and may increase its tolerance to cold stress.

August 29, 2016

Acknowledgements

It would not have been possible to write this thesis without the help and support of all the kind people around me. First of all, I would like to thank my supervisor Dr. Zhongmin Dong. The door to his office was always open whenever I ran into trouble or had a question about my research or writing. He consistently allowed this paper to be my own work, but steered me in the right direction whenever he thought I needed it. I would also like to thank my second supervisor Dr. J. Kevin Vessey. Without his support and good advice, I would not finish my graduate studies and thesis successfully, for which I extremely grateful.

Besides my advisors, I would like to thank the rest of my thesis committee, Dr. Pierre Jutras, not only for his insightful comments and encouragement, but also for the hard questions which gave me incentive to widen my research from various perspectives.

My sincere thanks also goes to the-financial, academic and technical support of Saint Mary's University and its staff: Dr. Houman Fei, Carmen Cranley, Matt Logan and Jing Yang, who gave me access to the laboratory and research facilities. Without their precious support, it would not have been possible to conduct this research.

I am most grateful to Chao Wang and Emily Peters my classmates at Saint Mary's University, for the stimulating discussions, for help when I was doing my lab work, and for all the fun we have had in the last two years.

Last but not least, I would like to thank my family: my parents and my brothers and sisters for supporting me spiritually throughout writing this thesis and in my life in general.

Abbreviations

Abbrev.	Full Forms
A. donax L.	*Arundo donax* L.
ANE	*Ascophyllum nodosum* extracts
A. nodosum	*Ascophyllum nodosum*
BRs	Brassinosteroids
DEGs	Differentially expressed genes
DMSO	Dimethyl sulfoxide
OD	Optical density
mL	Milliliter
N	Nitrogen
P	Phosphorus
S	Sulfur
Fe	Iron
Cu	Copper
Mn	Manganese
SOD	Superoxide dismutase
APX	Ascorbate Peroxidase
TLRs	Toll-like receptor

List of Table and Figures

General Introduction

Over the past several centuries, the discovery and use of fossil fuels have produced enormous social benefits as well as environmental pollution. Human activities are mainly responsible for increasing greenhouse gasses in the atmosphere. The majority of greenhouse gas emissions come from burning fossil fuels supporting heating, electricity and transportation purposes (Metz, 2007). Now, some developed countries are reducing their dependency on fossil fuels and tending to invest heavily in alternate fuels to protect ecological systems for sustainable development (Cottrell, 2015). Therefore, options for a long-term environmentally friendly renewable energy sources have to be developed, such as hydro, solar, wind, biomass, geothermal and so on (Omer, 2007). Biomass has the larger potential and can be considered as one of the best option for meeting the demand and insurance of future energy/fuel supply in a sustainable manner (Koçar, 2013). Agricultural biomass is a relatively broad category of biomass that includes the food based portion (oil and simple carbohydrates) and the non-food based portion (complex carbohydrates) of crops. The food-based portion of agricultural biomass includes crops, such as corn, sugarcane, and beets while the non-food portion includes leaves, stalks, corncobs, corn stover, orchard trimmings, rice husk, straw, etc. (Chandra, 2012). Perennial grasses (switchgrass, giant reed) and animal wastes are also non-food sources of agricultural biomass. Plant biomass has been known for decades to be one of the most promising renewable energy sources that can be used for the production of biofuels, which is an abundant resource having low CO_2 emissions and low cost (Zhao,

2009).

The feedstock for 99% biofuel production today is classified as “first generation,” which directly derives energy from starch, sugar or vegetable oil (Eijck, 2014). Concerns exist about the sourcing of “first generation” feedstock, including the impact it may have on biodiversity, land use, and competition with food crops (Naika, 2010). Therefore, it is recommended to have more efficient alternatives, “Second generation” or “next generation” (e.g. switchgrass, organic residues), produce biofuels from plant biomass referring largely to lignocellulosic materials, as this makes up the majority of the cheap and abundant non-food materials available from plants.

Arundo donax L., a new promising second generation biofuel crop grows well in a variety of environmental and soil conditions, producing high rates of biomass with little agricultural input (Papazoglou, 2007). However, there are still technical barriers to be solved for large-scale production, including how to improve its cold resistance in cold climates. The previous work on cultivating *A. donax* L. in Nappan, Nova Scotia indicated it could not survive the harsh Nova Scotian winter. Thus, this barrier needs to be overcome before the potential of *A. donax* L. can be realized in high latitude areas.

1.1 *Arundo donax* L.: A non-food biofuel crop

A. donax L. (giant reed) is a perennial and herbaceous plant originally cultivated in East Asia, that rapidly spread to the Mediterranean, North Africa, Southern Europe and North America in the 19th century (Perdue, 1958). *A. donax* L. is a sterile plant with asexual vegetative reproduction that allows it to spread quickly, and new plants can

generate every year directly from rhizomes, the underground stem of the plant (Ceotto, 2010). It has been traditionally used for musical instruments, but recently it became a promising second generation fuel crop by converting lignocellulosic biomass into ethanol through hydrolysis (Dhir, 2010).

1.1.1 As a feedstock to produce bioenergy and bio-based compounds

As a bioenergy crop, *A. donax* L. is important not only for its remarkable biomass productivity, but also for its low agronomic input. Its higher biomass production is good for efficient biological fermentation for biogas and bioethanol (Ragaglini, 2014). Besides the fermentation needs, the industrial use and bio-based compounds also play a key role (Tracy, 1998).

Biogas is gas from anaerobic digestion of various biomass types in digesters/bioreactors. It contains 50-75% methane (CH_4) and 25-50% other gasses such as hydrogen, carbon dioxide, nitrogen, ammonia, and hydrogen sulfide. *A. donax* is used for producing biogas through co-digesting with animal slurries (Corno, 2014). Available data indicate that *A. donax* has a higher anaerobic bio-gasification potential (ABP) than traditional energy crops such as corn, sorghum, rye and triticale (Schievano, 2015), due to its sterile productivity, there is no energy invested in seed production, resulting in increased energy within the plant biomass. For example, *A. donax* only produces about 75%, 94%, 77% and higher than 100% ABP of corn, rye, triticale and sorghum, respectively. However, its high biomass productivity per hectare determines that its bio-methane production is superior to those of other energy crops (Schievano, 2015).

Second generation bioethanol is obtained from lignocellulosic materials (Alvira,

2010). The principal advantages of second generation bioethanol are connected to the high biofuel yield per hectare, positive energetic balance and negative greenhouse gas emissions (Kheshgi, 2000). Thanks to the high production of biomass yield per hectare, *A. donax* L. can produce an extremely large amount of bioethanol. It was reported that compared with other bioenergy crops, *A. donax* L. produces around 11,000 L ha^{-1} that is 50% more than ethanol coming from sugar cane and sugar beet and 20% more than *Miscanthus* production (Zhang, 2011). Other promising energy crops like cassava produce 45% less ethanol in comparison with *A. donax* L.

Besides its high quality for biofuel, *A. donax* L. also has great potential for direct industrial use and bio-based compounds applications. In the past, *A. donax* L. was directly used in musical instruments, paper pulp and building materials (Papazoglou, 2005). New research shows more efficient techniques for the extraction and isolation of raw materials from *A. donax* L. for industrial usage (Seca, 2000). Some chemical compounds, such as Levulinic acid and indole compounds, can be extracted from *A. donax* L. and can be used as intermediates for pharmacological preparation, plastic production and insecticide formulation (Galletti, 2013).

1.1.2 The advantages and disadvantages of *Arundo donax* L.

Floral sterility is becoming an outstanding trait for a lignocellulosic plant: if the flowering state is delayed or prevented, it is a good period for effectively transforming stored energy into overall plant biomass (Ragauskas, 2006). *A. donax* L. is an excellent example of a plant exhibiting floral sterility. However, floral sterility has both advantages

and disadvantages for the plant itself. Because of the asexual vegetative reproduction, it may increase human labor input while cultivating into the field. This is still an obstacle for the large-scale of cultivation in farm's field.

The high environmental adaptability helps *A. donax* L. grow well in a variety soil conditions. To compare with other bioenergy crops, the biomass yield of *A. donax* L. is greater than any others per unit surface area. The first year aboveground dry matter yield is equal to or higher than 40 t ha^{-1}, sometimes reaching to 50 t ha^{-1} (Lewandowski, 2003). A study of three years aboveground dry matter yield of *A. donax* L. shows from 6.1 to 38.8 t ha^{-1}, higher than 2.5 to 26.9 t ha^{-1} of *Miscanthus x giganteus* (Greef et Deu.); and 24.7 t ha^{-1} of *Cynara cardunculus* L. var. *altilis* D.C. cv. (Mantineoa, 2009).

A. donax L. can grow in all types of soils, ranging from heavy clay to loose sands, soils with low fertility and high salinity (Iris Lewandowski, 2003). The only challenge for cultivating *A. donax* L. is cold temperature (Pompeiano, 2015). *A. donax* L. uses the C_3 pathway for carbon assimilation, has a high photosynthetic capacity associated with the absence of light saturation and has an optimum temperature in the range of 24 – 30°C (Rossa, 1998). Cold hardiness is a primary concern for giant reed adaptation ability because winter injury and late frosts may cause important yield decreases, limiting the growth and distribution of the species (Thomashow, 2001).

1.2 ANE: *Ascophyllum nodosum* extract

Ascophyllum nodosum is a brown alga commonly referred as seaweed (Ugarte, 2006). It is a dominant ocean species in North America and Europe, especially in coastal

area without ice scour (Rayorath, 2008). *A. nodosum* is the most researched seaweed species. The benefits of this seaweed as a source of organic matter and nutrients has led to its use as a soil conditioner for centuries (Temple, 1988). It has been reported in recent years that the liquefying extract from the brown seaweed (ANE) could enhance the nutrient uptake (N, P, K, and S) in soybeans under rainfed conditions (Rathorea, 2009). Liquid extracts obtained from *A. nodosum* (ANE) have been used for more than 60 years to promote plant vigor, root growth and development (Khan, 2009). The *A. nodosum* extract contains many macro- and micro- nutrients, and some bioactive components including amino acids and vitamins that can increase the seed germination, plant growth, yield, biomass production, elevated resistance to biotic and abiotic stress, and enhanced postharvest shelf-life of perishable products (Norrie, 2006).

1.2.1 The effects of ANE as a fertilizer on plant growth

In advancing the modern agricultural system, the goal is to increase production, reduce the negative environmental impact, and enhance the sustainability of the system. Marine bioactive substances extracted from marine algae are used in agricultural and horticultural crops, with many beneficial effects in the terms of enhancement of yield and quality have been reported (Pal, 2015). It is well documented that plants have a broad range of responses to ANE applications including soil treatment and foliar spray. The effects of ANE on plant growth can be divided into three aspects including increased plant shoot and root growth, promoted flowering and fruit set, and enhanced crop yield.

ANE, as a biostimulant, improves plant growth by promoting lateral root

formation and enhancing the volume of the root system. ANE application has enhanced biomass accumulation in tomato and cabbage seedlings by stimulating root growth (Crouch, 1992). Khana et al. showed that ANE-treated alfalfa root has an increased (69%) number of root nodulations per plant (Khana, 2013). To understand the mechanism of this action, early stages of root–rhizobia interactions were examined with positive results. Alfalfa (*Medicago sativa*) roots were treated with ANE and its organic compounds (organic sub-fractions methanol and chloroform) showed both the root hair growth of alfalfa and root nodules induced by *S. meliloti*, a synergistic soil bacterium with alfalfa, were improved (Khan, 2012). The results suggested that ANE and its components could promote the legume–rhizobia symbiotic relationship and plant signaling. ANE has been shown to benefit root and shoot growth in the model plant *Arabidopsis thaliana*, as root-tips of ANE treated samples grew dramatically in plants grown in medium. Additionally, the expression of *Arabidopsis thaliana* DR5: GUS, the DR5:GUS construct comprises a synthetic auxin-responsive promoter (DR5) fused to the GUS-encoding reporter gene (Yu, 2012), is a positive response to ANE components, indicating ANE may promote root growth by trigger auxin distribution in plants (Rayorath, 2007). The seaweed extracts may enhance general plant growth through improving efficiency of nutrient absorption by roots (Crouch, 1993).

Although plant roots and shoots share many developmental processes, they are entirely different plant organs and play different roles in plant growth. At opposite to the root, the major part of photosynthesis is exhibited by shoots (Fageria, 2013). Increasing chlorophyll content of tomato leaves may have been caused by betaines (components of

ANE) which is responsible for reducing chlorophyll degradation (Whapham, 1993). A soil application of ANE also improved plant growth and ultimately yield of onion through significantly greater shoot height and shoot number (Mandradia, 2012).

To examine the effect of ANE on fruit maturation, Fornes *et al*., used two early ripening mandarins (Clausellina and Marisol) and Navelina orange as fruit samples. Results showed ANE enhanced fruit maturation earlier than the control (Fornes, 1995). ANE can also trigger flowering and fruit set at the early stage of some crop plants (Arthur, 2003). For example, the treatments of ANE were applied in different stages of growing peppers to compare their effects on yield and quality. ANE treatments showed significant differences in fruit yield, the most significant change was in first bloom stage (Eris, 1995). The application of ANE can also enhance tomato fruit yield when sprayed at the vegetative stage, which resulted in 30% larger size fruits than those of the control treatment (Crouch, 1992). Seaweed extracts derived from *A. nodosum* showed increased fruit production and berry weight and its biomass production (including shoot dry matter and root dry matter) was increased 27% and 76% respectively (Spinelli, 2010).

Tomato seedlings treated with ANE triggered more flowers earlier than control plants (Crouch, 1993). The mechanism of ANE triggering plant flowering and fruit set at the early stage is unclear, but when ANE extracts were applied at an early growth stage in maize, the plant yield increased and the response was similar to that of auxin, an important plant hormone (Jeannin, 1991). The similar response to auxin of plant yield

could be evidence of triggering the plant hormone system. In many studies, when the flowering and fruit set is promoted, the total plant yield will have increased as well. For example, ANE-treated strawberry plants *cv. Camarosa* showed marked increases in fruit size and yield, but the pH of fruit juice showed no significant difference (Roussos, 2009).

The main purpose of ANE application in agriculture is increasing crop yield and nutrient contents. In a broccoli field experiments, the total phenolic, flavonoid and isothiocyanate contents were higher in ANE-treated plants than control plants, indicating that ANE cannot only enhance broccoli yield but can increase the amount of phytochemicals and overall nutritional quality of broccoli (Lola-Luz, 2013). The application of ANE on yield and nutritional content of olive trees as well as the quality of olive oil showed positive results: the K, Fe and Cu concentration increased in olive leaves treated with ANE, and the concentration of Mn in olive leaves decreased in the control (Chouliaras, 2009). Two successive winter seasons' field experiments have suggested that spraying ANE at a higher rate could significantly enhance photosynthetic pigments and P and K content of celeriac plant, in addition, the total sugar content and NO_3 content will also increase dramatically (Shehata, 2011).

1.2.2 The effects of ANE on plant resistance to abiotic and biotic stress.

1.2.2.1. Resistance to abiotic stress

Abiotic stresses including drought, extremely high and low temperatures, salinity and soil with reduced nutrients are main factors limiting the crop production: 96.5% of global rural land areas are affected by environmental stresses (Cramer, 2011). Application of ANE on many crops has been proven to alleviate abiotic stress (Arioli, 2015). Even though the mechanisms of ANE to help plants increase resistance to drought, salinity and extremely temperatures are unclear, more research helps to provide evidence.

A pot experiment was conducted to examine the effect of *A. nodosum* extract spraying on the *Amaranthus tricolor* plants under salt stress conditions. After irrigation with 1000 ppm salt water, the ANE-treated plants showed significantly increased length and diameter of the stem, root length, number of leaves and fresh and dry weight (Aziz, 2011). Salt stress may result in yield decreasing, and low-quality in many crop plants, such as eggplant. Hegazi *et al*., used ANE to alleviate the adverse results of salt stress. The highest concentration of ANE 5.0 cm^3 L^{-1} is most effective to resist the salinity. The results indicated the ANE treatment slightly increased the activity of SOD and APX (superoxidae dismutase and ascorbate peroxidase of tall fescue) that belong to antioxidant defense systems, but significantly decreased K content (Hegazi, 2015).

Generally, the application of ANE alleviates environmental stress and is attributed to the presence of sugar, micro- or macro-elements, but plant hormones play an important role as well (Rengasamy, 2010). To study the mechanisms of *A. nodosum* extracts enhancing the ability to resist abiotic stress, the whole transcriptome of *Arabidopsis* under

salt stress has been analyzed. A Pectin Methyl Esterase Inhibitor gene was found to resist the salt stress, and it may be used as a novel regulator of abiotic stress in plants (Wally, 2012).

Many young crops will face face drought-like conditions. The application of ANE on various young vegetables susceptible to drought extended the time before these vegetables began to succumb to their conditions. Additionally, ANE combined with fertilizer significantly improved the root and shoot development, root length and the volume of leaves (Neily, 2010). These results indicate the importance of ANE in commercial agricultural settings.

In addition, the application of ANE to alleviate drought stress may be caused by some bioactive molecules including antioxidants (Zhang, 1999). Plants endogenous antioxidants are responsive to the exogenous hormone-containing ANE application under water stress. Both the biochemical and the molecular of plants will change after ANE treatment. The glutathione reductase activity, superoxidae dismutase and ascorbate peroxidase of tall fescue under water stress, are increased after being treated with ANE treated. The application of ANE in this study may play a major role in plants' endogenous antioxidant enzymes activity under water stress and could increase the plants protection system against active oxygen species (Ayad, 1998). It is reported that the plants treated with ANE showed improved drought resistance through hormonal up regulation of the plant's defense system against the oxidative stress. The application of ANE plus humic

acid to creeping bentgrass cultivars subjected to drought improved the root mass and foliar α-tocopherol and zeatin riboside content (Zhang, 2003). Which was the first reported that ANE contain cytokinins and could increase the plant exogenous cytokinins to enhance the drought resistance

Temperature extremes, cold and heat, are the primary factors to affect in the rate of plant development and limiting crop productivity (Hatfield, 2015). Foliar application of ANE can reduce the visible injury after 72-96 hours of extreme heat conditions, while the treatment of ANE plus humic acid may decrease the heat injury and improve the post-transplant rooting of sod (Zhang, 2003). The foliar application of 10 mu M ANEC (ANE based cytokinins) on turf grass under extreme heat conditions may increase leaf trans-zeatin riboside content and nitratase activity (Zhang, 2009).

ANE and its lipophilic components (LPC) have increased freezing tolerance in *in vitro* and *in vivo* assays. These components may generate stress response genes to protect the plant from freezing stress, in addition to enhancing membrane integrity (Rayirath, 2009). Gene expression revealed the lipophilic component of ANE to improve freezing tolerance in *Arabidopsis thaliana*. The LPC improving plant freezing tolerance may increase the concentration of total soluble sugar and mediate the increase of osmotic protectants priming of the plant (Nair, 2012).

1.2.2.2.Resistance to disease and pest infection

Among biotic stresses, seaweed extracts may enhance plant defense against pest

and diseases directly and indirectly through the induction of some plant defense genes or enzymes. Jayaraman (2011) showed that the commercial extract of *A. nodosum* (StimplexTM) could reduce fungal diseases of greenhouse cucumbers by increasing the activity of the disease resistance-related enzymes (chitinase, β-1,3-glucanase, peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and lipoxygenase) and the expression of the related genes (chitinase, lipoxygenase, glucanase, peroxidase and phenylalanine ammonia lyase). At the same time, an accumulation of the phenolic compounds was observed after the treatment with ANE (Jayaraman, 2011). Root treatment of *Arabidopsis thaliana* with three treatments including ANE, chloroform and ethyl acetate fractions, to alleviate the symptoms on leaves. The ANE treatment induced the expression of jasmonic acid related gene transcripts to respond to the Pst DC3000 infection (Subramanian, 2011). Compared with other fungicides or fungal agents, ANE treatment of strawberry can reduce the total incidence of fruit rot and anthracnose, while increasing the fruit yield (Washington, 1999).

ANE also show the ability to protect plants against bacterium diseases. Application of the 1% ANE on cabbage seedlings in advance would produce an amount of microbes that can protect against *P. ultimum* (Dixon, 2002). More, the 0.2% ANE treated on greenhouse grown carrots shows greatly reduced disease severity compared with control (water). And the ANE-treated plants also induced a higher level of defense genes or proteins as well as increased some defense pathway marker genes (Jayaraj, 2008). Foliar application of 0.5% ANE would reduce the incidence of foliar pathogens

including *Alternaria solani* and *Xanthomonas campestris pv vesicatoria* up to 63% and 44% respectively (Ali, 2016).

ANE application also promotes plant health by positively affecting the rhizosphere microbial community. *Arabidopsis thaliana* treated with the alkaline extract of *A. nodosum* could reduce the production of females of the root-knot nematode (Wu, 1998). Similarity, ANE-treated tomato samples also had decreased the root knot nematodes (Crouch, 1993a). However, seaweed extracts are not dependent on rhizospheric microorganisms: the application of ANE may induce a plant hormonal response to nematode infection.

Generally, disease control is attained through the use of fungicides, insecticides and bactericides, which reduce the diseases but can also be toxic to plants. Elicitors activate the chemicals in the plant to trigger the plant defense activities. Common active elicitors include salicylic acid, methyl salicylate, benzothiadiazole, benzoic acid, chitosan, when affect the production of phenolic compounds and activation of various defense-related enzymes in plants (Sohal, 2013).

Molecule such as oligo- and polysaccharides and lipids in ANE show response to plant diseases. For example, an oligosaccharide extract from brown algae was prepared and applied to show response against tobacco mosaic virus (Vera, 2011). After a few hours of being treated, the oligo-treated tobacco plant showed salicylic acid (SA) and the phytoalexin scopoletin expressed several pathogenesis-related (PR) proteins (Klarzynski, 2003). The effect of two elicitors laminarin and carrageenan of algal polysaccharides on tobacco were indicated but only the carrageenans efficiently induced signaling and

defense gene expression in tobacco leaves, which encoden the genes of chitinase and proteinase, the pathways of ethylene, jasmonic acid and salicylic acid (Mercier, 2001).

1.2.3 Bioactive Compounds of ANE

Some components extracted from ANE, such as macro- and micro-element nutrients, polysaccharides, amino acids, vitamins, cytokinins, auxins, and abscisic acid (ABA)-like growth substances affect cellular metabolism in treated plants, which may stimulate growth and crop yield (Durand, 2003). Tasco-Forage, an extract from ANE, showed the ability to improve the antioxidant enzymes activities both in plants and animals. The components of ANE proven to increase free radical production (Allen, 2001). Although many of the chemical components of seaweed extracts and their modes of action remain unknown. It is assumed that some components exhibit synergistic activity (Fornes, 2002). ANE provided all the essential mineral and their dry weight in seaweed is 20-50% (Kazutoshi, 2002). Except for some elements of seaweed, such as potassium, sodium, calcium, magnesium, zinc, seaweed, especially the brown seaweed is abundant with cell wall polysaccharides, vitamins and proteins (Mabeau, 1993).

1.2.3.1.Growth hormones

According to studies of plant hormones derived from seaweed, there are four common plant growth hormones: Auxins, Abscisic acid, Cytokinins and Gibberellins. Only Auxins, Abscisic acid and Cytokinins often appear in *Ascophyllum nodosum*.

The first reported of auxins in marine algae gave a new picture of plant hormones

in algae (Van Overbeek, 1940). In the following years, auxins are found in brown, red, green algae (Buggeln, 1971). However, the content of indole-3-acetic acid (IAA) extracted from *A. nodosum* is very slight (Sanderson, 1987). Auxin may not be a significant compound in ANE treated plants to improve plant growth. As I mentioned above, when extracts were applied at an early growth stage of maize, the crop yield increased and this response was similar to that of auxin. The root-tips of ANE-treated samples dramatically increased and the *Arabidopsis thaliana* DR5: GUS expression is positive response to components of ANE, which may indicate that ANE promotes root growth by inducing auxins in plants.

Abscisic acid (ABA) was isolated from commercial brown algae *Ascophyllum nodosum*, at a concentration of 0.10–0.46 μg ABA per gram dry weight in ANE, more similar to the concentrations in higher plants (Boyer, 1987). ABA is common in algae, which can induce stomatal closure in drought, protein storage in seeds, gene transcription for proteinase inhibitors and inhibits shoot growth (Tanaka, 2006). The function of ABA in seaweed should be investigated more, as the role of ABA in terrestrial plants was indicated to encode a number of late embryogenesis abundant proteins, responsible for drought stress (Finkelstein, 2013).

Cytokinins were detected both in fresh seaweed and seaweed extracts (Brain, 1973). The formulation of cytokinins in seaweed commonly known as trans-zeatin and trans-zeatin riboside (Stirk, 1997). Cytokinins are a class of phytohormones that are responsible for the formation of roots and shoots, and plat a central role in the cell cycle (Werner, 2001). Thus, Cytokinins associated with ANE improved the potato yield and

protein content as well as increased the ripening of lime fruits (Kumar, 2014). Cytokinis also increased creeping bentgrass tolerance to heat (Chang, 2016). Through a soybean callus assay and a mung bean rooting bioassay, cytokinin-like and auxin-like activities from ANE extracts were compared. The results indicated that all the treatments were increasing the root development, but only the cytokinin-like activity enhanced the most of the callus growth (Staden, 1997).

1.2.3.2.Betaines

Blunden suggested that seaweed extract should have another component that functions similarly to cytokinins. Glycine betaine activity was found to be similar to cytokinins in many plants (Blunden G. C., 1986). ANE was applied through two methods: soil drench and foliar spray. All treated plants but one resulted in higher concentrations of cholorphyll in leaves. Very similar contents (γ-aminobutyric acid betaine 0.96 mg L−1, δ-aminovaleric acid betaine 0.43 mg L−1, glycinebetaine 0.34 mg L−1) were found in ANE and betaine treatments (Blunden G. J.-W., 1996). Moreover, betaines and their analogs can improve plant tolerance to some abiotic and biotic stress.

Glycnie betaine is an osmoprotectant in plants and animals, which many protect the plant photosynthetic apparatus to tolerate the abiotic stress (Holmström, 2000). Soil application of alkaline ANE on roots of tomato reduced the second stage of juveniles of *Meloidogyne javanica* and *M. iruognita* invading the roots (Wu, 1997). Improved methods were used to analyze common betaine and its analogs. The most common betaines including glycine betaine, γ-aminobutyric acid betaine, δ-aminovaleric acid betaine and laminine were found from *A. nodosum* and *Fucus* and *Laminaria* species.

1.2.3.3. Polysaccharides and derived oligosaccharides

Plant oligosaccharides can activate or inhibit plant growth, and the development is now clearly established (John, 1997). There are many unusual and complex polysaccharides isolated from seaweed that are not present in land plants, including the three main polysaccharides in brown seaweeds: fucoidan, alginate and laminaran that can trigger defense responses in plants, enhancing protection against pathogens (Lane, 2006).

Alginate as one of the main components in the cell walls of brown seaweeds where they can account for 17 to 45% of the seaweed's dry weight (Mabeau, 1987). Hien found the sodium alginate could promote the net rate of the photosynthesis and the ability of carbon assimilation in rice (Hien, 2000). Aftab reported that irradiated sodium alginate (ISA) sprayed on the *Artemisia annua* leaves at the concentration of 80 mg/L can increase growth, capacity of the photosynthesis and the artemisinin content in the leaves (Aftab, 2011). The alginate-derived oligosaccharides reduced the electrolyte leakage and the concentration of the malonaldehyde (MDA) in drought-treated tomato seedlings, and enhanced the content of the free proline and the total soluble sugar (Liu, 2013). Moreover, oligo-alginate has a dual beneficial effect on tobacco plants to enhance growth and defense against pathogens, suggesting that signaling pathways involved in plant growth and defense may be interconnected (Laporte, 2007).

Laminaran is a (1,3)-b -D glucan with b -(1,6) branching and has been proven to stimulate natural defense responses in plants and is involved in the induction of genes encoding different pathogenesis–related (PR) proteins with antimicrobial properties

(Zvyagintseva, 1999)

Although the effect of polysaccharides and their derived oligosaccharides on plant growth and the defense system is well studied, the potential plasma membrane receptors and the associated signaling pathways have not yet been identified. In *Arabidopsis thaliana* and rice genomes, some of plasma membrane receptors with protein kinase activity could be encoded with plant cell genomes and some are structurally related to animal TLRs. Thus, it is possible that some oligosaccharides may interact with a specific receptor structurally related to TLR4 and/or TLR2 or with another yet unidentified receptor with protein kinase activity (Shiu, 2004). In addition, it has been demonstrated that brassinosteroids, a kind of polyhydroxylated plant steroidal hormone, with suggested-involvement in stem elongation, leaf development, pollen tube growth, and photomorphogenesis (Clouse, 2002). Brassinosteroids bind to a specific receptor located in the plasma membrane (Nam, 2002).

Table 1. Major components of *Ascophyllum nodosum* (Craigie, 2011).

Compound	Content (% dry wt)	Content (% wt)
N-free carbohydrates		46-60
Alginic acid	15-30	
Laminaran	0-10	
Fucoidan	4-10	
Other carbohydrates	10	
Protein	5-10	5-10
Fats	2-7	
Tannins	2-10	
Organic	65.7	
Moisture		12-15
Crude ash	21.1	17-20
Klason lignin	18.6	
Total dietary	50.3	
Sugars	Fuc=6.60; Xyl=1.68; Gal=0.69; Glc=4.48; Man=3.84; Uronic acid=14.44	

1.3 Brassinosteroids (BR): growth hormones

Plant growth and development are governed by complex interactions between environmental signals and internal factors. The full range of growth responses induced by seaweed extracts implies the presence of more than one group of plant growth-promoting hormones (Mazorra, 2002). Brassinosteroids (BRs), are a group of plant steroids that are known to elicit remarkable growth responses and are found in almost every parts of plants, such as pollen, flower buds, fruits, seeds, vascular cambium, leaves, shoots and roots (Bajguza, 2003). Many studies of BRs show that they play an important role in a range of developmental processes, e.g. stem and root growth, floral initiation, and the development of flowers and fruits (Fariduddin, 2009)

The exogenous application of BRs modified antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, ascorbate peroxidase and non-enzymatic antioxidants to increase tolerance with low and/or high temperature stress, drought stress and moisture stress (Sairam, 1994; Li, 1998). When maize (*Zea mays*) seedlings treated with BR were subjected to water stress, the activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), as well as ascorbic acid and carotenoids contents increased (Vardhini, 2015). BRs enhanced the activity of CAT and reduced the activities of peroxidase and ascorbic acid oxidase under osmotic stress conditions in sorghum (*Sorghum vulgare*) (Vardhini, 2003).

It is still unclear whether BRs directly or indirectly modulate the responses of plants to oxidative stress. At present, some studies suggested BRs have a cross-talk with other phytohormones, to alternate the expression of some hormone biosynthetic genes

and/or signals, which influence the stress responses of plants (Bishop, 2002). In addition, recent studies have unveiled some interactions of plant immune system and growth regulating hormones like auxins, gibberellins, and ethylene (Bruyne, 2014). However, there is little evidence of BRs cross link with the immune system, but a point that there is a link of growth-promoting hormone responses to MAMP (microbe-associated molecular patterns) signaling is the LRR-RK BRI1-Associated Kinase 1 (BAK1), a leucine-rich repeat receptor-like kinase (Bajguz, 2009). BAK1, the most potent steroid hormone in plants, binds brassinolide (BL), identified as an inter-actor of the LRR-RK Brassonpsteroid-Insensitive 1 (BRI1). It is also suggested that BR biosynthesis and signaling can be rate-limiting modulators of BAK1-mediated MAMP responses (Belkhadir, 2012).

1.4 Mechanism of cold-induced oxidative stress injury

There is an almost a 50% decrease in productivity of major crops due to abiotic stresses (Cramer, 2011). Considering the abiotic stresses in Canada, cold temperature is the most prominent issue, resulting in millions of dollars decreased in revenue. Many commercial crops such as maize, cotton, soybean, tomato and some fruits (bananas, mangoes, grapes, oranges) are all sensitive to low temperature (Sharma, 2005).

Chilling and freezing interfere with both photosynthetic and non-photosynthetic processes in the cell. The low-temperature stress will induce chilling and freezing injuries in plants. Chilling injury occurred in susceptible species, such as tropical plants, which are sensitive to temperatures above the freezing point but below a critical threshold

temperature. The chilling-injured tissues will induce cellular dysfunctions including pitting, water soaking and reduce the crop yields and quality of harvest plants.

Freezing injury occurs as the temperature drops low enough for ice crystals to form within the tissue. The freezing-injured plants will have a drastic effect on the whole plant resulting in plant death. Acclimation is a developmental process in response to environmental conditions which leads to increased tolerance to low temperature stress (Shewfelt, 1992).

Actions have indicated that reactive oxygen species (ROS) can mediate at least one of the effects of low-temperature stress in plant system (Bowler, 1992). It is suggested the electron transport chains of mitochondria and chloroplasts like well-documented sources of ROS in plant cell (Osswald, 1994). So if under long-time oxidative stress conditions, ROS can cause lipid peroxidation, DNA damage and protein denaturation (Scandalios, 1993). To keep ROS at low steady-state levels, plant cells contain some antioxidant metabolites and enzymes (Asada, 1992). It is reported that overexpression of antioxidant enzymes, such as SOD (Gupta, 1993) can protect the cell against ROS. It was also suggested H_2O_2 can play a role in regulating chilling tolerance in maize (Prasad, 1994) and plant defense against pathogen infection (Chen, 1993).

The plant cell wall with a complex mechanism of cell expansion determines the cell size and shape. The plant cell can be divided into two types: type one is a structure of cellulose microfibrils mostly consisting of xyloglucans and amount of polysaccharides; type two mainly contains glucuronoarabinoxylans and β-glucan interact with cellulose micofibrils (Carpita, 2008). Those cell-wall related proteins, such as xyloglucan endo-β

transglucosylases/hydrolases (XET/XTHs; GH16), endo-1,4-β-D-glucanase (EGase, GH9), and expansins may stimulate cell wall expansion (Sénéchal, 2014). Cold stress will change the cell wall polysaccharide composition and cell wall-associated kinases (WAKs) that could bind either oligogalacturonides or pectin in cell walls (Zhang, 2012).

It is reported that some gene/protein, such as xyloglucan endo-β-transglucosylases/hydrolases (XET/XTH), pectin methylesterase (PME), hydroxycinnamyl alcohol dehydrogenase (CAD), cell wall peroxidases (PRX), arabinogalactan protein (AGP) and wall-associated kinase (WAK) in cell wall may response to cold (Gall, 2015).

1.5 Transcriptome

With the rapid development of massively parallel sequencing (next-generation sequencing) (Margulies, 2005) and the maturation of analytical tools during the last few years, the sequencing method situation has changed dramatically. Whole-genome or whole transcriptome analyses has become a realistic option for genetic non-model organisms, even for individual laboratories (Ellegren, 2012). The characterization of a species' transcriptome is descriptive and generates little biological insight, it is often an important first step and constitutes a valuable resource for further analyses. RNA-seq, also called whole-transcriptome shotgun sequencing, refers to the use of high-throughput sequencing technologies for characterizing the RNA content and composition of a given sample which is randomly decomposed into short reads of several hundred base pairs. To align the hundred pairs, there are two situations that need be solved. One is that the

transcripts or genomes' information is available, which can directly align to the existing reference genome (Wang, 2009); another is the sample without a reference transcripts or genome information, in the absence of transcripts of the sample that needs to be reconstructed from these reads (or read pairs), which is referred to *as de novo* assembly (Haas, 2013).

1.6 Electrolyte leakage

As cell membranes are the first target to respond to abiotic stresses, and the degree of the cell membrane injury stimulated by cold stress is often measured by electrolyte leakage from the cell (Bajji, 2001). Electrolyte leakage is an index of stress response in plant cells, it is used to instantaneously detect the injury factor triggered by stresses, including extreme temperatures, drought, pathogen attack and salinity (Lee, 2010). The mechanism of electrolyte leakage may be induced by the oxidative degradation of the lipid bilayer or the activation of K+-permeable channels, which K+ may play an important role in anabolic reaction to trigger catabolic processes and save energy for adaptation and repair needs (Demidchik, 2014).

1.7 Chlorophyll content

Chlorophyll, including chlorophyll a and b, which is an import pigment involved in plant photosystems that can help to covert light to energy. Photosynthetic efficiency is determined by the amount of solar radiation absorbed by the plant. Thus, the chlorophyll content indicates the nutritional status and direct response to environmental stresses

(Gitelson, 2003).

Objectives

1. To explore the regulation of plant cold tolerance and plant growth promoting genes expressions effect of ANE by gene expression changes.

2. To compare the effect of ANE and BR on plant gene expressions by transcriptome analysis.

3. To evaluate the effect ANE on *A. donax* L. cold stress tolerance by measuring electrolyte leakage, chlorophyll content after 24 hours' freezing treatment.

4. To assess the effect of ANE and chitosan on *A. donax* survival rate over the winter.

Material & Methods

In vitro propagated plants of *Arundo donax* L. (provided by Nile Fiber Atlantic Canada Inc.) were chosen for this study, based on their phenotype and their lignocellulosic content, which determines their worth as biomass for ethanol production. Plants were propagated through tissue culture propagation for ten weeks in a growth chamber (Fig 2a). Plants were placed into 36-hole trays filled with a peat-based mix and grown in the greenhouse for 4 weeks at 22°C with a 12 h photoperiod (Fig 2b). Plants were watered daily to prevent wilt and fertilized weekly with a Hoagland's solution (S1). Two independent experiments were conducted to characterize the plants' response to cold stress.

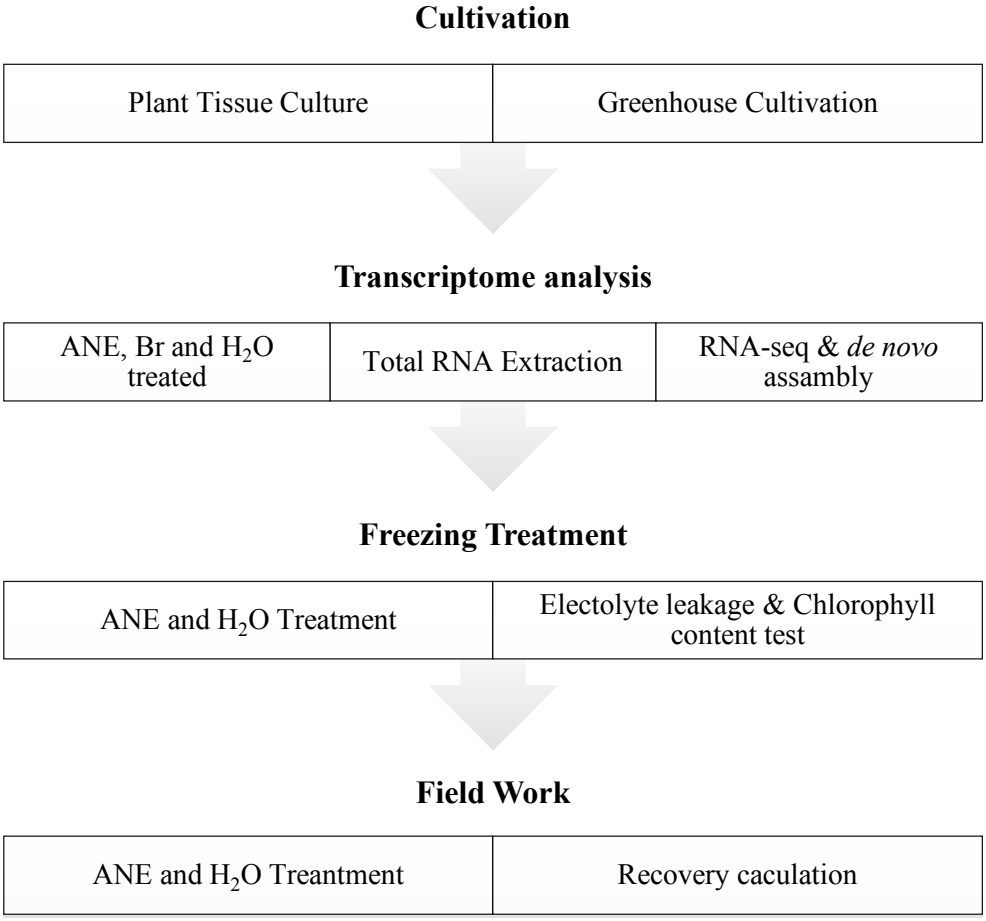


Figure 1. The workflow of present study.

a

b

Figure 2. The plant tissues were transferred into new medium for root growth (a); The

ten-weeks plants were transferred into greenhouse (b).

2.1. Transcriptome analysis

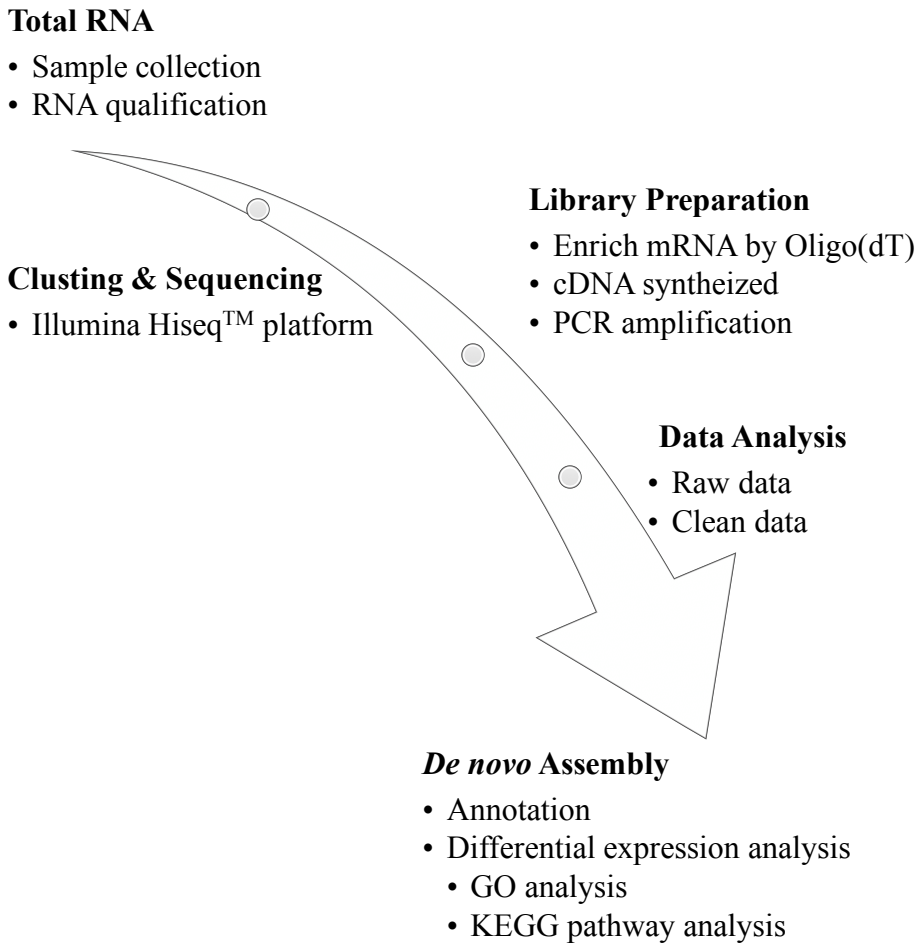


Figure 3. The workflow of *A. donax* L. transcriptase analysis in present study.

2.1.1. Sample collection and preparation

2.1.1.1.RNA quantification and qualification

The quantification and qualification of RNA extracted from *A. donax* L. is necessary for further analysis. First, the RNA degradation and contamination was monitored on 1% agarose gels. Then, the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA) was used to check the RNA purity. RNA concentration and integrity was measured using Qubit® RNA Assay Kit in Qubit® 2.0 Flurimeter (Life Technologies, CA, USA) and the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

2.1.1.2.Library preparation for Transcriptome sequencing

After the quality control (QC) procedure, 1.5 μg RNA per sample was prepared for sample preparations. Following the manufacturers' recommendations, sequencing libraries were generated using NEBNext® UltraTM RNA Library Prep Kit and index codes were added to attribute sequences to each sample. Briefly, the Poly-T oligo-attached magnetic beads were used to purify mRNA from total RNA.

To carried out fragmentation, the divalent cations were used under increased temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). The first strand of cDNA was synthesized by applying a random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-) while the second strand of cDNA was synthesized using Polymerase I and RNase H. The exonuclease/polymerase activities help remaining

overhangs to convert into blunt ends. For hybridization, the NEBNext Adaptor with hairpin loop structure was ligated after adenylation of 3' ends of DNA fragments. To select 150~200 bp cDNA fragments preferentially, using the AMPure XP system (Beckman Coulter, Beverly, USA) to purify the library fragments. Before PCR, the size-selected and adaptor-ligated cDNA were mixed with 3 μl USER Enzyme (NEB, USA) at 37°C for 15 minutes followed by 5 minutes at 95 °C. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. Finally, the Agilent Bioanalyzer 2100 system applied to assess the PCR products and library quality. The workflow chart is as follows (Fig 4).

2.1.1.3.Clustering and sequencing

TruSeq PE Cluster Kit v3-cBot-HS (Illumia) was used for clustering the index-coded samples on the Cluster Generation System. After successfully clustering samples, they were sequenced to generate paired-end reads and the library preparations were available on an Illumina Hiseq platform.

2.1.2. Data analysis

2.1.2.1. Quality control

The original raw data (raw reads) from Illumina HiSeqTMare transformed to Sequenced Reads by base calling. Raw data are recorded in a FASTQ file, which contains sequence information and corresponding sequencing quality information. Raw data of FASTQ format were processed through in-house Perl scripts. The containing adapter,

ploy-N contained reads and low-quality reads were removed from raw data to generate clean data (clean reads). At the same time, the quality standards were calculated to evaluate the clean data, such as Q20, Q30, GC-content and sequence duplication level. The clean, high quality data with high quality is key for the downstream analyses.

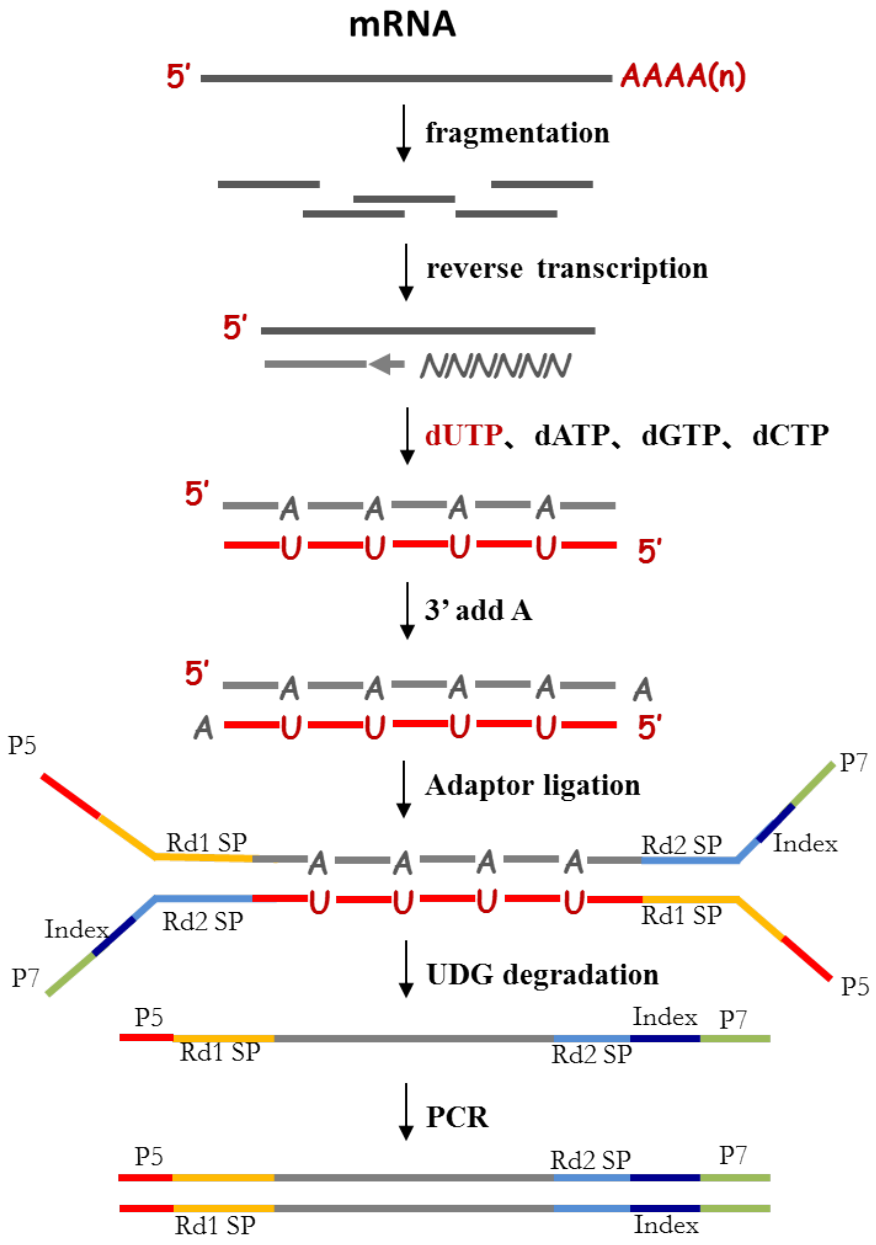


Figure 4. The library preparation and construction workflow of RNA-seq.

2.1.2.2.*De novo* Transcriptome assembly

Differing from the "Mapping first" approaches, such as Scripture or Cufflinks, a reference (genome) is needed first for mapping. If the genome is available, this is the most sensitive way for transcriptome assembly. "Assembly first" (*de novo*) approaches are more suitable for samples without a reference genome (Grabherr, 2011). Trinity, a new and robust *de novo* reconstruction is used for *A. donax* L. transcriptomes. It is built in the three super software modules: Inchworm, Chrysalis and Butterfly (Fig 5). In the first step, Inchworm decomposes all the reads into overlapping k-mers (in practice, k = 25), selects the most frequent seeding k-mer in the dictionary and extends the seed in each direction to form a contig assembly (Fig 5a). Second, Chrysalis clusters minimally overlapping Inchworm contigs into sets of connected components, and constructs complete de Bruijn graphs for each component. A collection of Inchworm contigs based on each component that are likely to be derived from alternative splice forms or closely related paralogs (Fig 5b). Finally, Chrysalis with the original reads and paired ends generates the individual de Bruijn graphs, which should be reconciled during the Butterfly component, where full-length, linear transcripts are reconstructed. It reconstructs distinct transcripts for splice isoforms and paralogous genes, and resolves ambiguities stemming from errors or from sequences >k bases long that are shared between transcripts (Fig 5c)

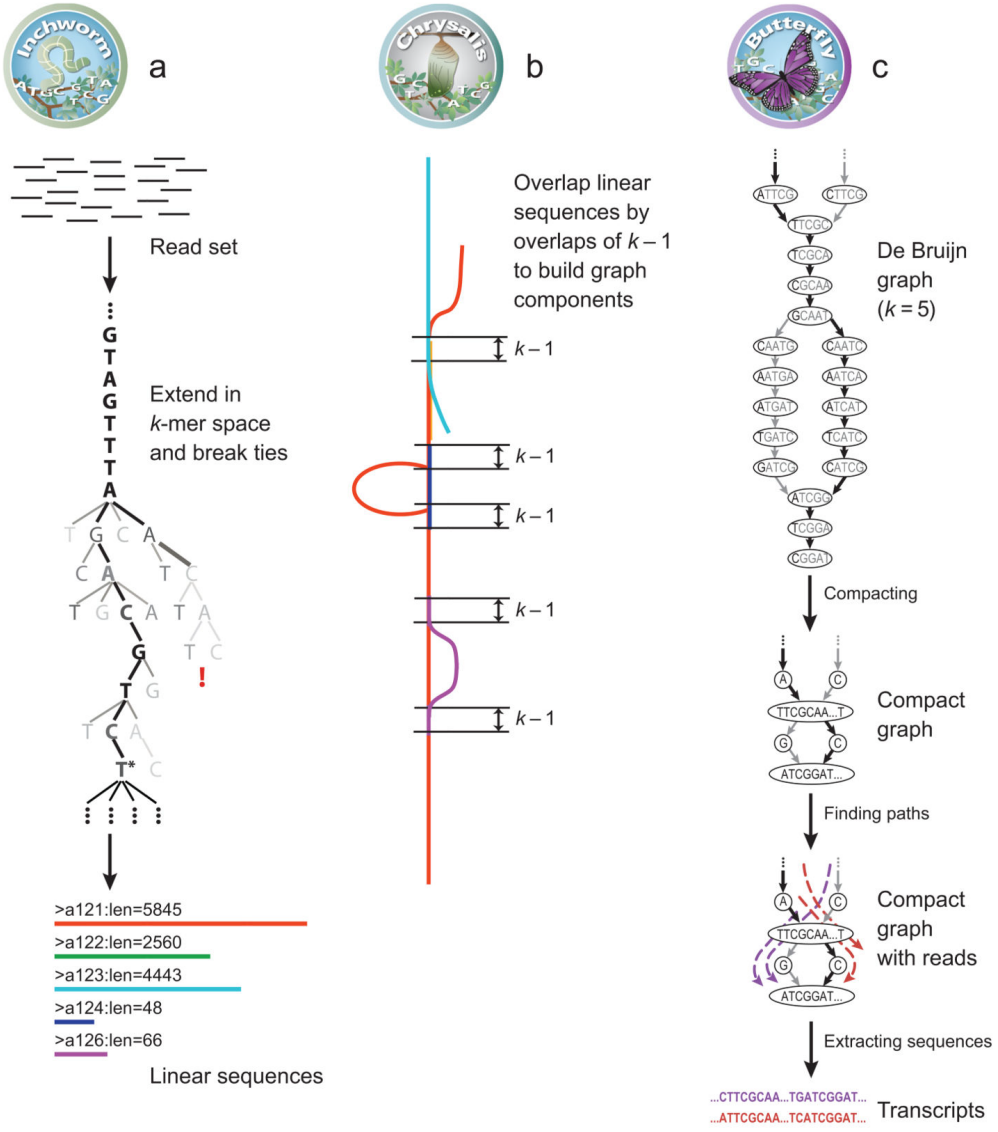

Inchworm
a
Read set
GTAGTTTA
Extend in
k-mer space
and break ties
!
>a121:len=5845
>a122:len=2560
>a123:len=4443
>a124:len=48
>a126:len=66
Linear sequences
Chrysalis
b
Overlap linear
sequences by
overlaps of k – 1
to build graph
components
k – 1
k – 1
k – 1
k – 1
k – 1
Butterfly
c
De Bruijn
graph
(k = 5)
Compacting
Compact
graph
Finding paths
Compact
graph
with reads
Extracting sequences
...CTTCGCAA...TGATCGGAT...
...ATTCGCAA...TCATCGGAT...
Transcripts

Figure 5. Overview workflow of Trinity modular method. (a) Inchworm assembles the read data set (short black line, top) by greedily searching for paths in a k-mer graph (middle), resulting in a collection of linear contigs (color lines, bottom), with each k-mer present only once in the contigs. (b) Chrysalis pools contigs if they share at least one k-1-mer and reads span the join, and builds individual de Bruijn graphs from each pool (colored lines). (c) Butterfly takes each de Bruijn graph from Chrysalis (top), and trims spurious edges and compacts linear paths (middle). It then reconciles the graph with reads (dashed colored arrows, bottom) and pairs (not shown), and outputs one linear sequence for each splice form and/or paralogous transcript reflected in the graph (bottom, colored sequences). (Grabherr, 2011).

2.1.2.3.Gene Function annotation

Gene function was annotated based on the following databases: Nr (NCBI non-redundant protein sequences); Nt (NCBI non-redundant nucleotide sequences); Pfan (Protein Family); KOG/COG (Clusters of Orthologous Groups of proteins); Swiss-Prot (A manually annotated and reviewed protein sequence database); KO (KEGG Ortholog database); GO (Gene Ontology).

NR, NT, SwissProt, KOG were used NCBI blast 2.2.28+. For NR, NT and SwissProt databases, the e-value threshold is 1e-5 (Each unigene shows top 10 alignment results), and 1e-3 for KOG. The top 10 alignment results will be shown for each unigene; Pfam, the most comprehensive database of protein domains and families, consists of two components: Pfam-A and Pfam-B. Pfam-A represents high quality, manually curated families. To comprehensively understand the known proteins, the PRODOM database is often used as a supplement. These automatically generated entries are called Pfam-B (Finn, 2006). GO is the established standard for the functional annotation of gene products, the annotations are based on the protein results from the databases NR and Pfam: Blast2GO v2.5 (Götz, 2008), the e-value threshold is 1e-6. KEGG, a robust database, is used to understand high-level functions and utilities of the biological system, including the cell, the organism and the ecosystem. It contains KEGG PATHWAY, KEGG DRUG, KEGG DISEASE, KEGG MODULE, KEGG GENES, KEGG GENOME and KO system (KEGG ORTHOLOG) combines each KEGG annotation system. KEGG has established a complete KO annotation system which can accomplish the function annotation of the genome/transcriptome of a newly sequenced species, the e-value

threshold is 1e-10.

2.1.2.4.Quantification of gene expression levels

Gene expression levels were estimated by RSEM (Dewey, 2011) for each sample: Clean data were mapped back onto the assembled transcriptome, and read count for each gene was obtained from the mapping results.

2.1.2.5.Differential expression analysis

For the samples without biological replicates: prior to differential gene expression analysis, for each sequenced library, the read counts were adjusted by the edge R program package through one scaling normalized factor. Differential expression analysis of two samples was performed using the DEGseq (2010) R package. The P value was adjusted using q value (Storey, 2003). qvalue<0.005 & |log2(foldchange)|>1 was set as the threshold for significantly differential expression. For differentially expressed gene, if log2 Foldchange>0, we defined it is up-regulated and vice versa.

To analyze the differential gene expression genes (DEGs), volcano plots can be used to infer the overall distribution of differentially expressed genes: the threshold is normally set as [log2(Fold Change)] > 1 and q-value < 0.005; The Venn diagram presents the number of genes that are uniquely expressed differentially within each group, with the overlapping regions showing the number of genes that are expressed in two or more groups. Cluster Analysis is used to find genes with similar expression patterns under various experimental conditions. By clustering genes with similar expression patterns, it

may be possible to discern unknown functions of previously characterized genes or the function of unknown genes. In hierarchical clustering, areas of different colors denote different groups (clusters) of genes, and genes within each cluster may have similar functions or take part in the same biological process.

2.1.2.6.GO enrichment analysis

Gene Ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) was implemented by the GOseq R packages based on the Wallenius non-central hyper-geometric distribution (Young, 2010), which can adjust for gene length bias in DEGs.

2.1.2.7.KEGG pathway enrichment analysis

KEGG (Kanehisa, 2008) is a database resource for understanding high-level functions and utilities of the biological system, such as the cell, the organism and the ecosystem, using molecular-level information (especially large-scale molecular datasets) generated by genome sequencing and other high-throughput experimental technologies (http://www.genome.jp/kegg/). KOBAS (Mao, 2005) software was used to test the statistical enrichment of differential expression genes in KEGG pathways.

2.1.3. Freezing Treatment

After four weeks of growing in the greenhouse at Saint Mary's University, *A.*

donax L. plants were sprayed with 15mL of 3ml/L ANE and H_2O. All the plants were treated with ANE or water during 12 hours prior to being transferred into -4℃ growth chamber for 24 hours. After 24 hours, the leaves were ready for the next experiment of completely randomized design. After all of the experiments on plants leaves, the plants were transferred to the green roof under natural climatic conditions.

2.1.4. Electrolyte Leakage

This electrolyte leakage method is based on (Sukumaran, 1972) and later modified by (Ristic, 1993). Firstly, fully developed leaves were excised and cut into 1.0 cm^2 discs. For this experiment, I used the second leaves counting from the top to bottom. It is important to have the same weight and development stages of leaves during this experiment, as the freezing stress is based on leaf order and development stage (Takagi, 2003). Then, the discs were placed into tubes with 16 mL deionized water. The tubes were then covered and shaken using a horizontal shaker (150 cycles / minute) at 25 °C for 1 hour. After shaking, the solutions were firstly measured by an electrical conductivity meter. The results were marked as Lt. Before the second measurement (Ltm), the tubes were autoclaved in 121 °C for 15 minutes. Finally, the percentage of electrolyte leakage as the ratio of the conductivity before and after autoclaving was calculated. The formula is:

Rt % = (Lt/Ltm)100

2.1.5. Chlorophyll

The extraction with dimethylsulfoxide (DMSO) has proven more efficient for the analysis of chlorophyll content of plants (Hiscox, 1979). 100mg leaves of *A. donax* L. were prepared and 7 mL DMSO added. Then all the tubes with leaves inside were incubated for 24 hours. Filled up DMSO to each tubes at 10 mL after incubation. Finally, 1.0 mL samples of chlorophyll extract were transferred to a cuvette and measured at 645 nm and 663 nm of OD value. Calculation used Arnon's (1949) equation:

Chla ($g\ l^{-1}$) = 0.0127 × A663 – 0.00269 × A645;

Chlb ($g\ l\text{-}^{1}$) = 0.0229 × A645 – 0.00468 × A663;

Tot Chl ($g\ l^{-1}$) = 0.0202× A645 + 0.00802 × A663.

2.1.6. Field Work

All the plants were moved after freezing treatments to the green roof and planted in three big boxes under normal climatic conditions. The boxes where located on the green roof above the library at Saint Mary's University. Before the winter, the soil was drenched with 3 mL/L ANE, 75 ppm chitosan and H_2O and then above-ground biomass was harvested 5 cm above the soil surface. To monitor the soil temperature during the winter, electronic thermometers were placed in the middle and at the edge of one of the three boxes. The boxes were then covered with plastic to mimic the soil conditions in a Nova Scotian winter. (Fig 7).

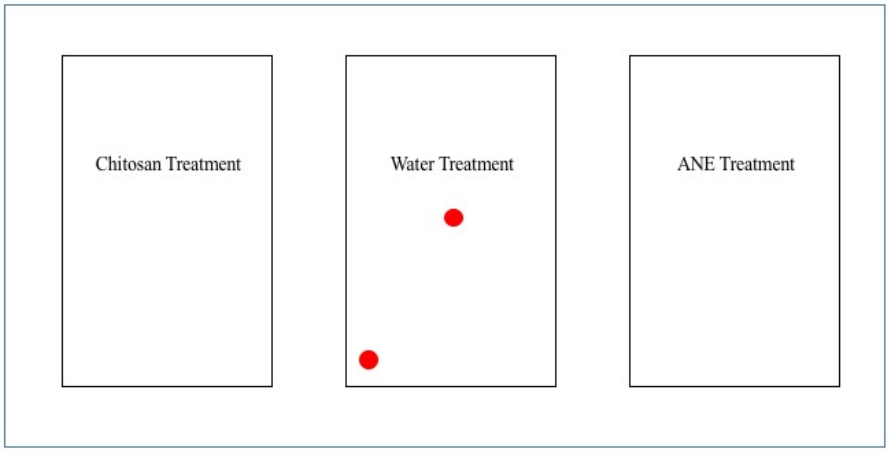


Figure 6. The chitosan, water and ANE-treated *A. donax* L. after freezing treatment were cultivated in three boxes; The red dots represent the thermometers.

Figure 7. To achieve the field soil conditions in the winter, the boxes were covered a layer of plastic.

Results

3.1. Transcriptome analysis

3.1.1. Transcriptome sequencing and *De novo* assembly of the *Arundo donax* L.

3.1.1.1. Illumina transcriptome sequencing

The original raw data from Illumina HiSeqTM 2500 were transformed to Sequenced Reads by base calling. Overall, 208,414,413 paired-end (PE) reads were obtained for filtering. All the reads with adaptor contamination, low quality nucleotides or the uncertain nucleotides constituted more than 10% of the total reads (N>10%) and were removed. Thus, the downstream analyses are based on clean reads (totaling 199,502,584 (95.7%)) after filtering. To evaluate the corrected sequencing data by average sequencing rate (read error), percentages of bases whose correct base recognition rates are greater than 99% and 99.9% in total bases (Q20, Q30 which an error probability of 0.01 and 0.001). GC content is the percentages of G and C in total bases. In total, there are 62,525,110 row reads of ANE; 74,012,716 of BR raw reads and 71,876,612 of H_2O raw reads were trimmed. After trimming, the file sizes of clean bases were 7.45G for ANE, 8.89G for BR and 8.59G for H_2O with only 0.03% error rates. The quality of Q20, Q30 and GC content in all treatments were accepted for further downstream *de novo* transcript assembly (Table 2).

3.1.1.2. *De novo* transcriptome assembly

For samples with no reference genome, clean reads need to be assembled to get a

reference sequence for the following analysis. Clean reads were reconstructed by Trinity, which is the software chosen to complete the transcriptome reconstruction process without a reference genome, to get a full-length transcriptome. The longest transcript of each gene is selected as the unigene. Our assembly generated 186,282 transcripts that further translated into 123,000 unigenes for downstream analyses. The length distribution of transcripts and unigenes ranged from 201bp to 1.3kb (Fig 8). The most number of transcripts and unigenes are located in 200-500bp, with a mean length transcript of 758bp and an N50 (the average length of sequences) of 1263bp of transcripts and unigenes' mean length is 572 with 837bp N50 (Table 3)

Table 2. Summary of Illumina transcriptome sequencing data for *Arundo donax* L.

Sample	**Raw Reads**	**Clean Reads**	**Clean Bases**	**Error(%)**	**Q20(**	**Q30(%)**	GC **Content(%)**
ANE	62,525,110	59,612,862	7.45G	0.03	95.27	90.46	56.3
H_2O	71,876,612	68,749,188	8.59G	0.03	95.38	90.76	53.97
BR	74,012,716	71,140,534	8.89G	0.03	95.73	91.45	50.54

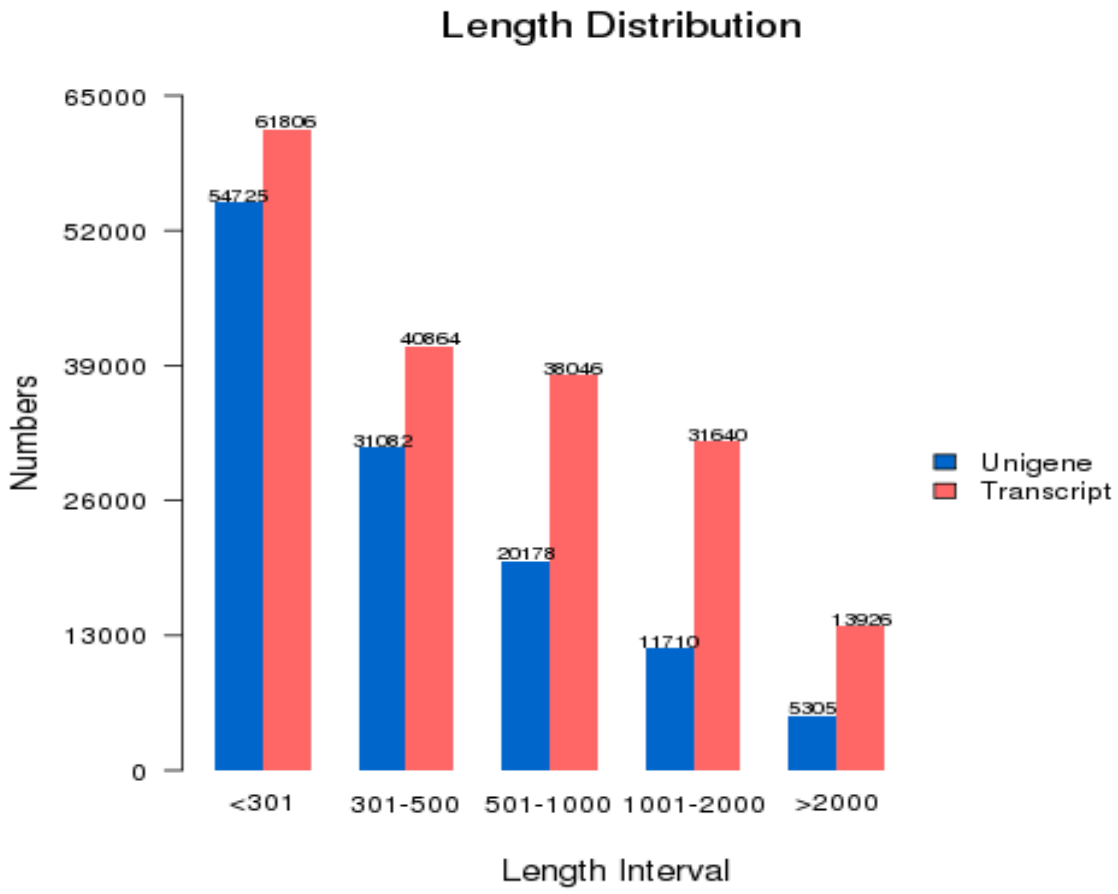


Figure 8. Length of transcripts and unigenes. X-axis indicates length interval of transcript and unigene; Y-axis indicates the number of transcript and unigene in each length interval.

Table 3. Summary of the *de novo* assembly of *Arundo donax* L.

Sample	Min length	Mean length	Median length	Max length	N50	Total Nucleotides
Transcripts	201	758	433	12,938	1263	141,272,636
Unigenes	201	572	326	12,938	837	70,379,041

3.1.2. Gene Functional Annotation.

3.1.2.1. Gene functional annotation

To achieve the comprehensive understanding of gene functional annotation, the 123,000 unigenes were analyzed by seven databases (Nr, Nt, Pfam, KOG/COG, Swiss-Prot, KO, GO). The successfully annotated genes are shown in Table 4.

Based on the top annotation via Nr database, the results showed a significant similarity of *Setaria italica* (35.5%), then *Sorghum bicolor* (15.7%), *Zea mays* (15.3%) and the possibility of other species is 22.5%. The E-value of the top matches is 13.8% of the unigenes with higher score (E=0). Of the unigenes, 70.1% showed similarity greater than 80%, 26 % showed similarities between 60 – 80 % and only 4 % had similarities less than 60% (Fig 9).

3.1.2.2. KOG classification

To understand the functional information of *A. donax* L. transcriptome, the assembled unigenes were matched against the Eukaryotic Orthologous Groups (KOG) database. 12,899 unigenes were annotated in the KOG database with 26 classifications (Fig 10). There are 2,443 unigenes located in category R (18.9%), which refers to "General function mechanism", a category that doesn't specify a certain functional group. The remaining unigenes were annotated into the following KOG cateogries: "Posttranslational modification, protein turnover, chaperones", "Signal transduction mechanisms", "Translation, ribosomal structure and biogenesis", "Intracellular trafficking,

secretion, and vesicular transport".

Table 4. The ratio of successfully annotated genes.

	Number of Unigenes	Percentage (%)
Annotated in NR	49116	39.93
Annotated in NT	42118	34.24
Annotated in KO	14350	11.66
Annotated in SwissProt	28426	23.11
Annotated in PFAM	30212	24.56
Annotated in GO	32555	26.46
Annotated in KOG	12899	10.48
Annotated in all Databases	6323	5.14
Annotated in at least one Database	59705	48.54
Total Unigenes	123000	100

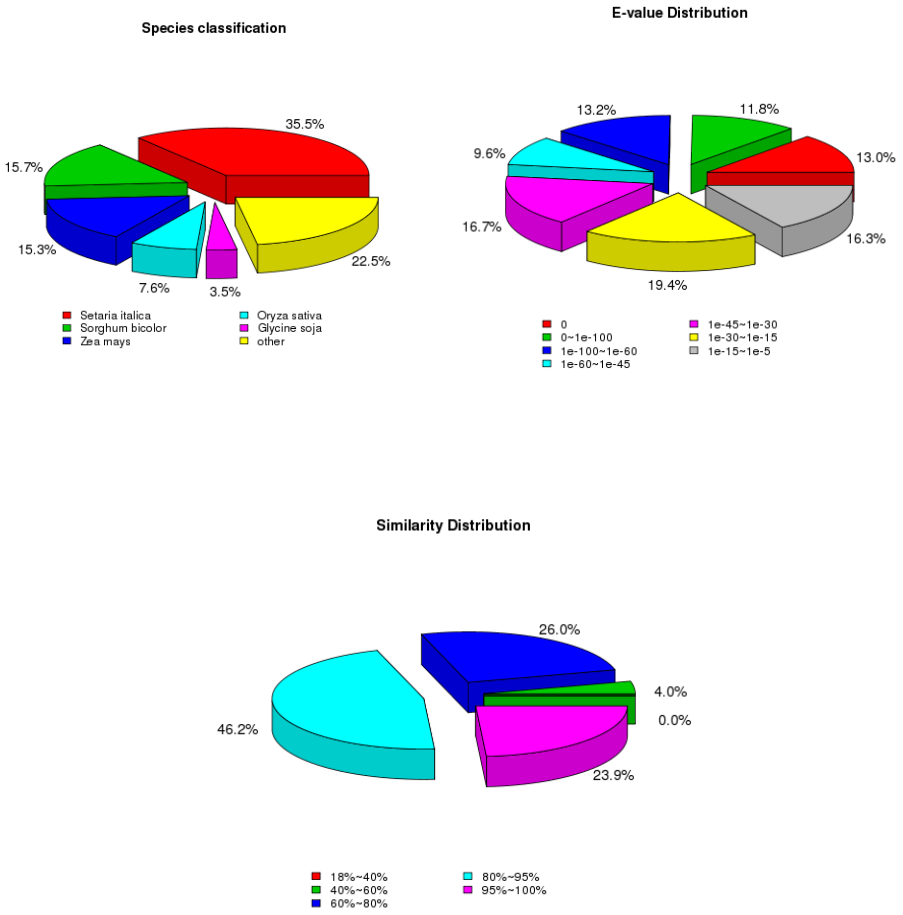


Figure 9. Species, E-value, and similarity distributions of assembled transcriptome against Nr database are shown.

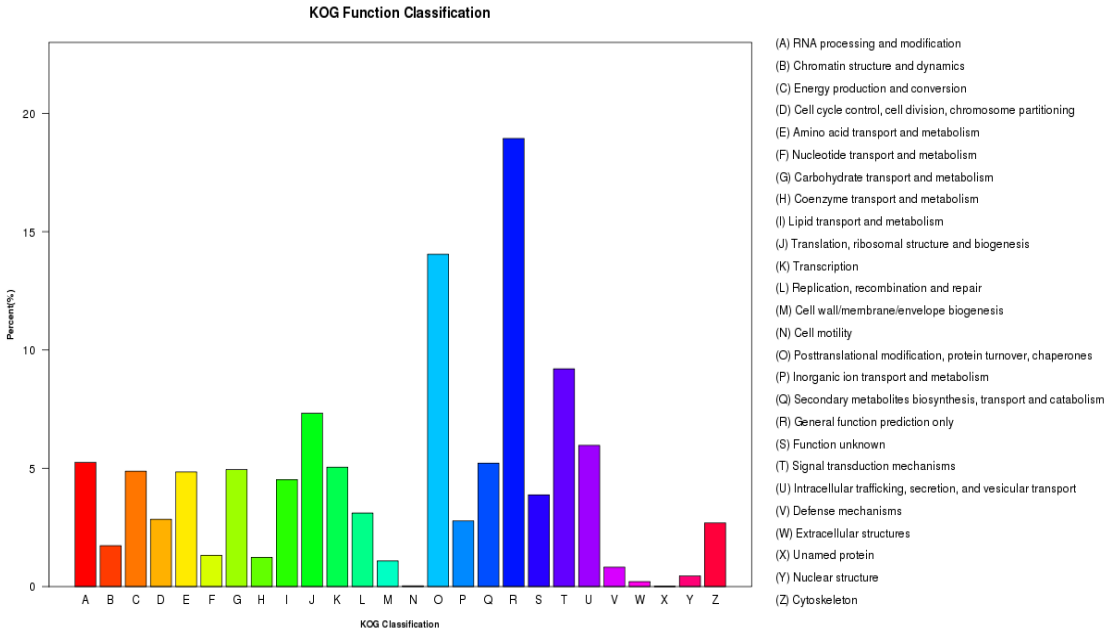


Figure 10. KOG Classification. X-axis is the names of the 26 KOG group; Y-axis is the percentage of genes annotated under this group in the total annotated genes.

3.1.3. Gene expression analysis

3.1.3.1.Quality analysis of RNA-seq data.

Transcriptomes reconstructed by Trinity were used as a reference (ref). RSEM is the software used to do the alignment. The summary of mapping results is shown in Figure 3. In total, there are 41,384,416 (69.42%) clean reads of ANE were mapped in 59,612,862 total clean reads, 49,583,946 (69.70%) clean reads of BR were mapped in 71,140,534 total clean reads and for the H_2O treatment, 47,319,336 (68.83%) clean reads of 68,749,188 were totally mapped (Fig 11). All the data were analyzed between three groups, ANE vs BR, ANE vs H_2O and BR vs H_2O. The scatter plots and Pearson correlation (Fig 12) indicated that the gene expression of ANE treatment is more similar to BR treatment than the ANE treatment compared with H_2O.

3.1.3.2. The differential expression analysis of *Arundo donax* L.

In total, there are maximum 71,267 unigenes that showed differential expression, only 664 genes were defined as DEGs (differentially expressed genes), which is significantly expressed genes, in ANE and BR when compared to H_2O treatments. According to the threshold without biological replicate, this experiment is set as: |log2 (FoldChange)| > 1 and q value < 0.005. Between ANE and H_2O treatment, 212 genes were DEGs, including 145 up-regulated genes, and 67 down-regulated genes. Only 166 genes were DEGs in BR versus H_2O, including 69 up-regulated genes and 97 down-regulated genes (Fig 13). Between ANE versus H_2O and BR versus H_2O, there are 26

different up-regulated genes in common, and 23 down-regulated genes in common (Fig 14).

Comparing ANE versus H_2O and BR versus H_2O, there are 26 up-regulated and 23 down-regulated genes that overlap between the two treatments (Tables 5 and 6). Except the most ribosomal proteins, the chloroplast, RubisCo and Chitinase are all showing up-regulated in ANE and BR treatments, which may indicate both ANE and BR could steer plant development.

Clustering analysis was used to find the differences in patterns of total differential expressed genes clustering in ANE, BR and H_2O treatments. The heat map shows the hierarchical clustering of differentially expressed genes in all three treatments (Fig 15). The total differential expressed genes in the BR treatment are closer to the control (H_2O), than the ANE treatment. However, there are three sections A, B and C shown in the heat map, showing the different patterns of differential expressed genes in ANE, BR and control. The A section presents the down-regulated genes after BR treatment that is most similar with control; B section shows the most dissimilar genes between ANE and BR treatments; C section is obviously similarly expressed genes between ANE and BR treatments. The sections in Figure 15 were analyzed to show the genes' molecular function, the results are shown in fig 16. In total, there were 68 genes in the A section, and the molecular functions can statistically be divided into 12 categories, whereas 46 genes in the B section have 12 categories as well. The C section contains 49 genes but only divided into 10 categories. Except for the uncharacterized proteins in those three sections, the "oxidoreductase activity" both in A section and B sectionand the "structural

constituent of ribosome" in C section show the most molecular functions. The oxidoreductase activity including many child enzymes activity is the basic activity in plant (i.e. photosynthesis and respiration). The most similar up-regulated genes in ANE and BR belongs to "structural constituent of ribosome", which is the action of a molecule that contributes to the structural integrity of the ribosome.

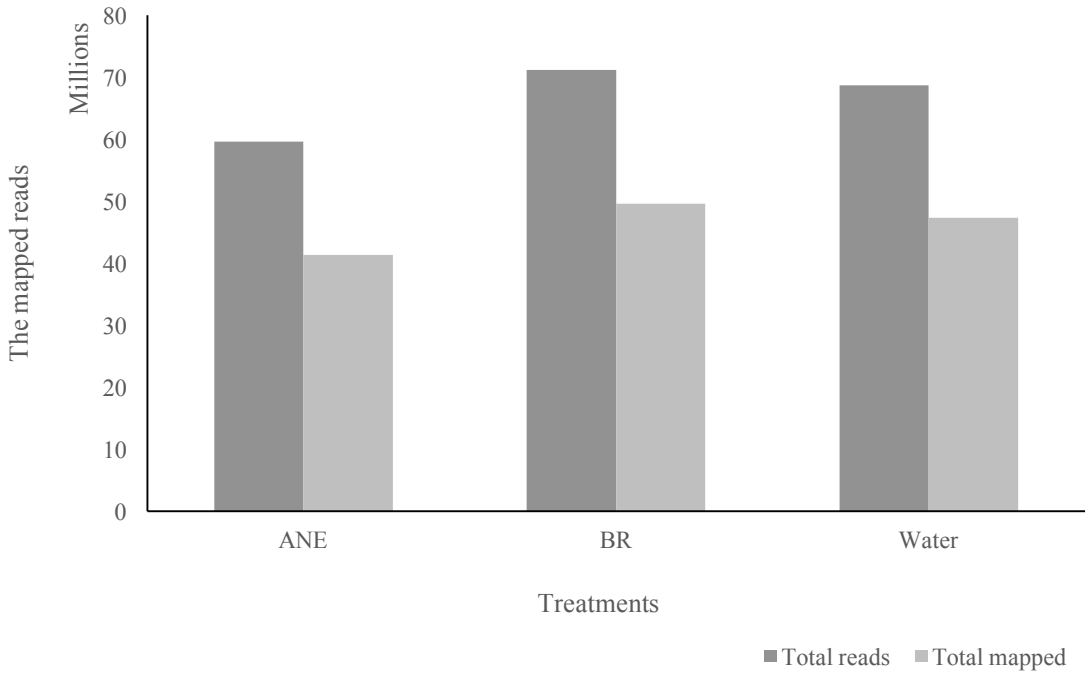


Figure 11. Overview of the Alignment Situation.

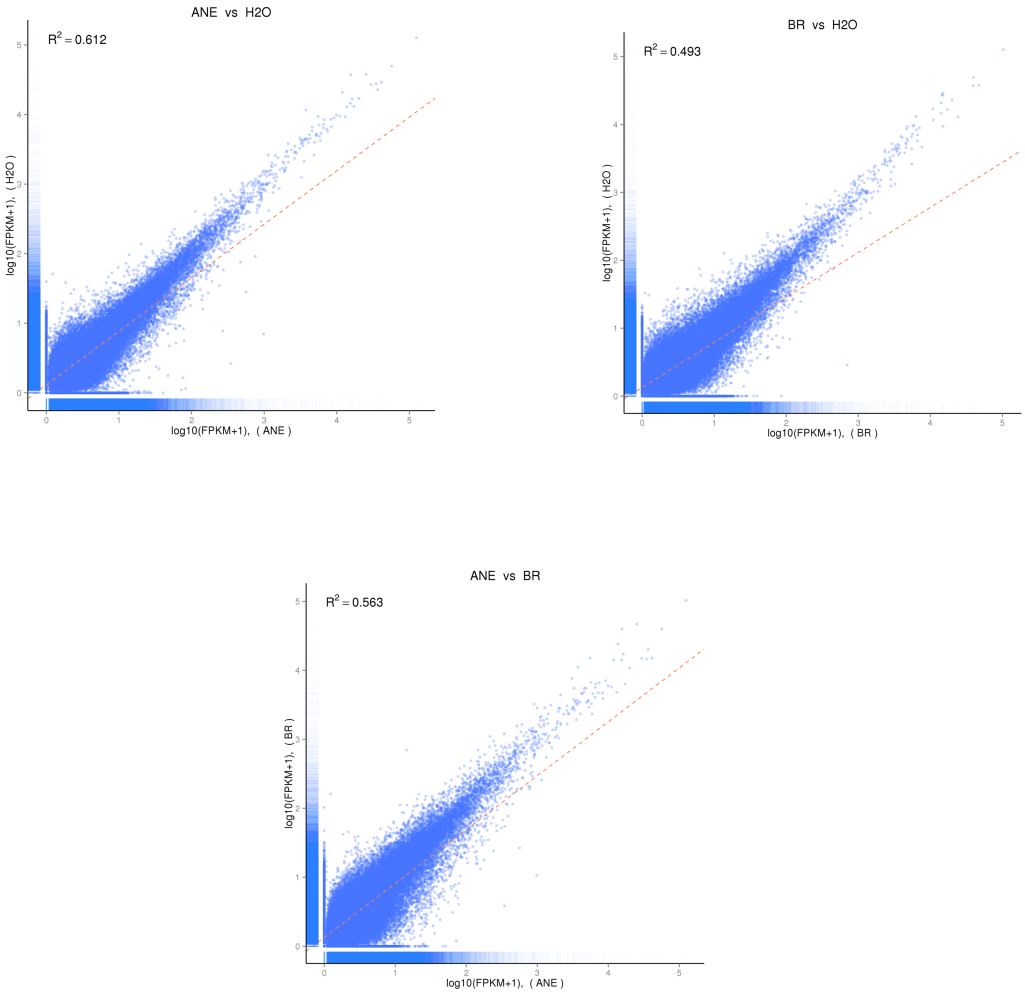


Figure 12. The correlation between ANE, H_2O and BR treatments without biological replicates of *A. donax* L.

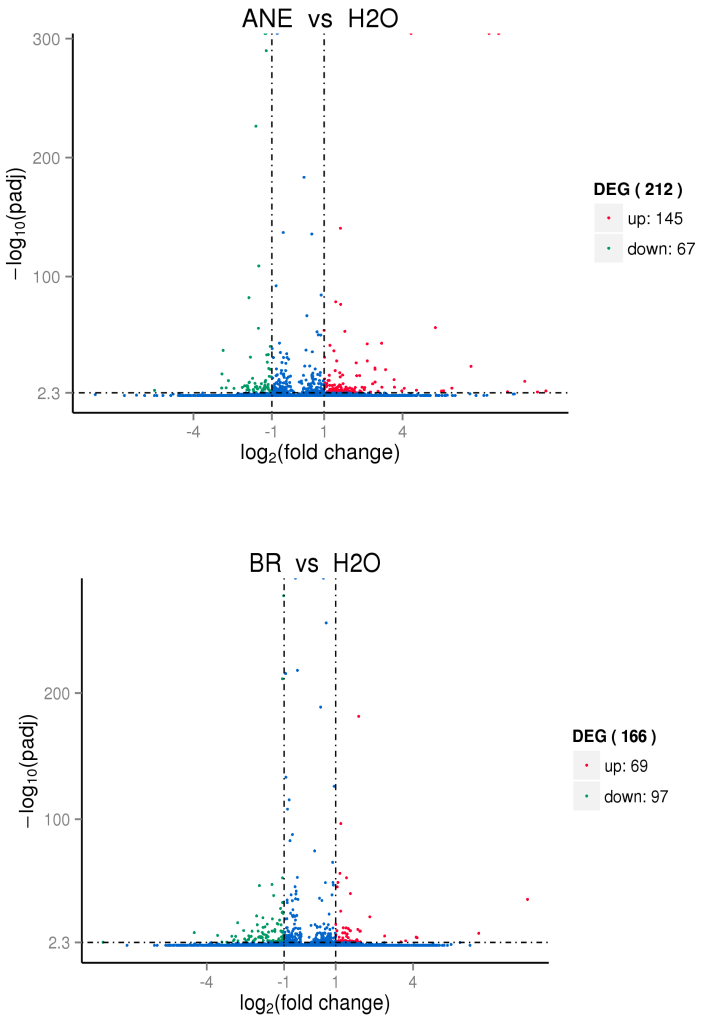
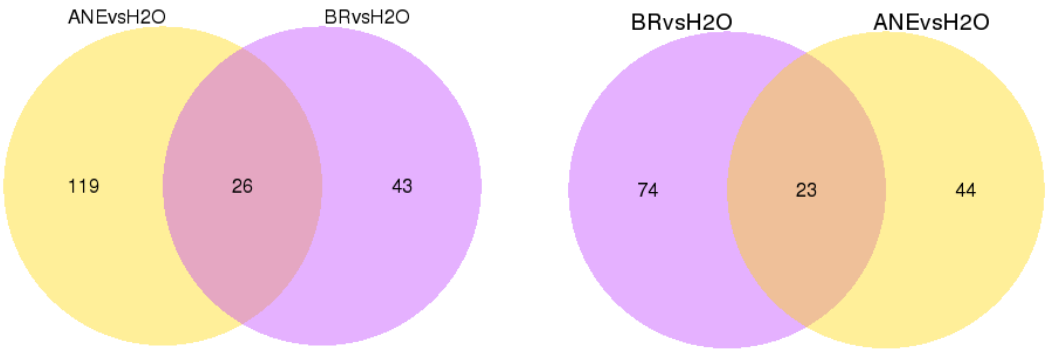


Figure 13. The differential expressed genes of ANE, BR treatments compare with H_2O treatments. The horizontal axis represents fold changes of gene expression, and the vertical axis represents the statistically level. The red dots are significantly up-regulated genes and the green dotes are down-regulated genes.



up-regulated down-regulated

Figure 14. Venn diagram of differentially expressed genes (DEGs). The number in one color circle present the total number of DEGs, and the number in overlap presents the DEGs in common.

Table 5. All up-regulation genes in ANE Vs H_2O and BR Vs H_2O treatments.

Genes	Gene Length	Swissprot Evalue	Swissprot Description
c80893_g1	2194	6.79E-32	BTB/POZ and TAZ domain-containing protein 2
c66400_g1	1699	--	--
c102414_g1	1079	4.83E-36	Extracellular ribonuclease LE
c80903_g1	4029	1.19E-14	Protein CHLOROPLAST IMPORT APPARATUS
c80756_g1	1474	5.21E-108	60S ribosomal protein L18a
c76749_g2	942	3.29E-32	Low molecular mass early light-inducible protein
c70968_g1	1363	1.23E-27	Ribonuclease 3
c83404_g2	1081	5.29E-57	Elongation factor 1-delta 2
c75964_g3	659	--	--
c79624_g1	1630	1.40E-82	40S ribosomal protein S19
c84718_g1	2099	0	RuBisCO large subunit-binding protein subunit alpha, chloroplastic (Fragment)
c81787_g1	1209	1.23E-121	40S ribosomal protein S8
c82106_g1	1193	3.54E-78	60S ribosomal protein L19-1
c64145_g1	857	1.44E-70	31 kDa ribonucleoprotein, chloroplastic
c84537_g2	1566	6.08E-101	60S ribosomal protein L13-2
c65609_g1	649	--	--
c79280_g2	1269	6.35E-38	40S ribosomal protein S15
c81237_g2	1865	2.03E-08	Ethylene-responsive transcription factor ERF112
c64937_g1	4493	2.81E-101	Methylsterol monooxygenase 1-1
c83699_g1	1469	1.18E-142	60S ribosomal protein L7a
c69863_g1	967	--	--

c75936_g1	803	1.87E-57	60S ribosomal protein L44
c109468_g1	1103	8.02E-131	Chitinase 11
c70474_g1	988	1.67E-19	Horcolin
c79623_g2	1349	9.72E-173	60S ribosomal protein L5-1
c83465_g2	2322	0	Chaperonin 60 subunit beta 2, chloroplastic

Table 5. All down-regulation genes in ANE Vs H_2O and BR Vs H_2O treatments.

Genes	Gene Length	Swissprot Evalue	Swissprot Description
c55188_g1	1558	2.20E-112	Senescence-specific cysteine protease SAG39
c68571_g1	2446	0	Vacuolar-processing enzyme
c26155_g1	1216	1.65E-06	UPF0301 protein Plut_0637
c69279_g1	1829	0	Serine carboxypeptidase 2
c84130_g1	2694	0	Probable beta-D-xylosidase 7
c81295_g1	2472	1.05E-61	Photosynthetic NDH subunit of subcomplex B 5, chloroplastic
c84581_g1	1165	2.58E-129	Xyloglucan endotransglucosylase/hydrolase protein 22
c78062_g1	1762	1.28E-174	Photosynthetic NDH subunit of subcomplex B 1, chloroplastic
c78553_g1	3861	--	--
c78664_g1	4497	2.29E-117	Transposon Ty3-G Gag-Pol polyprotein
c70536_g1	1657	7.89E-10	Uncharacterized protein MJ1232
c73907_g1	1318	--	--
c72638_g1	2609	9.43E-45	Putative disease resistance protein RGA3

c75803_g1	3902	1.28E-58	Disease resistance protein RPP13
c77024_g1	3052	0	Probable galactinol--sucrose galactosyltransferase 2
c70255_g1	1867	2.88E-147	Probable high-affinity nitrate transporter 2.4
c75426_g1	2371	0	Sucrose:sucrose 1-fructosyltransferase
c76249_g1	1127	--	--
c82609_g1	2599	1.05E-135	Uncharacterized protein ycf45
c79475_g2	1490	0	Probable isoaspartyl peptidase/L-asparaginase 2
c79481_g1	1962	--	--
c83222_g1	3152	0	Probable metal-nicotianamine transporter YSL6

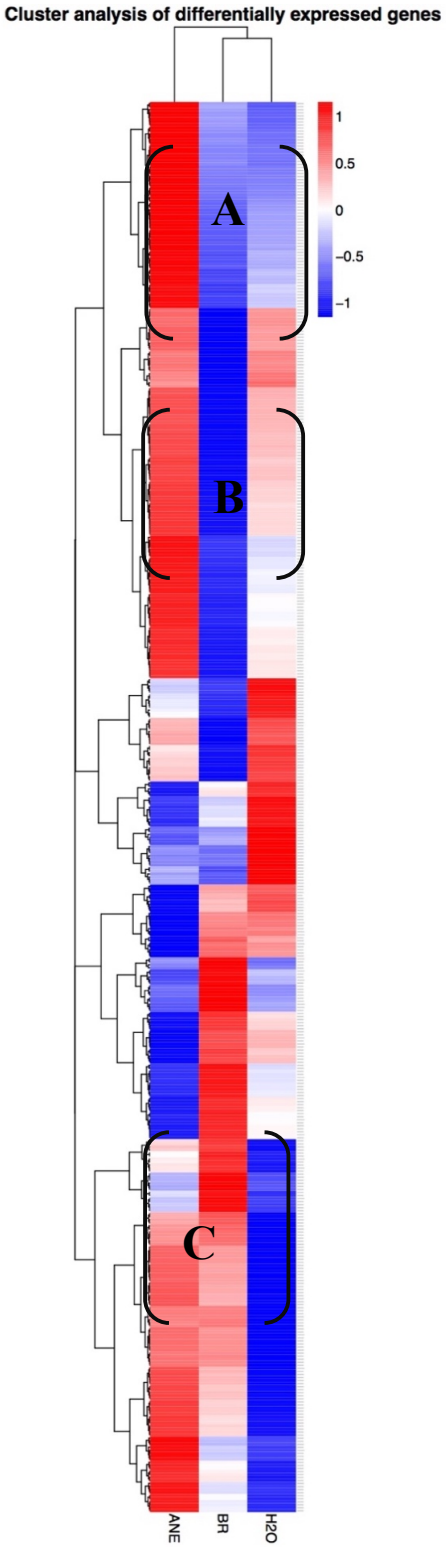
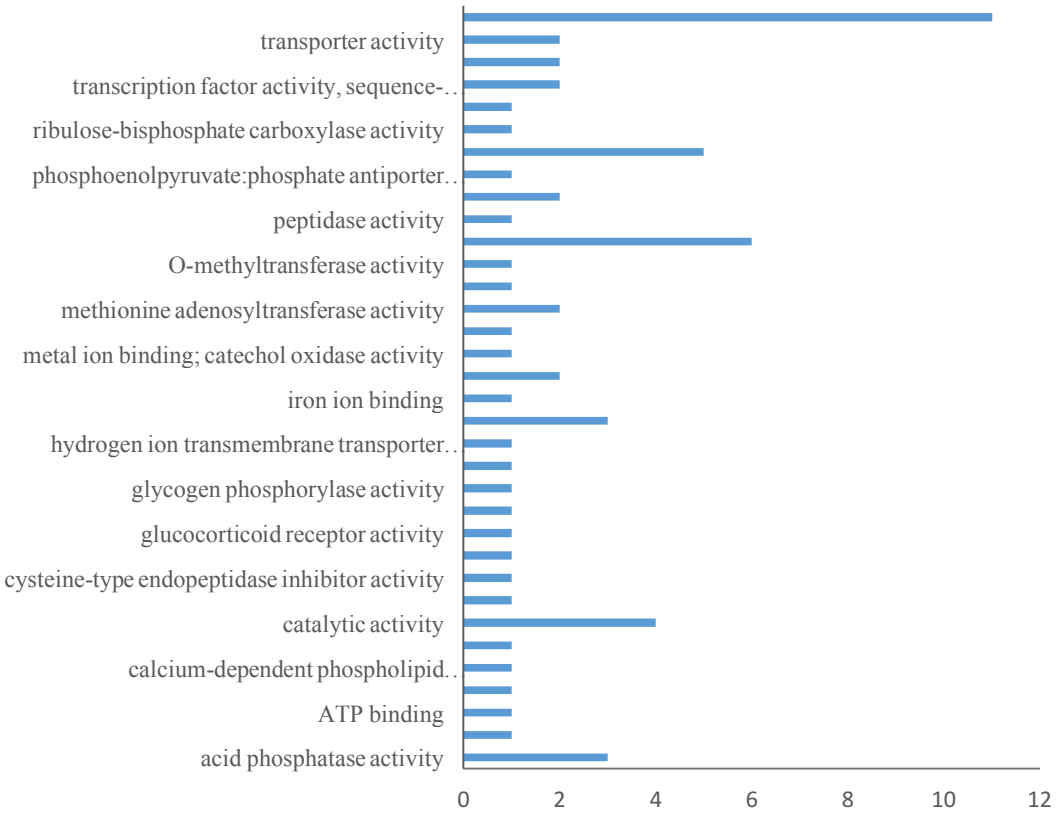
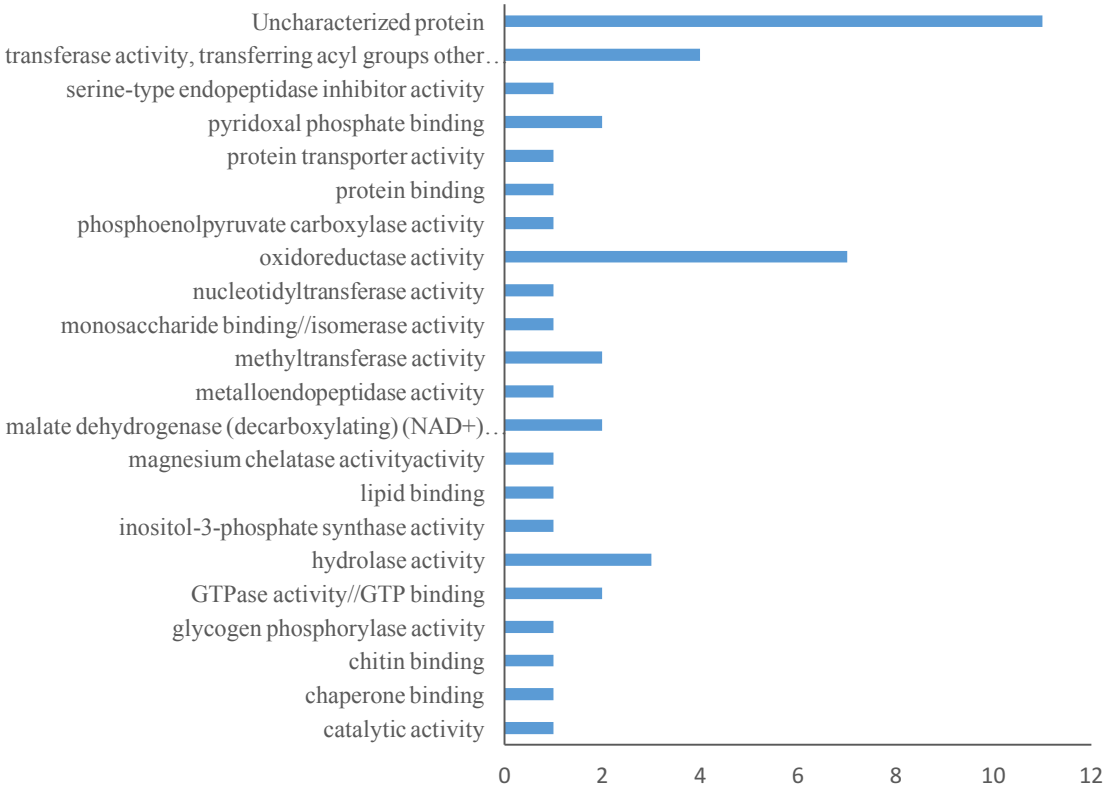


Figure 15. Heat map of cluster analysis of DEGs.

The genes molecuar function ditribution of Fig. 15 section A



The genes molecuar function ditribution of Fig. 15 section B



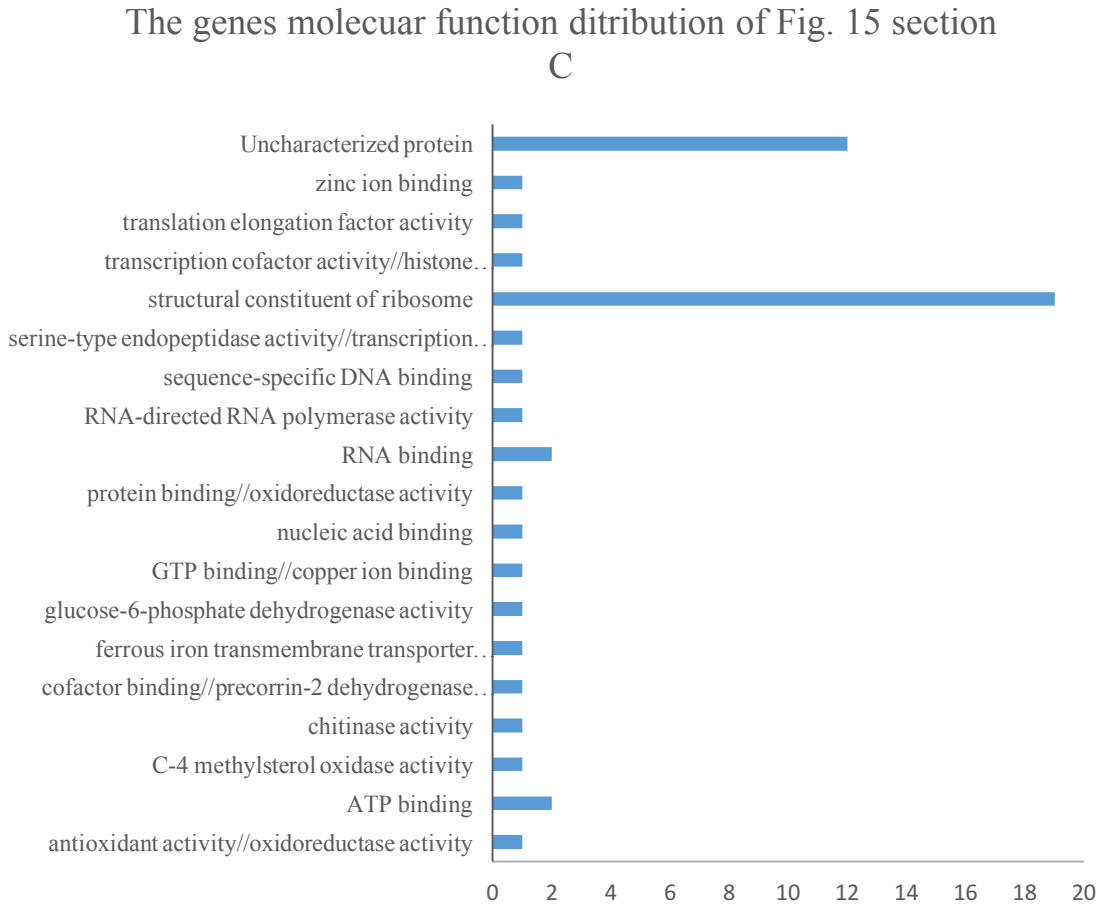


Figure 16. The detail comparisons of cluster analysis. A: the area where the gene expressions of BR treatment is more similar to water; B; the area where the gene expressions of ANE and BR treatments are significantly difference; C; the area where ANE and BR treatments have the similar gene expressions.

3.1.3.3.The genes analysis

All the up-regulated DEG genes in ANE vs H_2O and BR vs H_2O were analyzed and summarized (Tables 7, 8, 9). There are 146 significant up-regulated genes in ANE vs H_2O treatment, and 70 in BR vs H_2O treatment. In the ANE vs H_2O up-regulated genes, the top 6 up-regulated genes related to plant growth and plant defense are c78010_g1 (PLP2), c36935_g1(MTR_069s1001), c76292_g1(TPS6), c83073_g1(EXPA4), c81715_g4(GPT2), and c80903_g1(CIA2). PLP2 was originally found in insect cells, and then expressed in cucumber, tobacco and bean leaves, this protein involved in lipolytic acyl hydrolase (LAH) activity, which contributes to resistance to Cucumber mosaic virus, possibly providing the fatty acid precursors for biosynthesis of oxylipins (Camera, 2009). Besides, TPS6 shown expression in defense against herbivores and microbial pathogens (Kollner, 2008). Those two genes both highly increased involved in biotic stress. Vitamin C exists in various plant showing some effects on photosynthesis and transmembrane electron transport (Wheeler, 1998). EXPA4 causes loosening and extension of plant cell wall (Choi, 2003); GPT2 as substrate for fatty acid biosynthesis and NADPH generation (H. H. Kunz, 2010); CIA2 involved in protein import and translation efficiencies (Sun, 2009), RuBisCO large subunit-binding protein subunit alpha (Rlspsa) is in the assembly of RuBisCo in higher plant chloroplast (Demirevska, 2008). All of those relating to the plant development.

The abiotic stresses-inducible genes can be divided into two groups: one is directly related to plant resistance cells such as anti-freezing protein, osmotic regulatory protein, some enzymes for synthesizing betaine and proline; the other is regulating gene

or signal transduction (Ciarmiello, 2011).

In ANE vs H_2O treatment, there are 12 up-regulated DEGs annotated with plant cold response. Those genes are c78602_g1 (ANN4), c59812_g1 (Olp), c83155_g3 (HSP70), c84137 (RH3), c73125_g2 (UspAp), c78602_g1(WRKY21), c109468_g1(Cht11), c80526_g1(DHAR2), c75292_g3(PER24). ANN4, described affect by the osmotic stress, which is may involved in cold response (Lee S. L., 2004). Olp belongs to the Pathogenesis-related protein 5 (PR-5) family, which is induction in many plants abiotic or biotic stresses (Patade, 2013). HSP70 was detected in all cells, most of this family genes are interacting with other genes under stress condition. Their effects on the other cell is still little understood (Neven, 1992). RH3 participate in metabolism of RNA, it response many cellular functions including abiotic stress (Macovei, 2012). UspAp short for Universal stress protein A-like protein, those proteins are directly response to the abiotic stresses, especially to the extremely higher or lower temperature (Jung, 2015); c78602_g1(WRKY21), c109468_g1(Cht11), c80526_g1(DHAR2), c75292_g3(PER24) are transfer factors or enzymes to involved in the abiotic responses. WRKY transcription factor family showing response to abiotic stress, biotic stress, senescence. WRKY 21 is described that may have involved in cold stress (Zhou, 2008). Cht11, PER24 and DHAR2 are enzymes involved in stress responses (Xu, 2007) (Lee D. H., 2000) (Yoshida, 2006).

Compare to the ANE vs H_2O up-regulation results, the most up-regulated genes response to plant growth in BR treatment, 3 of them are same genes showing in DEGs as ANE treatment, including CIA2, GPT2 and RubisCo large submit-binding protein.

c83968_g1(NPF), c80893_g1(BT) indicated that encoded by auxin-responsive genes, or induced the auxin during the plant growth (Robert, 2009) (Chiba, 2015). c81464_g1(APL) gene encoded with Myb coiled-coil-type transcription factor was import for phloem in Arabidopsis (Bonke, 2003). Glutaredoxin-C1 was found in BR treatment, which suggested has biochemical roles: the reduction of disulphide bonds and the binding of iron–sulphur clusters (Rouhier, 2010). Only the Chitinase is the same up regulated gene as ANE treatment.

Table 7. The up-regulated gene expressions show plant development response after ANE treatment.

Gene ID	Log2. Fold change	P value	Protein Name	Description
c78010_g1	8.0179	3.50E-06	Patatin-like protein 2 (PLP2)	Possesses non-specific lipolytic acyl hydrolase (LAH) activity.
c36935_g1	7.313	0	Cell wall-associated hydrolase (MTR_069s1001)	
c76292_g1	6.6186	2.14E-28	(S)-beta-macrocarpene synthase (TPS6)	Involved in the biosynthesis of the bicyclic sesquiterpene (S)-beta-macrocarpene.
c70975_g1	3.2064	2.70E-10	L-ascorbate oxidase (Vc)	May be involved in a redox system involving ascorbic acid.

c83073_g1	2.9461	8.88E-15	Expansin-A4 (EXPA4)	Causes loosening and extension of plant cell wall
c81715_g4	2.5559	6.45E-10	Glucose-6-phosphate/phosphate translocator 2, chloroplastic (GPT2)	An substrate for fatty acid biosynthesis and NADPH generation
c80903_g1	2.3604	1.67E-20	Protein CHLOROPLAST IMPORT APPARATUS 2 (CIA2)	Involved in protein import and translation efficiencies
c84718_g1	1.4654	2.71E-06	RuBisCO large subunit-binding protein subunit alpha, chloroplastic (Fragment)	Involved in the assembly of RuBisCo in higher plant chloroplast

Table 8. The up-regulated gene expressions show cold resistance response after ANE treatment.

Gene ID	Log2. Fold change	P value	Protein Name	Description
c78602_g1	2.5209	9.06E-10	Probable WRKY transcription factor 21 (WRKY21)	WRKY 21 is described that may have involved in cold stress
c79513_g1	2.1856	5.49E-06	Annexin D4 (ANN4)	described affect by the osmotic stress, which is may involved in cold response
c59812_g1	1.7137	1.33E-05	Osmotin-like protein (Olp)	Belongs to the Pathogenesis-related protein 5 (PR-5) family, which is induction in many plants abiotic or biotic stresses
c83155_g3	1.6277	5.27E-11	Heat shock cognate 70 kDa protein (HSP70)	Most of this family genes are interacting with other genes under stress condition

c84137_g1	1.5323	1.35E-10	DEAD-box ATP-dependent RNA helicase 3, chloroplastic (RH3)	RH3 participate in metabolism of RNA, it response many cellular functions including abiotic stress
c109468_g1	1.3953	2.65E-12	Chitinase 11 (Cht 11)	Random hydrolysis of N-acetyl-beta-D-glucosaminide (1->4)-beta-linkages in chitin and chitodextrins
c73125_g2	1.3319	4.21E-07	Universal stress protein A-like protein (UspAp)	Directly response to the abiotic stresses, especially to the extremely higher or lower temperature
c80526_g1	1.0941	4.31E-11	Glutathione S-transferase (DHAR2)	Involved in the redox homeostasis, especially in scavenging of ROS under oxidative stresses
c75292_g3	1.0559	2.55E-10	Peroxidase 24 (PER24)	Removal of H_2O_2, oxidation of toxic reductants, biosynthesis and degradation of lignin, suberization, auxin catabolism, response to environmental stresses such as wounding,

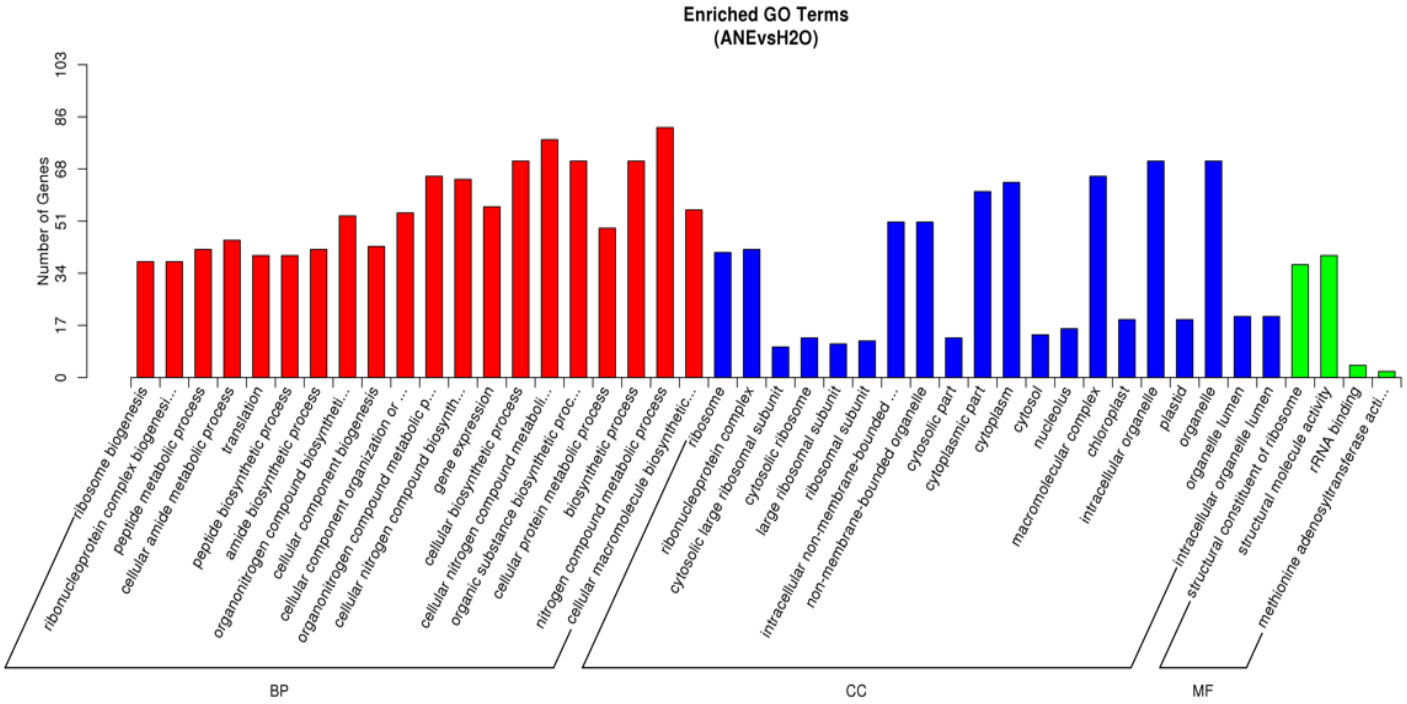
Table 9. The up-regulated gene expressions show plant development and cold resistance after BR treatment.

Gene ID	Log2. Fold change	P.value	Protein Name	Description
c80903_g1	1.9594	7.65E-15	Protein CHLOROPLAST IMPORT APPARATUS 2. (CIA2)	Involved in the general chloroplast protein import pathway regulation, including protein import and protein translation efficiencies.
c83968_g1	1.63	1.68E-06	Protein NRT1/ PTR FAMILY 4.4 (NPF)	Nitrate transporters belongs to NRT1 family, which transport a broad spectrum of di/tripeptides
c80893_g1	1.5765	4.14E-15	BTB/POZ and TAZ domain-containing protein 2 (BT)	Enhances responses to auxin in post germination and vegetative development.
c80815_g1	1.5531	7.17E-07	Glucose-6-phosphate 1-dehydrogenase 2, chloroplastic	The main function of this enzyme is to provide reducing power (NADPH) and pentose phosphates for fatty acid and nucleic acid synthesis which are involved in membrane synthesis and cell division

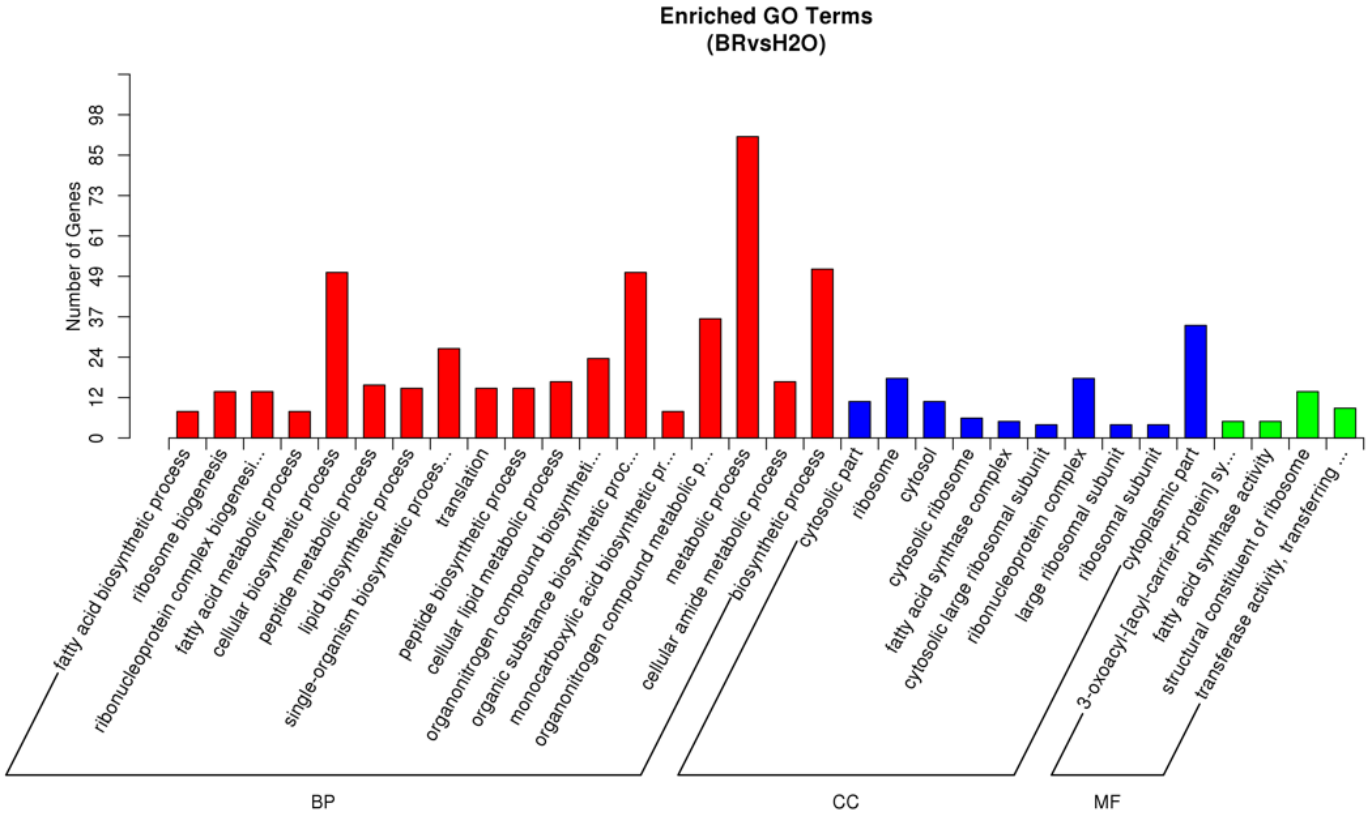
c81464_g1	1.3697	4.59E-07	Myb family transcription factor APL	Has a dual role both in promoting phloem differentiation and in repressing xylem differentiation in vascular develop.
c84718_g1	1.2807	1.01E-05	RuBisCO large subunit-binding protein subunit alpha	Involved in the assembly of RuBisCo in higher plant chloroplast
c109468_g1	1.1353	9.24E-10	Chitinase 11	Random hydrolysis of N-acetyl-beta-D-glucosaminide (1->4)-beta-linkages in chitin and chitodextrins
c71462_g1	1.0331	8.88E-06	Glutaredoxin-C1	Has a glutathione-disulfide oxidoreductase activity in the presence of NADPH and glutathione reductase

3.1.3.4.Go analysis

Gene Ontology (GO) is a major bioinformatics initiative to unify the presentation of gene and gene product attributes across all species. GO terms distribution of *A. donax* L. transcripts are most abundant in biological process (BP) and cellular component (CC) both in ANE versus H_2O and BR versus H_2O treatments. In ANE treatment, BP ontology transcripts were mainly in “metabolism process” and “cellular process”, which showed 81 and 78 transcripts respectively while CC were mainly in “cell” and “intracellular” showing 62 and 60, respectively (S1). In BR treatment, the most transcripts of CC are 48 “cell” and 44 “intracellular part” and BP is mainly in “metabolic process” and “organic substance metabolic process”, which show 91 and 80 transcripts respectively (S2). There are some transcripts showing no similarity to any protein, which may belong to long non-coding RNAs or unknown genes.


Enriched GO Terms
(ANEvsH2O)
Number of Genes
0
17
34
51
68
86
103
ribosome biogenesis
ribonucleoprotein complex biogenesi...
peptide metabolic process
cellular amide metabolic process
translation
peptide biosynthetic process
amide biosynthetic process
organonitrogen compound biosynthetic...
cellular component biogenesis
cellular component organization or ...
organonitrogen compound metabolic p...
cellular nitrogen compound biosynth...
gene expression
cellular biosynthetic process
cellular nitrogen compound metaboli...
organic substance biosynthetic proc...
cellular protein metabolic process
biosynthetic process
nitrogen compound metabolic process
cellular macromolecule biosynthetic...
ribosome
ribonucleoprotein complex
cytosolic large ribosomal subunit
cytosolic ribosome
large ribosomal subunit
ribosomal subunit
intracellular non-membrane-bounded ...
non-membrane-bounded organelle
cytosolic part
cytoplasmic part
cytoplasm
cytosol
nucleolus
macromolecular complex
chloroplast
intracellular organelle
plastid
organelle
organelle lumen
intracellular organelle lumen
structural constituent of ribosome
structural molecule activity
rRNA binding
methionine adenosyltransferase acti...
BP
CC
MF

A


Enriched GO Terms
(BRvsH2O)
Number of Genes
0
12
24
37
49
61
73
85
98
fatty acid biosynthetic process
ribosome biogenesis
ribonucleoprotein complex biogenesi...
fatty acid metabolic process
cellular biosynthetic process
peptide metabolic process
lipid biosynthetic process
single-organism biosynthetic proces...
translation
peptide biosynthetic process
cellular lipid metabolic process
organonitrogen compound biosyntheti...
organic substance biosynthetic proc...
monocarboxylic acid biosynthetic pr...
organonitrogen compound metabolic p...
metabolic process
cellular amide metabolic process
biosynthetic process
cytosolic part
ribosome
cytosol
cytosolic ribosome
fatty acid synthase complex
cytosolic large ribosomal subunit
ribonucleoprotein complex
large ribosomal subunit
ribosomal subunit
cytoplasmic part
3-oxoacyl-[acyl-carrier-protein] sy...
fatty acid synthase activity
structural constituent of ribosome
transferase activity, transferring ...
BP
CC
MF

B

Figure 17. GO enrichment bar chart of DEGs. The x-axis shows GO term in the sub-level of the GO three main domains, and the y-axis shows the number of the differential expression genes annotated in this term and the ratio between this number and the total number of annotated differential expression genes. From left to right is the three main GO domains: biological process, cell composition and molecular function. 17 (A) is GO terms in ANE vs H_2O treatments; 17 (B) is GO terms in BR vs H_2O treatments.

3.1.3.5.KEGG pathway analysis

The interactions of multiple genes may be involved in certain biological functions. KEGG (Kyoto Encyclopedia of Genes and Genomes) is a collection of manually curated databases dealing with genomes, biological pathways, diseases, drugs, and chemical substances (Fig 18). Pathway enrichment analysis identifies significantly enriched metabolic pathways or signal transduction pathways associated with differentially expressed genes compared with the whole genome background. The top 20 enrichment pathways of commonly up-regulated transcripts in ANE vs H_2O treatments were enriched in pathway: “Ribosome”, “Photosynthesis - antenna proteins”, “Legionellosis”, “Cysteine and methionine metabolism”, “Phenylpropanoid biosynthesis”, “Antigen processing and presentation”, “Porphyrin and chlorophyll metabolism”. The difference is that the enriched pathways of BR vs H_2O treatments were only “Ribosome” and “Oxidative phosphorylation”. There is no similar pathway between ANE and BR treatments, except the “Ribosome” pathway. The internal and external environmental signal can affect the plant growth and plant stress defense. Thus, the “Photosynthesis- antenna proteins” and “porphyrin and chlorophyll metabolism”, which involve plant photosynthesis, might be the reason that ANE can stimulate the plant development. The “Ribosome” pathway is most enriched in both ANE and BR treatments indicating these two treatments may have positive effects in plant cell or protein development.

The “Photosynthesis- antenna proteins” pathway is the subunit of photosystem I and II containing some antenna light-harvesting proteins. The light-harvest proteins in green plants take actions in peripheral antenna systems, which will improve the efficient

absorption of light energy (Fig 19).

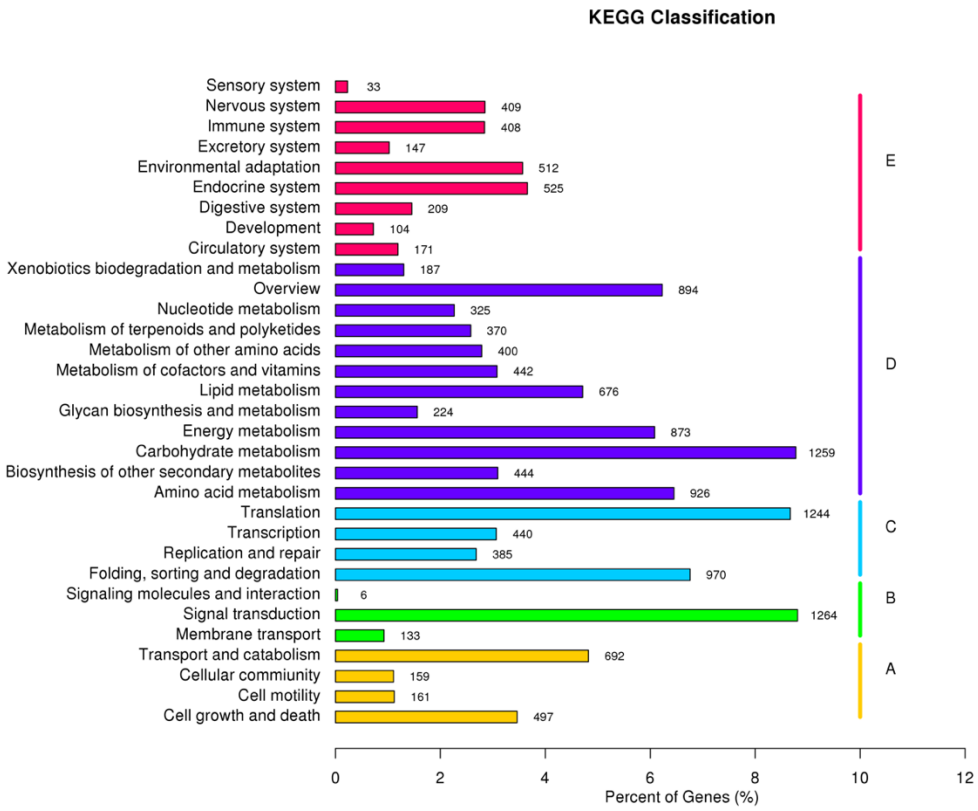


Figure 18. KEGG Classification. Y-axis is the names of KEGG pathways; X-axis is the number of the genes annotated in the pathway and the ratio between the number in this pathway and the total number of annotated genes. The KEGG metabolic pathways gene involved in are divided into 5 branches: A: Cellular Processes, B: Environmental Information Processing, C: Genetic Information Processing, D: Metabolism, E: Organismal Systems.

Table 10. Significant KEGG pathways of commonly up-regulated transcripts in BR Vs H_2O treatments.

Pathway term	Rich factor	Q-value	Gene number
Ribosome	0.023060797	8.58E-11	11
Oxidative phosphorylation	0.014869888	0.002604951	4
Type I diabetes mellitus	0.038461538	0.196042896	1
Pathogenic Escherichia coli infection	0.020408163	0.234623469	1
Steroid biosynthesis	0.014084507	0.234623469	1
Nitrogen metabolism	0.012658228	0.234623469	1
Legionellosis	0.009708738	0.234623469	1
Photosynthesis	0.009433962	0.234623469	1
Alanine, aspartate and glutamate metabolism	0.009174312	0.234623469	1
Pentose phosphate pathway	0.008928571	0.234623469	1
Glutathione metabolism	0.006024096	0.291584932	1
Parkinson’s disease	0.005347594	0.291584932	1
RNA degradation	0.004672897	0.291584932	1
Huntington’s disease	0.004464286	0.291584932	1
Tuberculosis	0.004291845	0.291584932	1
Alzheimer’s disease	0.004166667	0.291584932	1
Spliceosome	0.003278689	0.3364707	1
Carbon metabolism	0.002008032	0.447490178	1
Biosynthesis of amino acids	0.001984127	0.447490178	1

Table 11. Significant KEGG pathways of commonly up-regulated transcripts in ANE Vs H_2O treatments.

Pathway term	Rich factor	Q value	Gene number
Ribosome	0.075471698	1.40E	36
Photosynthesis - antenna proteins	0.173076923	1.42E-08	9
Legionellosis	0.029126214	0.23547971	3
Cysteine and methionine metabolism	0.015706806	0.52566414	3
Phenylpropanoid biosynthesis	0.01171875	0.52566414	3
Antigen processing and presentation	0.02173913	0.52566414	2
Porphyrin and chlorophyll metabolism	0.019230769	0.52566414	2
Estrogen signaling pathway	0.01754386	0.52566414	2
Oxytocin signaling pathway	0.01459854	0.52566414	2
AMPK signaling pathway	0.012195122	0.53483301	2
PI3K-Akt signaling pathway	0.010989011	0.53483301	2
Phenylalanine metabolism	0.010204082	0.53483301	2
Steroid hormone biosynthesis	0.142857143	0.52566414	1
Mineral absorption	0.043478261	0.52566414	1
Type I diabetes mellitus	0.038461538	0.52566414	1
Sesquiterpenoid and triterpenoid	0.03125	0.53483301	1
Valine, leucine and isoleucine biosynthesis	0.027027027	0.53483301	1
Selenocompound metabolism	0.025	0.53483301	1
Pantothenate and CoA biosynthesis	0.021276596	0.53483301	1
Pathogenic Escherichia coli infection	0.020408163	0.53483301	1

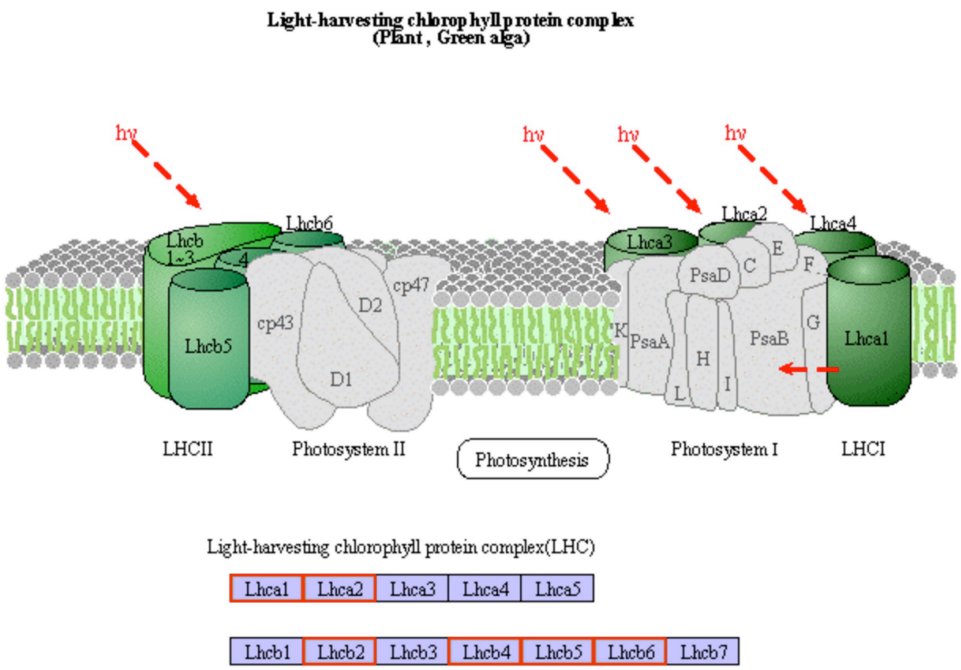


Figure 19. The up-regulated gene expressions in photosynthesis-antenna proteins pathway.

The red squares are presenting the up-regulated gene expressions.

3.2. Freezing Treatment Analysis

3.2.1. Electrolyte Leakage Test

Before cold treatment, the leakage of leaves was measured 12 hours after being treated with ANE and water treatments. The leakage of ANE is 12.57%, slightly higher than water (7.94%), p value is $0.053 > 0.05$, which means the ANE treatment is not good for plant, well harm to plant. More, the two electrolyte leakage tests measured the leakage after freezing conditions (-4°C) for 24 hours, and both the first and the second results showed the leaves treated with water treatments leakage increased a lot. In the freezing treatments, the leakage of water was 10.07% and 9.55%, showing much higher than 7.94% leakage in the cold treatment. To the contrary, the ANE treatment showed no change, but the leakage was still higher than water treatments overall (Fig. 20).

Comparing the change in electrolyte leakage before and after the freezing treatment, Figure 21 shows the ANE treatment exhibited a leakage decrease (-2.03 %) between experiments while the water treatment shows a large increase in leakage (23.55 %). That indicates ANE may have positive effect to increase plants cold resistance in other way.

More, 1mL/L solution of ANE was sprayed on leaves to replicate the experiment with a lower concentration of treatment solution. The average leakage of ANE treatment is 50.61% after cold stress, which is much lower than 83.57% of water treatments, p value is $0.019 < 0.05$, the average leakage between ANE and water treatments are significantly different (Fig.23).

3.2.2. Chlorophyll Test

Similar to the electrolyte leakage test, the chlorophyll content of the ANE-treated *A. donax* L. and H_2O as control were tested before and after freezing conditions. The results showed no significant difference between two treatments overall (Fig 22).

Again, when spraying 1ml/L ANE on leaves, the chlorophyll content shows positive results comparable to the electrolyte leakage results. Although the average chlorophyll content of ANE and water treatments (C1 and C2) have no difference before the cold stress, the average 0.018ml/g of ANE chlorophyll content after cold stress was much lower than 0.0086ml/g that of water treatments, p value is 0.056>0.05 (Fig.24).

3.2.3. Field Work

The measured soil temperature range was -2.5°C to -1.6°C (Table 11) in which falls within the range of changes in annual mean soil temperature during the twentieth century (−3° to 3°C) from place to place in Canada (Zhang, 2005). This illustrates that the green roof recovery experiment is similar to the conditions of field work. The recovery rate (sprout number emergence in spring) showed that the ANE treatments rate of recovery (70%) is slightly higher than water and chitosan, both are 60% recovery.

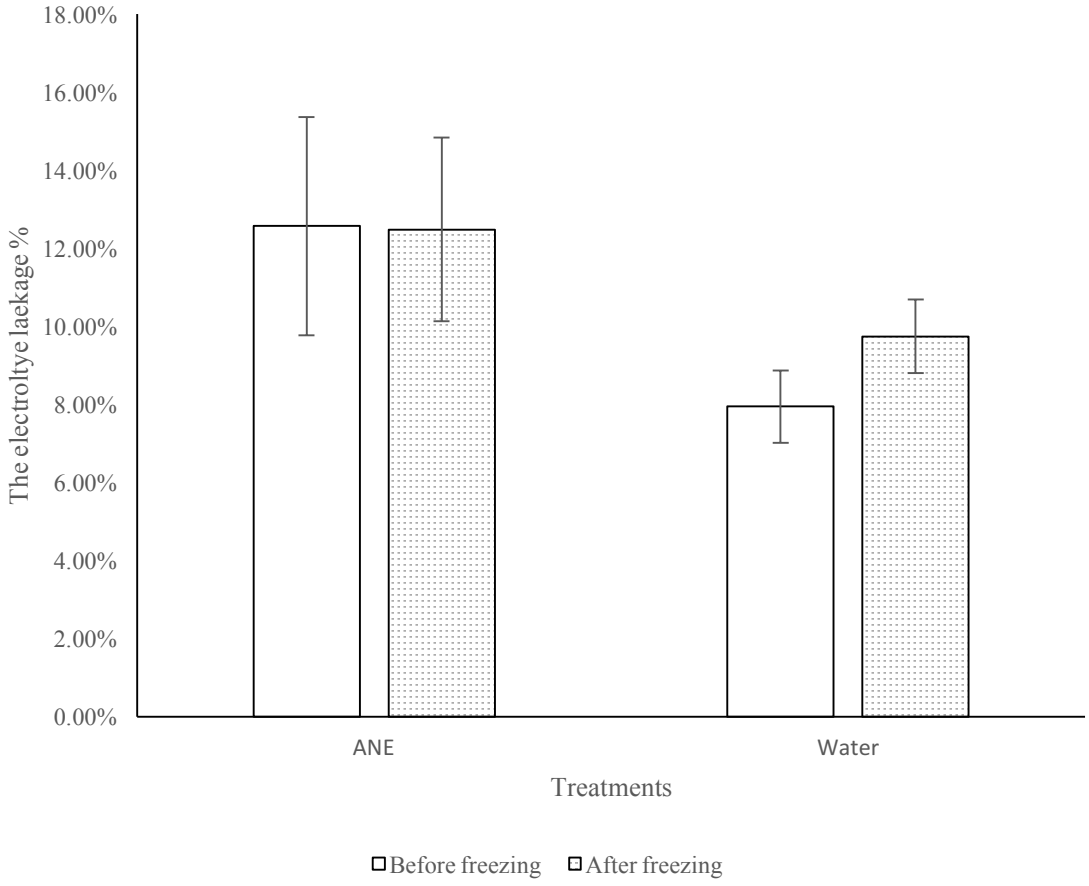


Figure 20. The electrolyte leakage of ANE-treated (3ml/L) and water-treated *A. donax* L. before freezing stress and after.

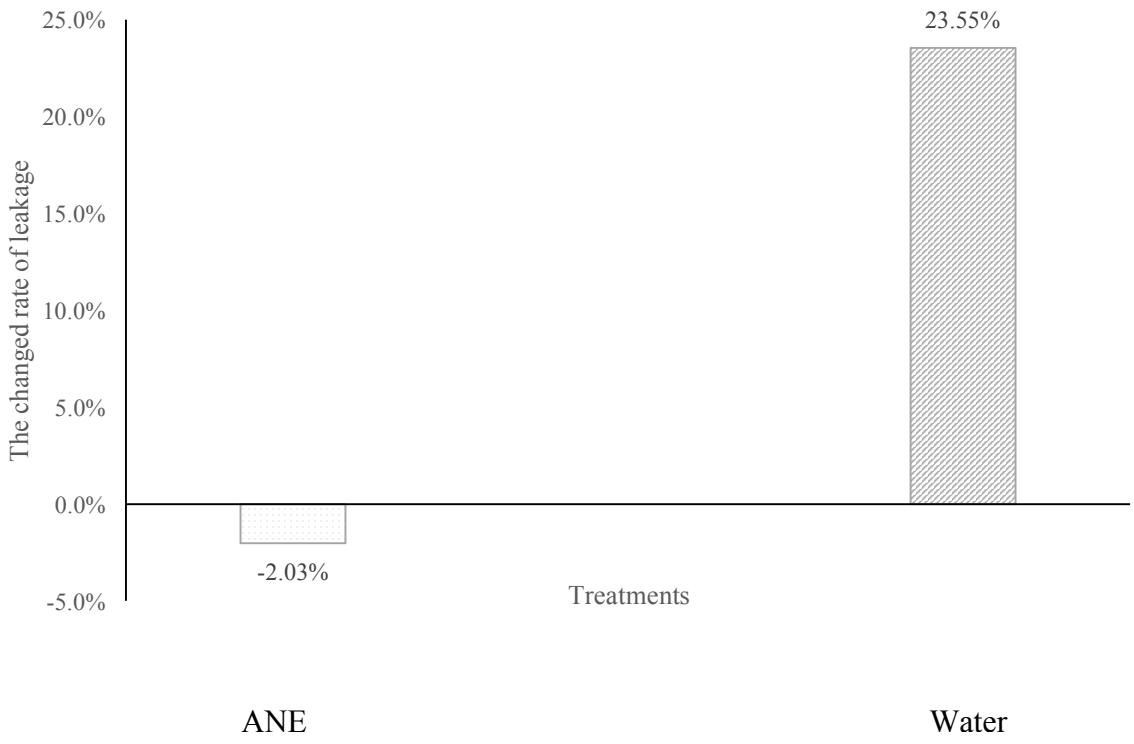


Figure 21. The changed electrolyte leakage of ANE-treated compared with water-treated *A. donax* L. before freezing treatment and after.

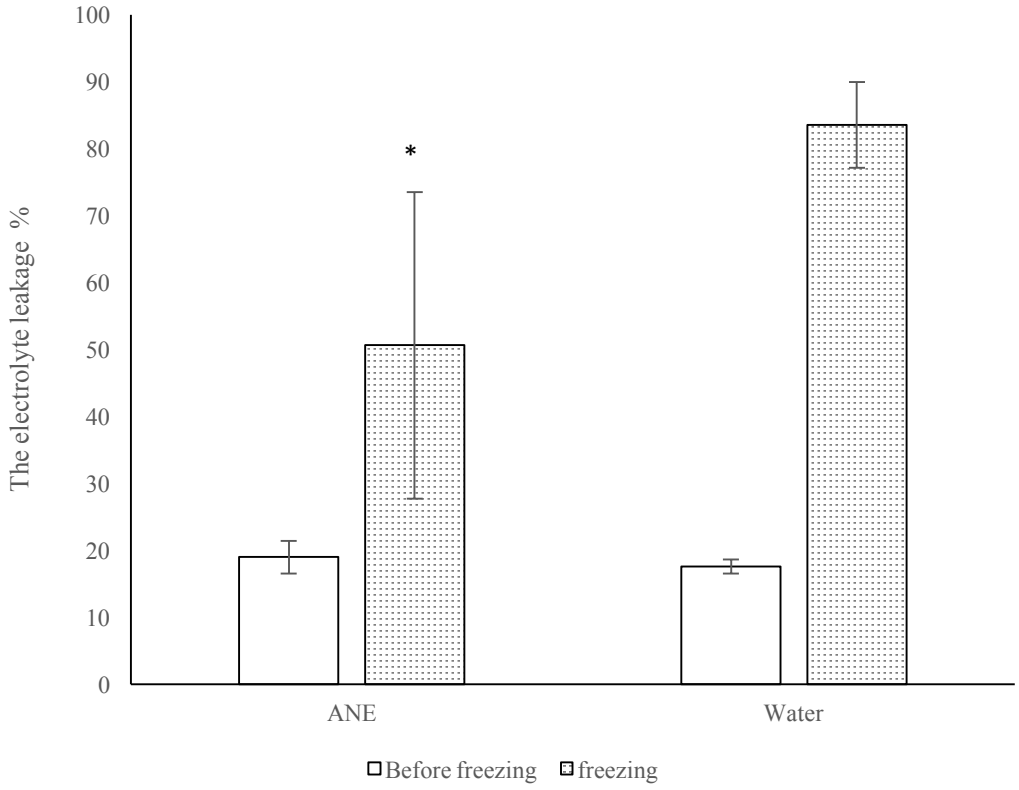


Figure 22. The electrolyte leakage of ANE-treated (1ml/L) and water-treated *A. donax* L. before and after 24 hours freezing treatments. $P<0.05$.

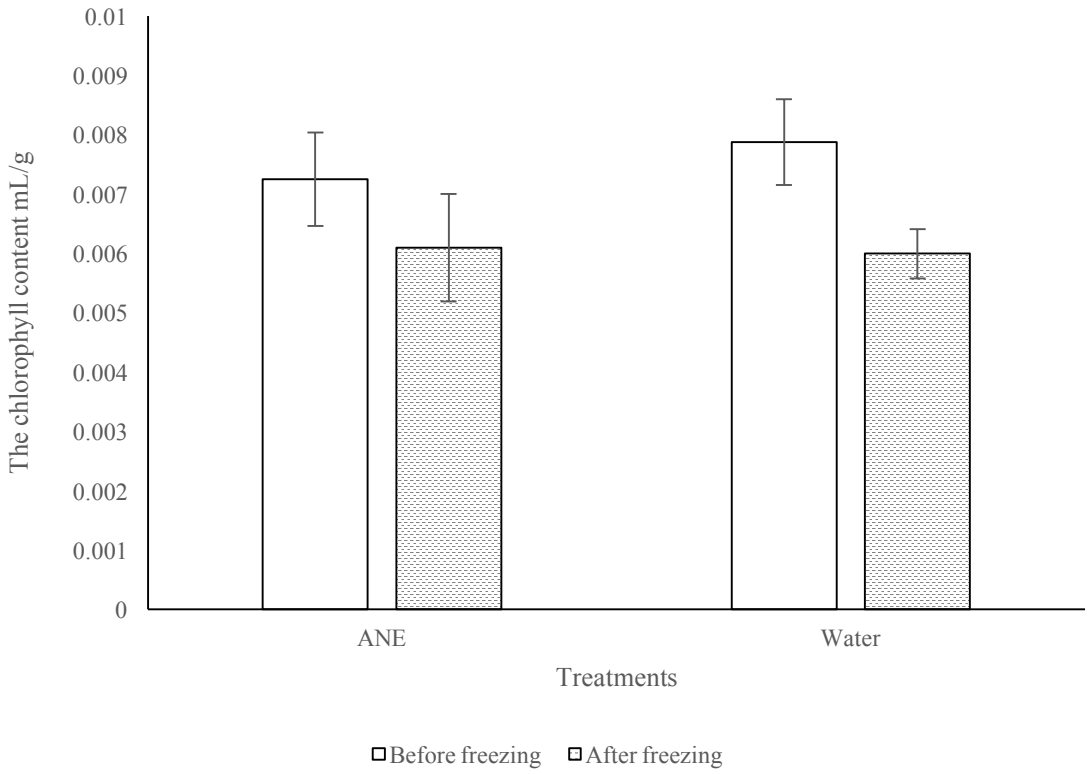


Figure 23. The chlorophyll content of ANE (3mL/L) and water-treated *A. donax* L. before freezing treatment and after freezing treatments. P >0.05

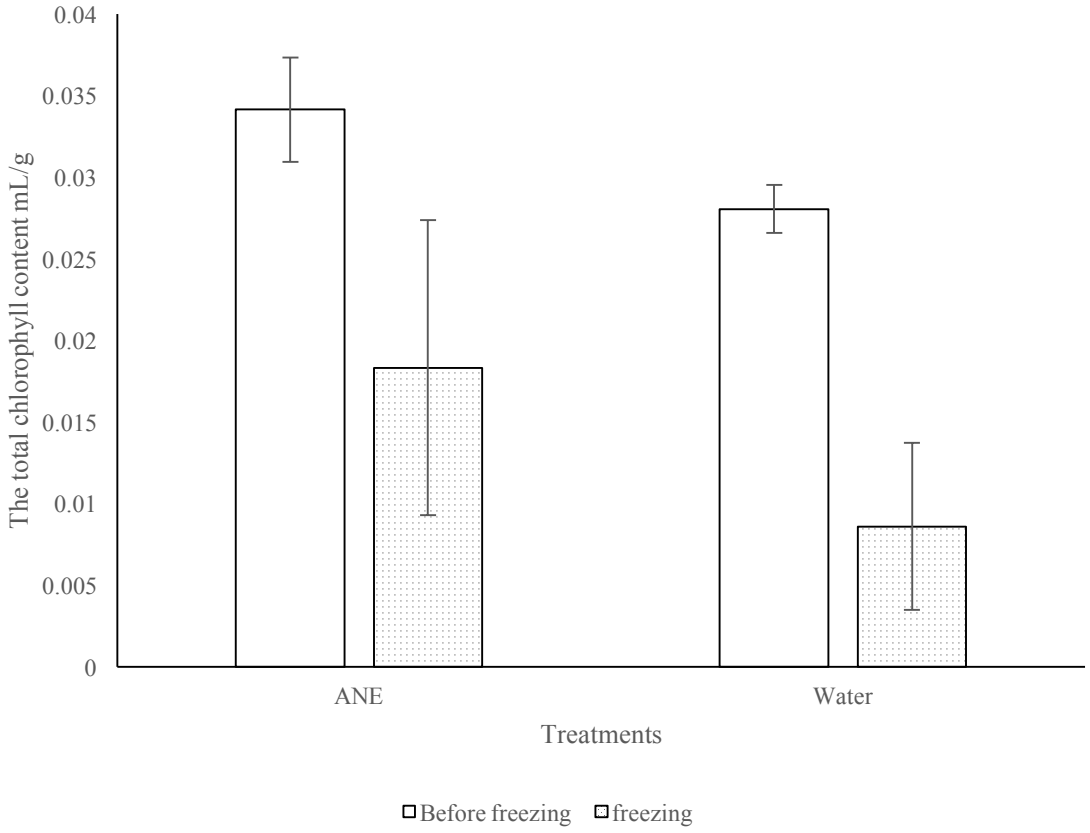


Figure 24. The chlorophyll content of ANE-treated (1ml/L) and water-treated *A. donax* L. after and before 24 hours freezing treatment. P>0.05.

H_2O treatments

ANE treatments

Chitosan treatments

Figure 25. The sprout situation of H_2O, ANE and Chitosan-treated *A. donax* L. in the spring, 2016.

Table 12. The changes of temperature from Dec. 14, 2015 to May. 14, 2016.

Date	Air-temperature	Soil-temperature	
1 (Dec. 14, 2015)	0/-2 °C	2.3°C	2.9°C
2 (Feb. 14, 2016)	-10/-16°C	-2.5°C	-1.6°C
3 (May. 14, 2016)	17/13°C	16.3°C	15.6°C

Table 13. The sprout number of *A. donax* L. in May 14, 2016.

Treatment	Plant Numbers, before winter	Sprout Numbers, after winter	Survival rate (%)
ANE	20	14	70%
H_2O	20	12	60%
Chitosan	20	12	60%

Discussion

4.1. Transcriptome analysis

4.1.1. Sequencing & reconstruction assembly

Only 47 sequences of *A. donax* L. have been found in Genbank. Because my plant is a kind of grass, and its completely lack of information for analyzing this species genetically (Sablok, 2014). Thus, the sequencing analysis and reconstruction methods seem to be more important than any other species with genomes. Compare to microarray, RNA-seq is more a powerful tool to develop a genomic resource for any interesting species. Trinity, the reconstruction tool for species without reference genome, has been reported to present an efficiently assembly than Velvet according to the de-Bruijin's graph theory (Grabherr, 2011). The clean reads, the GC content, sequencing error (Q20, Q30) are performed with Phred score to evaluate the sequencing (Jiang, 2011). The results of application of those methods to the *A. donax* L. analysis are acceptable. Due to lack of a reference genome, the putative unigenes have been mapped with closer species. The most similar species is *Setaria italica* (35.5%), followed by *Oryza sativa* (7.6%), *Sorghum bicolor* (15.7%), *Zea mays* (15.3%). *Setaria italic* are able to more efficiently fix carbon in drought, high temperatures, and limitations of nitrogen or CO_2. It has been demonstrated that plants like that are always being bioenergy crops because of its efficient conversion of solar light into biomass and energy (Brutnell, 2010). This result suggests that *A. donax* could become a new prospective bioenergy crop genetically.

4.1.2. Gene functional analysis

4.1.2.1. Growth development

Functional annotation of the assembled transcripts provides a huge picture of one specie's genomic resources. All the changed genes expression after ANE, BR treatments make it easy to compare genes involved in traits of biological process and molecular function or some economic and ecological importance. According to the previous experiment, it was found all the pathways to synthetize BR were up-regulated after 12 hours ANE treatment in soybeans. Thus, it might suggest that ANE promotes plant growth by promoting BR production. However, the annotation and DEGs analysis did not showing any connection between ANE and BR treatments.

Volcano and Venn diagrams directly show some gene expression increased and decreased after treatments (Fig 13 and 14), and some of them are overlapped between ANE and BR treatments. Among the genes with significant up-regulated expression, 7 genes in ANE treatment and 5 genes in BR treatments are related to plant development. Chloroplastic glucose-6-phosphate/phosphate translocator 2 (GPT2) and protein c chloroplast import apparatus 2 (CIA2) are the genes both found in ANE and BR treatments. GPT2 is the transporter, responsible for transport of Glc6P into plastids of heterotrophic tissues, which is involved in starch biosynthesis (Kunz, 2010); CIA is a regulation gene involved in the general chloroplast protein import pathway regulation, including protein import and protein translation efficiencies (Sun, 2009). Those genes are all related to plant photosynthesis promoting plant growth. Besides, there are two genes associated with cell wall, one is cell wall-associated hydrolase (MTR-069s1001), another

is expansin-A4 (EXPA4). EXPA4 was identified many years ago, associated with studies of the mechanism of plant cell wall enlargement. It is response to the cell growth of plant resulting loosening the cell wall disrupting non-covalent bonding between cellulose microfibrils and matrix glucans (Cosgrove, 2000). The higher increased expression of cell wall associated hydrolase gene is recently founded in *Medicago truncatula* without knowing function. It might take some action like expansins in the cell wall.

The up-regulation genes between ANE and BR are not many, there is weakly evidence to illustrate the relationship between ANE and BR. But ANE and BR can improve *A. donax* L., and ANE are more effect to help plant development in genetic level.

4.1.2.2.Cold resistance

To assess the effect of ANE and BR treatments on *A. donax* L. gene expressions, 145 significantly up-regulated genes of ANE and 69 genes of BR treatments were analyzed in all 71,267 changed genes data. Eleven of 145 up-regulated genes in ANE were associated with plants cold stress, with only one of 11 found in BR treatment.

Abscisic acid-like, stress-like and Glutathione-like genes were found up-regulated expression after ANE treatment. Chitinase 11 was the only one were showing both in ANE and BR treated plants related to cold resistance. Chitinase is the enzyme to degrade chitin that is the second abundant polysaccharides in nature (Hamid, 2013). Plant chitinase presents in plant to regulate the development and response to environment stresses, such as drought, cold and high salinity (Kasprzewska, 2003). Chitinase is induced by many pathogens or elicitors that is important involving plant defense system

(Nakamura, T. 2008). Application of ANE were used in pathogen infected carrots, which induce some defense-related enzymes, including peroxidase, and chitinase (Jayaraj J, 2007). This indicated that ANE enhanced disease resistance in carrot through inducing defense genes expression or enzymes activities. Chitinase has expression both in BR-treated and ANE treatment, which may indicate that BR and ANE treatment could improve *A. donax* L. cold tolerance. It is shown chitinase inhibitor cold stress. Interestingly, it has been reported that plant antifreeze proteins (AFPs) can inhibit plants ice crystal growth in extreme cold temperatures, but they are not unique proteins. Some AFPs found in winter rye are chitinase, glucanase and thaumatin (Yeh, 2000). The chitinase-APFs extracted from winter rye did not directly response to cold, but accumulated as part of plant growth and development (Griffith, 2004).

4.2. Electrolyte leakage

Cell membranes will be damaged by the low temperature. Electrolyte leakage is a way to test the cell membrane penetrability, the degree of cell membrane damage is reflected by the intracellular electrolyte leakage rate (Liu, 2013). In the present experiment, the electrolyte leakage of ANE treatment was much higher than the control in normal temperatures and slightly higher than control after freezing stress. This means the ANE may have negative effect on plant no matter in normal or cold stress. However, compared with the results between non-freezing and freezing condition, the ANE treatment was showing protection after freezing, because of no increased electrolyte leakage. In contrast, the leakage of water increased significantly after cold stress.

According to the genes expression results, gene expressions related to the cell well were higher increased, including cell wall-associated hydrolyse protein and expansin may result in the loosening of cell wall for developing purpose. This can also increase the cell membrane penetrability leading the increasing electrolyte leakage.

The concentration of ANE used were 3ml/L, the same as instructed on the label of the ANE product (@ Acadia Seaplants Company), which was good for soybean in an other project in our lab, seems to have harmed to *A. donax* L. The 1ml/L ANE solution was used and indicated that higher concentration could be a negative factor to effect on electrolyte leakage of ANE applying on to *A. donax* L..

4.3. Chlorophyll content

Chlorophyll content can reflect nutrient stress in general, and in particular can show the nitrogen or sulfur stress (Haboudane, 2002). Mostly, cold stress will prevent the synthesis of chlorophyll formation in plant leaves, inducing the reduce of chlorophyll content (Nouri, 2015). The previous studies have shown ANE promote the production of chlorophyll comparison with control (water). However, there was no significant difference between ANE and control in this experiment. Due to the cold stress, the chlorophyll content decreased slightly in cold tolerate plants and significantly in cold sensitive plants. *A. donax* L., the C_3 plant is more advantage over the C_4 plant in cold condition. Thus, the chlorophyll content of ANE-treated in non-freezing and after freezing should higher than H_2O in the initial hypothesis. The negligible difference between ANE and H_2O may cause by the time to effect the plant. The expression of

several genes showing up-regulated of chloroplast or photosynthesis. Might the 12 hours ANE-treatment did not induce any significant physiological changes.

Similar as electrolyte leakage results, the 1ml/L ANE showed a significant decreasing comparing with water treatments after 24 hours' freezing stress. Although the content of ANE before cold stress did not showing any difference with control, either. The reason might the 12-hours ANE treatment didn't take effect on the plants.

4.4. Field work

The green roof growth condition is effected by sunlight coming through the around buildings, which means each treatment may have different sunlight absorption. The *A. donax* L. recovery is little higher than water and chitosan, but not significantly. May because the position of the ANE box is at the shade of the building. The optimum growth condition is very important condition for plants growth outside.

Conclusion

Ascophllum nodosum is a brown algae and its commercial product extraction can increase plant growth, elevated resistance to biotic and abiotic stress, and enhanced post harvest shelf-life of perishable products (Arioli, 2015). Brassinosteroids (BRs), are a group of plant steroids that are known to elicit remarkable growth responses. A previous study in our lab indicated that after ANE treatment, the expression of many genes related to plant growth were regulated, especially in BRs biosynthesis pathway (Chai, 2014). Almost all of the enzymes in this pathway were showing up-regulated. Thus, it was hypothesized that ANE regulates plant growth through inducing BR synthesis. The present study investigated the effect of ANE and Br treatments on *Aroudo donax* L. gene expression, chlorophyll content and electrolyte leakage.

The results indicate that there were many genes related to plant development and cold resistance up-regulated after ANE treatment (3 ml/L) compared with BR treatment and water control. The gene expressions of ANE and BR are significantly different suggesting that ANE regulates many plant development pathways, not only the BR synthesis pathway.

High concentration of ANE (3 ml/L) had no effect on the chlorophyll and foliar electrolyte leakage, while 1 ml/L ANE treatment significantly decreased foliar electrolyte leakage and increased chlorophyll content after freezing stress.

In conclusion, ANE has great effects on *A. donax* L. stress-defense system and growth development in gene levels, and increased plant cold stress resistance at low ANE concentration (1 ml/L).

References

Aftab, T. K. (2011). Enhancing the growth, photosynthetic capacity and artemisinin content in Artemisia annua L. by irradiated sodium alginate. Radiation Physics and Chemistry, 80(7), 833-836.

Ajay Arora, R. K. (2002). Oxidative stress and antioxidative system in plants. Current Science, 82(10), 1227-1238.

Allen, V. G. (2001). Tasco: Influence of a brown seaweed on antioxidants in forages and livestock—A review 1. J. Anim. Sci., 79, 21-31.

Alvira, P. T.-P. (2010). Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. Bioresource Technology, 101(13), 4851-4861.

Arioli, T. M. (2015). Applications of seaweed extracts in Australian agriculture: past, present and future. Journal of Applied Phycology, 27(5), 2007-2015.

Arioli, T. M. (2015). Applications of seaweed extracts in Australian agriculture: past, present and future. Journal of Applied Phycology, 27(5), 2007-2015.

Arthur, D. G. (2003). Effect of a seaweed concentrate on the growth and yield of three varieties of Capsicum annuum. South African Journal of Botany, 69(2), 207-211.

Asada, K. (1992). Ascorbate peroxidase – a hydrogen peroxide-scavenging enzyme in plants. Physiologia Plantarum, 85(2), 235-241.

Ayad, J. Y. (1998). The effect of seaweed (Ascophyllum nodosum) extract on antioxidant activities and drought tolerance of tall fescue (Festuca arundinacea Schreb.).

Aziz, N. M. (2011). Growth, flowering and chemical constituents performence of Amaranthus tricolor plants as influenced by seaweed (Ascophyllum nodosum) extract application under salt stress conditions. J Appl Sci Res(7), 1472-1484.

Bajguz, A. H. (2009). Effects of brassinosteroids on the plant responses to environmental stresses. Plant Physiol Biochem, 47(1), 1-8.

Bajguza, A. T. (2003). The chemical characteristic and distribution of brassinosteroids in plants. Phytochemistry, 62(7), 1027-1046.

Bajji, M. K.-M. (2001). The use of the electrolyte leakage method for assessing cell

membrane stability as a water stress tolerance test in durum wheat. Plant Growth Regulation (00), 1-10.

Belkhadir, Y. J.-P. (2012). Brassinosteroids modulate the efficiency of plant immune responses to microbe-associated molecular patterns. Proc Natl Acad Sci U S A., 109(1), 297-302.

Bishop, G. J. (2002). Brassinosteroids and Plant Steroid Hormone Signaling. Plant Cell, 14, s97-s110.

Blunden, G. C. (1986). The characterisation and quantitative estimation of betaines in commercial seaweed extracts. Bot. Marina, 29, 155-160.

Blunden, G. J.-W. (1996). Enhanced leaf chlorophyll levels in plants treated with seaweed extract. Journal of Applied Phycology, 8(6), 535-543.

Bonke, M. T.-T. (2003). APL regulates vascular tissue identity in Arabidopsis. Nature, 426, 181-186.

Bowler, C. M. (1992). Superoxide Dismutase and Stress Tolerance. Annual Review of Plant Physiology and Plant Molecular Biology, 43, 83-116.

Boyer, G. L. (1987). Identification of abscisic acid in the seaweed Ascophyllum nodosum. Phytochemistry, 27(5), 1521-1522.

Brain, K. R. (1973). Cytokinin activity of commercial aqueous seaweed extract. Plant Sci Lett, 1, 241-245.

Brutnell, T. P. (2010). Setaria viridis: A Model for C4 Photosynthesis. The Plant Cell, 22, 2537-2544.

Buggeln, R. a. (1971). Evaluation of Evidence for the Presence of Indole-3-Acetic Acid in Marine Algae. Planta, 8, 503-508.

C.M.J. Williams, T. B. (2008). Use of saline land and wastwater for growing a potential biofuel crop (*Arundo donax* L.). Irrigation Australia 2008 Conference. Melbourne: Irrigation Australia 2008 Conference.

Camera, S. L. (2009). The Arabidopsis Patatin-Like Protein 2 (PLP2) Plays an Essential Role in Cell Death Execution and Differentially Affects Biosynthesis of Oxylipins and Resistance to Pathogens. MPMI, 22(4), 469-481.

Carpita, N. C. (2008). Maize and sorghum: genetic resources for bioenergy grasses. Trends in Plant Science, 13(8), 415-420.

Ceotto, E. C. (2010). Shoot cuttings propagation of giant reed (*Arundo donax* L.) in water and moist soil: The path forward? Biomass and Bioenergy, 34(11), 1614-1623.

Chandra, R. T. (2012). Methane production from lignocellulosic agricultural crop wastes: A review in context to second generation of biofuel production. Renewable and Sustainable Energy Reviews, 16(3), 1462-1476.

Chai, Lihong. (2014). From communication. (A visiting Professor in Saint Mary's University)

Chen, Z. S. (1993). Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. Science, 262(5141), 1883-1886.

Chiba, Y. S. (2015). Identi cation of Arabidopsis thaliana NRT1/PTR FAMILY (NPF) proteins capable of transporting plant hormones. J Plant Res, 128, 679-686.

Choi, D. L.-T. (2003). Regulation of Expansin Gene Expression Affects Growth and Development in Transgenic Rice Plants. The Plant Cell, 15, 1386-1398.

Chouliaras, V. T. (2009). The effects of a seaweed extract in addition to nitrogen and boron fertilization on productivity, fruit maturation, leaf nutritional status and oil quality of the olive (Olea europaea L.) cultivar Koroneiki. Science of Food and Agriculture, 89(6), 984-988.

Ciarmiello, L. F. (2011). Plant Genes for Abiotic Stress. In A. S. Venkateswarlu, Abiotic Stress in Plants - Mechanisms and Adaptations (pp. 283-308). Rijeka: InTech.

Corno, L. P. (2014). *Arundo donax* L.: A non-food crop for bioenergy and bio-compound production. Biotechnology Advances, 32(8), 1535-1549.

Cosgrove, D. J. (2000). Loosening of plant cell walls by expansins. Nature, 407, 321-326.

Cottrell, J. S. (2015). "Fossil Fuel to Renewable Energy: Comparator Study of Subsidy Reforms and Energy Transitions in African and Indian Ocean Island States. Incheon: United Nations Office for Sustainable Development.

Craigie, J. S. (2011). Seaweed extract stimuli in plant science and agriculture. J Appl Phycol, 23, 371-393.

Cramer, G. R. (2011). Effects of abiotic stress on plants: a systems biology perspective. BMC Plant Biology (11), 163-177.

Crouch I. J., v. S. (1993a). Effect of seaweed concentrate from Ecklonia maxima (Osbeck) Papenfuss on Meloidogyne incognita infestation on tomato. J. Appl Phycol, 5, 37-43.

Crouch, I. J. (1992). Effect of seaweed concentrate on the establishment and yield of greenhouse tomato plants. Journal of Applied Phycology, 4(4), 291-296.

Crouch, I. J. (1993). Effect of seaweed concentrate fromEcklonia maxima (Osbeck) Papenfuss onMeloidogyne incognita infestation on tomato. Journal of Applied Phycology, 5(1), 37-43.

Crouch, I. J. (1993). Evidence for the presence of plant growth regulators in commercial seaweed products. Plant Growth Regulation, 13(1), 21-29.

Demidchik, V. S. (2014). Stress-induced electrolyte leakage: the role of K+-permeable channels and involvement in programmed cell death and metabolic adjustment. Journal of Experimental Botany.

Demirevska, K. S.-S. (2008). Rubisco and some chaperone protein responses to water stress and rewatering at early seedling growth of drought sensitive and tolerant wheat varieties. Plant Growth Regul, 56, 97-106.

Dewey, B. L. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics, 12, 323.

Dhir, S. K. (2010). Optimization and transformation of *Arundo donax* L. using particle bombardment. African Journal of Biotechnology, 9(39), 6460-6469.

Dixon, G. &. (2002). Suppressing Pythium ultimum induced damping-off in cabbage seedlings by biostimulation with proprietary liquid seaweed extracts Managing soil-borne pathogens: A sound rhizosphere to improve productivity in intensive horticultural systems. Proceedings of the XXVI International Horticultural Congress, (pp. 11-17). Toronto.

Dominguez, P. G. (2015). ASR1 transcription factor and its role in metabolism. Plant Signaling & Behavior, 10(4), e992751-1-e992751-5.

Eijck, J. V. (2014). Current and future economic performance of first and second generation biofuels in developing countries. Applied Energy, 135(15), 115-141.

Ellegren, H. S.-B. (2012). The genomic landscape of species divergence in Ficedula flycatchers. Nature(491), 756-760.

Eris, A. S. (1995). The Effects of Seaweed Extract (Ascophyllum nodosum) on Yield and Quality Criteria in Peppers. Acta horticulturae(412), 185-192.

Fageria, N. K. (2013). The Role of Plant Roots in Crop Production. Boca Raton: CRC Press.

Fariduddin, Q. K. (2009). Effect of 28-homobrassinolide on the drought stress-induced changes in photosynthesis and antioxidant system of Brassica juncea L. Acta Physiologiae Plantarum, 31(5), 889-897.

Fornes, F. S.-P. (1995). Effect of a seaweed extract on citrus fruit maturation. International Symposium on Quality of Fruit and Vegetables: Influence of Pre- and Post- Harvest Factors and Technology (p. 379). ActaHortic.

Fornes, F. S.-P. (2002). Effect of a Seaweed Extract on the Productivity of 'de Nules' Clementine Mandarin and Navelina Orange. Botanica Marina, 45, 486-489.

Götz, S. e. (2008). High-throughput functional annotation and data mining with the Blast2GO suite. Nucl. Acids Res., 36(10), 3420-3435.

Gall, H. L.-M. (2015). Cell wall metabolism in response to abiotic stress. Plants(4), 112-166.

Galletti, A. M. (2013). From giant reed to levulinic acid and gamma-valerolactone: A high yield catalytic route to valeric biofuels. Applied Energy, 102, 157-162.

Gitelson, A. A. (2003). Relationships between leaf chlorophyll content and spectral reflectance and algorithms for non-destructive chlorophyll assessment in higher plant leaves. Journal of Plant Physiology(160), 271-282.

Grabherr, M. G. (2011). Trinity: reconstructing a full-length transcriptome without a genome from RNA-Seq data. Nat Biotechnol, 29(7), 644-652.

Griffith, M. Y. (2004). Antifreeze proteins in overwintering plants: a tale of two activities. Trends in Plant Science, 9(8), 399-405.

Gupta, A. S. (1993). Increased resistance to oxidative stress in transgenic plants that overexpress chloroplastic Cu/Zn superoxide dismutase. Proc. Nati. Acad. Sci. USA, 90, 1629-1633.

H. H. Kunz, R. E.-I. (2010). The role of plastidial glucose-6-phosphate/phosphate translocators in vegetative tissues of Arabidopsis thaliana mutants impaired in starch biosynthesis. Plant Biology, 12, 115-128.

Haas, B. J. (2013). De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. Nature Protocols(8), 1494-1512.

Haboudane, D. M. (2002). Integrated narrow-band vegetation indices for prediction of crop chlorophyll content for application to precision agriculture. Remote Sens Environ., 81(2), 416-426.

Hamid, R. K. (2013). Chitinases: An update. J Pharm Bioallied Sci., 5(1), 21-29.

Hatfield, J. L. (2015). Temperature extremes: Effect on plant growth and development. Weather and Climate Extremes, 10, 4-10.

Hegazi, A. N.-S. (2015). Alleviation of Salt Stress Adverse Effect and Enhancing Phenolic Anti-oxidant Content of Eggplant by Seaweed Extract. Gesunde Pflanzen(67), 21-31.

Hien, N. Q. (2000). Growth-promotion of plants with depolymerized alginates by irradiation. Radiation Physics and Chemistry, 59(1), 97-101.

Hiscox, J. D. (1979). A method for the extraction of chlorophyll from leaf tissue without maceration. Canadian Journal of Botany, 57, 1332-1334.

Holmström, K. O. (2000). Improved tolerance to salinity and low temperature in transgenic tobacco producing glycine betaine. J Exo Bot, 51(343), 177-185.

I. Jeannin, J.-C. L.-F.-G. (1991). The Effects of Aqueous Seaweed Sprays on the Growth of Maize. Botanica Marina, 34, 496-473.

Iris Lewandowski, J. M. (2003). The development and current status of perennial rhizomatous grasses as energy crops in the US and Europe. Biomass and Bioenergy, 25(4), 335-361.

Jayaraj J, P. Z. (2007). Combined expression of chitinase and lipid transfer protein genes in transgenic carrot plants enhances resistance to foliar fungal pathogens. . Plant Cell Rep., 26(9), 1539-46.

Jayaraj, J. W. (2008). Seaweed extract reduces foliar fungal diseases on carrot. Crop Protection, 27(10), 1360-1366.

Jayaraman, J. N. (2011). Commercial extract from the brown seaweed *Ascophyllum nodosum* reduces fungal diseases in greenhouse cucumber. Journal of Applied Phycology, 23(3), 353-361.

Jeannin, I. L.-C.-G.-F. (1991). The effects of aqueous seaweed sprays on the growth of maize. Botanica Marina, 34(6), 469-474.

Jiang, L. S. (2011). Synthetic spike-in standards for RNA-seq experiments. Genome research 21, 1543-1551.

John, M. R. (1997). Cell signalling by oligosaccharides. Trends in Plant Science, 2(3), 111-115.

Jordan Vacheron, G. D.-L.-L.-D.-C. (2013). Plant growth-promoting rizobacteria and root system functing. Frontiers in Plant Science(4), 356.

Jung, Y. J. (2015). Universal Stress Protein Exhibits a Redox-Dependent Chaperone Function in Arabidopsis and Enhances Plant Tolerance to Heat Shock and Oxidative Stress Front. Front. Plant Sci., 6, 1141.

Kanehisa, M. A. (2008). KEGG for linking genomes to life and the environment. Nucleic Acids research, 36, D480=D484.

Kasprzewska, A. (2003). Plant chitinases--regulation and function. Cell Mol Biol Lett., 8(3), 809-24.

Kazutoshi, N. (2002). Seaweeds Kaiso; Bountiful harvest from the seas: Sustenance for health and well being by preventing common lifestyle related diseases. Japan Seaweed Association.

Khan, W. R. (2009). Seaweed Extracts as Biostimulants of Plant Growth and Development. Journal of Plant Growth Regulation, 28(4), 386-399.

Khan, W. Z. (2012). Commercial Extract of *Ascophyllum nodosum* Improves Root

Colonization of Alfalfa by Its Bacterial Symbiont Sinorhizobium meliloti. Communications in Soil Science and Plant Analysis, 43(8), 2425-2436.

Khana, W. P. (2013). *Ascophyllum nodosum* Extract and Its Organic Fractions Stimulate Rhizobium Root Nodulation and Growth of Medicago sativa (Alfalfa). Communications in Soil Science and Plant Analysis, 44(5), 900-908.

Kheshgi, H. S. (2000). The potential of biomass fuels in the context of global climate change: Focus on transprotation fuels. Annual Review of Energy and the Environment, 25, 199-244.

Klarzynski, O. D.-C. (2003). Sulfated Fucan Oligosaccharides Elicit Defense Responses in Tobacco and Local and Systemic Resistance Against Tobacco Mosaic Virus. Molecular Plant-Microbe Interactions, 16(2), 115-122.

Koçar, G. C. (2013). An overview of biofuels from energy crops: Current status and future prospects. Renewable and Sustainable Energy Reviews, 28, 900-916.

Kollner, T. G. (2008). Protonation of a Neutral (S)- -Bisabolene Intermediate Is Involved in (S)- -Macrocarpene Formation by the Maize Sesquiterpene Synthases TPS6 and TPS11. The Journal of Biological Chemistry, 283(30), 20779-20788.

Kunz HH, H. R. (2010). The role of plastidial glucose-6-phosphate/phosphate translocators in vegetative tissues of Arabidopsis thaliana mutants impaired in starch biosynthesis. Plant Biology, 12(s1), 115-128.

Lane, C. E. (2006). A multi-gene molecular investigation of the kelp(Laminariales, phaeophyceae) supports substantial taxonomic re-organization. Journal of Phycology, 42(2), 493-512.

Laporte, D. V. (2007). Structurally unrelated algal oligosaccharides differentially stimulate growth and defense against tobacco mosaic virus in tobacco plants. Journal of Applied Phycology(19), 79-88.

Lee, B. H. (2010). Phenotypic Analysis of Arabidopsis Mutants: Electrolyte Leakage after Freezing Stress. Cold Spring Harbour Protocols.

Lee, D. H. (2000). Chilling stress-induced changes of antioxidant enzymes in the leaves of cucumber: in gel enzyme activity assays. Plant Science, 159, 75-85.

Lee, S. L. (2004). Proteomic Identification of Annexins, Calcium-Dependent Membrane Binding Proteins That Mediate Osmotic Stress and Abscisic Acid Signal Transduction in Arabidopsis. Plant Cell, 16(6), 1378-1391.

Lewandowski, I. S. (2003). The development and current status of perennial rhizomatous grasses as energy crops in the US and Europe. Biomass Bioenergy, 25, 335–361.

Li, L. V. (1998). Effects of plant growth regulators on the antioxidant system in callus of two maize cultivars subjected to water stress. Plant Growth Regulation, 24(1), 55-66.

Liu, W. Y. (2013). The Low Temperature Induced Physiological Responses of Avena nuda L., a Cold-Tolerant Plant Species. . The Scientific World Journal, 658-793.

Lola-Luz, T. H. (2013). Effect on yield, total phenolic, total flavonoid and total isothiocyanate content of two broccoli cultivars (Brassica oleraceae var italica) following the application of a commercial brown seaweed extract (*Ascophyllum nodosum*). Agricultural and Food Science, 23(1), 28-37.

Luciana G. Angelini, L. C. (2009). Comparison of *Arundo donax* L. and Miscanthus x giganteus in a long-term field experiment in Central Italy: Analysis of productive characteristics and energy balance. Biomass and Bioenergy, 33(4), 635-643.

Mabeau, S. F. (1993). Seaweed in food products: biochemical and nutritional aspects. Trends in Food Science & Technology, 4(4), 103-107.

Mabeau, S. K. (1987). Isolation and Analysis of the Cell Walls of Brown Algae: Fucus spiralis, F. ceranoides, F. vesiculosus, F. serratus, Bifurcaria bifurcata and Laminaria digitata. Journal of Experimental Botany, 38(194), 1573-1580.

Macovei, A. V. (2012). A new DEAD-box helicase ATP-binding protein (OsABP) from rice is responsive to abiotic stress. Plant Signaling & Behavior, 7(9), 1138-1143.

Mandradia, B. D. (2012). Effect of seaweed extract on growth and yield of onion. International Journal of Farm Sciences, 2(1), 59-64.

Mantineoa, M. D. (2009). Biomass yield and energy balance of three perennial crops for energy use in the semi-arid Mediterranean environment. Field Crops Research, 114(2), 204-213.

Mao, X. C. (2005). Automated genome annotation and pathway identification using the KEGG Orthology (KO) as a controlled vocabulary. Bioinformatics, 21, 3787-3793.

Margulies, M. e. (2005). Genome sequencing in microfabricated high-density picolitre reactors. Nature (437), 376-380.

Mazorra, L. M.-B. (2002). Influence of Brassinosteroids on Antioxidant Enzymes Activity in Tomato Under Different Temperatures. Biologia Plantarum, 45(4), 593-596.

Mercier, L. L.-T.-T. (2001). The algal polysaccharide carrageenans can act as an elicitor of plant defence. New Phytologist, 149(1), 43-51.

Metz, B. D. (2007). Climate Change 2007 Mitigation. New York: Cambrige University Press.

Michael P. Fuller, S. B. (1998). Application of seaweed extract improves winter hardiness of winter barley. Journal of Experimental Botany .

Mittler, R. (2002). Oxidative stress, antioxidants and stress tolerance. Trends in Plant Science, 7(9), 405-410.

Naika, S. G. (2010). Production of first and second generation biofuels: A comprehensive review. Renewable and Sustainable Energy Reviews, 14(2), 578-597.

Nair, P. K. (2012). Transcriptional and metabolomic analysis of Ascophyllum nodosum mediated freezing tolerance in Arabidopsis thaliana. BMC Genomics, 13, 643.

Nam, K. H. (2002). BRI1/BAK1, a Receptor Kinase Pair Mediating Brassinosteroid Signaling. Cell, 110(2), 203-212.

Nathalie Durand, X. B. (2003). The effect of marine bioactive substances (N PRO) and exogenous cytokinins on nitrate reductase activity in Arabidopsis thaliana. Physiologia Plantarum, 119(4), 489-493.

Neily, W. S. (2010). Commercial Extract from the brown seaweed *Ascophyllum Nodosum* (Acadian) improves early establishment and helps resist water stress in vegetable and flower seedlings. HortScience, 45, 105-106.

Neven, L. G. (1992). Association of 70-Kilodalton Heat-Shock Cognate Proteins with Acclimation to Cold. Plant Physiol, 99(4), 1362-1369.

Norrie, J. &. (2006). Benefits of *Ascophyllum nodosum* marine-plant extract applications to Thompson seedless grape production. Acta Hortic, 243-248.

Nouri, N.-Z. M. (2015). Abiotic Stresses: Insight into Gene Regulation and Protein Expression in Photosynthetic Pathways of Plants. International Joural of Molecular Sciences, 16(9), 20392-20416.

Omer, A. M. (2007). Energy, Environment and Sustainable Development. Renewable and Sustainable Energy Reviews, 12(9), 2265-2300.

Osswald, E. F. (1994). Mechanisms of oxygen activation during plant stress. Proceedings of the Royal Society of Edinburgh. Section B. Biological Sciences, 102, 131-154.

Pal, A. D. (2015). Effect of seaweed saps on growth, yield, nutrient uptake and economic improvement of maize (sweet corn). Journal of Applied and Natural Science, 7(2), 970-975.

Papazoglou, E. G. (2007). Impact of high cadmium and nickel soil concentration on selected physiological parameters of *Arundo donax* L. European Journal of Soil Biology, 43(4), 207-215.

Papazoglou, E. K. (2005). Photosynthesis and growth responses of giant reed (Arundo donax L.) to the heavy metals Cd and Ni. Environmental International, 31(2), 243-249.

Patade, V. Y. (2013). Cold tolerance in Osmotin transgenic tomato (Solanum lycopersicum L.) is associated with modulation in transcript abundance of stress responsive genes. SpringerPlus, 2, 117.

Perdue, R. E. (1958). Arundo donax—Source of musical reeds and industrial cellulose. Economic Botany, 12(4), 368-404.

Pompeiano, A. V. (2015). Freeze tolerance and physiological changes during cold acclimation of giant reed [Arundo donax (L.)]. Grass and Forage Science, 70(1), 168-175.

Prasad, T. K. (1994). Evidence for Chilling-Induced Oxidative Stress in Maize Seedlings and a Regulatory Role for Hydrogen Peroxide. The Plant Cell, 6(1), 65-74.

Ragaglini, G. D. (2014). Suitability of giant reed (Arundo donax L.) for anaerobic

digestion: effect of harvest time and frequency on the biomethane yield potential. Bioresource Technology, 152, 107-115.

Ragauskas, A. J. (2006). The path forwardf for biofuels and biomaterials. Science, 311(5760), 484-489.

Rathorea, S. C. (2009). Effect of seaweed extract on the growth, yield and nutrient uptake of soybean(Glycine max) under rainfed conditions. South African Journal of Botany(75), 351-355.

Rayirath, P. B.-W. (2009). Lipophilic components of the brown seaweed, Ascophyllum nodosum, enhance freezing tolerance in Arabidopsis thaliana. Planta, 230(1), 135-147.

Rayorath, P. J. (2007). Rapid bioassays to evaluate the plant growth promoting activity of Ascophyllum nodosum (L.) Le Jol. using a model plant, Arabidopsis thaliana (L.) Heynh. Journal of Applied Phycology, 20(4), 423-429.

Rayorath, P. K. (2008). Extracts of the Brown Seaweed Ascophyllum nodosum Induce Gibberellic Acid (GA3)-independent Amylase Activity in Barley. J Plant Growth Regul, 27, 370-379.

Rengasamy, S. S. (2010). Effect of seaweed liquid fertilizer on the growth, biochemical constituents and yield of Tagetes erecta, under field trial. Journal of Phytology, 2(6), 61-68.

Ristic, Z. A. (1993). Changes in leaf ultrastructure and carbohydrates inArabidopsis thaliana L. (Heyn) cv. Columbia during rapid cold acclimation. Protoplasma, 172(2), 111-123.

Robert, H. S.-S. (2009). BTB and TAZ domain scaffold proteins perform a crucial function in Arabidopsis development. The Plant Journal, 58, 109-121.

Rossa, B. T. (1998). *Arundo donax* L. (Poaceae) — a C3 Species with Unusually High Photosynthetic Capacity. Plant Biology, 111(3), 216-221.

Rouhier, N. (2010). Plant glutaredoxins: pivotal players in redox biology and iron–sulphur centre assembly. New Phytologist, 186(2), 365-372.

Roussos, P. D.-K. (2009). Strawberry fruit quality attributes after application of plant

growth stimulating compounds. Scientia Horticulturae, 119(2), 138-146.

Sablok, G. F. (2014). Sablok, G., Fu, Y., Bobbio, V., Laura, M., Rotino, G. L., Bagnaresi, P., … Varotto, C. (2014). Fuelling genetic and metabolic exploration of C3 bioenergy crops through the first reference transcriptome of *Arundo donax* L. Plant Biotechnology Journal, 12(5), 554-567.

Sairam, R. (1994). Effects of homobrassinolide application on plant metabolism and grain yield under irrigated and moisture-stress conditions of two wheat varieties . Plant Growth Regulation (14), 173-181.

Sanderson, K. J. (1987). Auxin in a seaweed extract: Identification and quantitation of indole-3-acetic acid by gas chromatography-mass spectrometry. J Plant Physiol, 129, 363-367.

Scandalios, J. G. (1993). Oxygen Stress and Superoxide Dismutases. Plant Physiology, 101(1), 7-12.

Schievano, A. D. (2015). Biogas from dedicated energy crops in Northern Italy: electric energy generation costs. GCB Bioenergy, 7(4), 899-908.

Seca, A. M. (2000). Structural Characterization of the Lignin from the Nodes and Internodes of Arundo donax Reed. Journal of Agricultural & Food Chemistry, 48(3), 817-824.

Sénéchal, F. W. (2014). Homogalacturonan-modifying enzymes: structure, expression, and roles in plants. Journal of Experimental Botany, 65(18), 5125-5160.

Sharma, P. S. (2005). The molecular biology of the low-temperature response in plants. BioEssays, 27(10), 1048-1059.

Sharp, R. A. (2001). A new approach to seaweed management in Eastern Canada: the case of Ascophyllum nodosum. Cah. Biol. Mar, 42, 63-70.

Shehata, S. M.-A.-Y.-G. (2011). Effect of Foliar Spraying with Amino Acids and Seaweed Extract on Growth Chemical Constitutes, Yield and its Quality of Celeriac Plant. European Journal of Scientific Research, 58(2), 257-265.

Shewfelt, R. L. (1992). Response of plant membranes to chilling and freezing. Plant Membranes, 192-219.

Shiu, S. H. (2004). Comparative Analysis of the Receptor-Like Kinase Family in Arabidopsis and Rice. Plant Cell, 16(5), 1220-1234.

Sohal, M. T. (2013). Role of Elicitors in Inducing Resistance in Plants against Pathogen Infection: A Review. ISRN Biochemistry, 2013, 10.

Spinelli, F. F. (2010). A novel type of seaweed extract as a natural alternative to the use of iron chelates in strawberry production. Sci Hortic. Scientia Horticulturae, 125(3), 262-269.

Staden, W. A. (1997). Comparison of cytokinin-and auxin-like activity in some commercial seaweed extracts. J App Phy, 8, 503-508.

Stirk, W. A. (1997). Isolation and identification of cytokinins in a new commercial seaweed product made from Fucus serratus L. J Appl Phycol, 9, 327-330.

Storey, J. D. (2003). The positive false discovery rate: A Bayesian interpretation and the q-value. Annals of Statistics, 31, 2013-2035.

Subramanian, S. S. (2011). Extracts of the marine brown macroalga, Ascophyllum nodosum, induce jasmonic acid dependent systemic resistance in Arabidopsis thaliana against Pseudomonas syringae pv. tomato DC3000 and Sclerotinia sclerotiorum. European Journal of Plant Pathology, 131(2), 237-248.

Sukumaran, N. W. (1972). An excised leaflet test for evaluating potato frost tolerance. HortScience, 7, 467-468.

Sun CW, H. Y. (2009). CIA2 coordinately up-regulates protein import and synthesis in leaf chloroplasts. Plant Physiol, 150(2), 879-888.

Sun, C. H. (2009). CIA2 Coordinately Up-Regulates Protein Import and Synthesis in Leaf Chloroplasts. Plant Physiology, 150(2), 879-888.

Takagi, T. N. (2003). The Leaf-Order-Dependent Enhancement of Freezing Tolerance in Cold-Acclimated Arabidopsis Rosettes is not Correlated with the Transcript Levels of the Cold-Inducible Transcription Factors of CBF/DREB1. Plant and Cell Physiology, 44(9), 922-931.

Tanaka, Y. S. (2006). Ethylene Inhibits Abscisic Acid-Induced Stomatal Closure in Arabidopsis. Plant Physiology, 140(1), 396.

Temple, W. D. (1988). Effects of kelp (Macrocystis integrifolia) on soil chemical properties and crop response. Plant and Soil, 105(2), 213-222.

Thomashow, M. F. (2001). So what's new in the field of plant cold acclimation? lots! Plant Physiology , 125, 89-93.

Tracy, J. L. (1998). Suitability of classical biological control for giant reed (*Arundo donax*) in the United Stateds (Vol. 17). Holtville: University of California Cooperative Extension.

Ugarte, R. A. (2006). Changes in the brown seaweed Ascophyllum nodosum (L.) Le Jol. plant morphology and biomass produced by cutter rake harvests in southern New Brunswick, Canada. Eighteenth International Seaweed Symposium. 18, pp. 351-359. Bergen: Journal of Applied Phycology.

Van Overbeek, J. (1940). Auxin in marine algae. Plant Physiol, 15, 291-299.

Vardhini, B. V. (2003). Amelioration of osmotic stress by brassinosteroids on seed germination and seedling growth of three varieties of sorghum. Plant Growth Regulation, 41(1), 25-31.

Wally, M. N. (2012). Analysis of Seaweed Extract-induced Transcriptome Leads to Identification of a Negative Regulator of Salt Tolerance in Arabidopsis. HortScience, 47(6), 704-709.

Wang, Z. G. (2009). RNA-Seq: a revolutionary tool for transcriptomics. Nature Reviews Genetics (10), 57-63.

Washington, W. S. (1999). Effect of fungicides, seaweed extracts, tea tree oil, and fungal agents on fruit rot and yield in strawberry. Australian Journal of Experimental Agriculture, 39(4), 487-494.

Werner, T. M. (2001). Regulation of plant growth by cytokinin. PNAS, 98(18), 10487-10492.

Whapham, C. A. (1993). Significance of betaines in the increased chlorophyll content of plants treated with seaweed extract. Journal of Applied Phycology, 5, 231.

Wheeler, G. L. (1998). The biosynthetic pathway of vitamin C in higher plants. Nature, 393, 365-369.

Wu, Y. J. (1997). The role of betaines in alkaline extracts of Ascophyllum nodosum in the reduction of Meloidogyne javanica and M. incognita infestations of tomato plants. Fundam. appl. Nematol, 20(2), 99-102.

Wu, Y. J. (1998). Suppression of fecundity of the root-knot nematode, Meloidogyne javanica, in monoxenic cultures of Arabidopsis thaliana treated with an alkaline extract of Ascophyllum nodosum. Journal of Applied Phycology(10), 91.

Xu, F. F. (2007). Chitinases in Oryza sativa ssp. japonica and Arabidopsis thaliana. Journal of Genetics and Genomics, 34(2), 138-150.

Yoshida, S. T. (2006). Cytosolic Dehydroascorbate Reductase is Important for Ozone Tolerance in Arabidopsisthaliana. Plant Cell Physiol, 47(2), 304-308.

Young, M. D. (2010). Gene ontology analysis for RNA-seq: accounting for selection bias. . Genome Biol., 11(2), R14.

Zhang, C. Q. (2011). Carbon consequences and agricultural implications of growing biofuel crops on marginal land in China. Environmental Science & Technology, 45(24), 10765-10772.

Zhang, F. H. (2012). Genome-wide gene expression profiling of introgressed indica rice alleles associated with seedling cold tolerance improvement in a japonica rice background. BMC Genomics(13), 461-476.

Zhang, X. (1999). Hormone-Containing Products' Impact on Antioxidant Status of Tall Fescue and Creeping Bentgrass Subjected to Drought. Crop Science, 40(5), 1344-1349.

Zhang, X. E. (2003). Cytokinin-Containing Seaweed and Humic Acid Extracts Associated with Creeping Bentgrass Leaf Cytokinins and Drought Resistance. Crop Science, 44(5), 1737-1745.

Zhang, X. E. (2003). Seaweed Extract, Humic Acid, and Propiconazole Improve Tall Fescue Sod Heat Tolerance and Posttransplant Quality. HortScience, 38(3), 440-443.

Zhang, X. W. (2009). Optimizing Dosages of Seaweed Extract-Based Cytokinins and Zeatin Riboside for Improving Creeping Bentgrass Heat Tolerance. Crop Science,

50, 316-320.

Zhang, Y. W. (2005). Soil temperature in Canada during the twentieth century: Complex responses to atmospheric climate change. J. Geophys. Res., 110, D03112.

Zhao, Y. D. (2009). Biomass yield and changes in chemical composition of sweet sorghum cultivars grown for biofuel. Field Crops Research, 111(1-2), 55-64.

Zhaolong Wang, J. P. (2003). Responses of Cytokinins, Antioxidant Enzymes, and Lipid Peroxidation in Shoots of Creeping Bentgrass to High Root-zone Temperatures. Journal of the American Society for Horticultural Science, 128(5), 648-655.

Zhou, Q. T.-M. (2008). Soybean WRKY-type transcription factor genes, GmWRKY13, GmWRKY21, and GmWRKY54, confer differential tolerance to abiotic stresses in transgenic Arabidopsis plants. Plant Biotechnology Journal, 6, 486-503.

Zvyagintseva, T. N. (1999). A new procedure for the separation of water-soluble polysaccharides from brown seaweeds. Carbohydrate Research, 322(1), 32-39.