TECHNISCHE UNIVERSITÄT MÜNCHEN

Lehrstuhl für Biochemische Pflanzenpathologie

"Transcriptional and physiological changes in ragweed (*Ambrosia artemisiifolia L.*) upon abiotic stress"

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Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und der technischen Universität München zur Erlangung des akademischen Grades eines

Doktor der Naturwissenschaften

genehmigten Dissertation.

Vorsitzende : Univ. – Prof. Dr. R. Matyssek

Prüfer der Dissertation : 1. Univ. – Prof. Dr. J. Durner

2. Univ. – Prof. Dr. G. Müller-Starck

Die Dissertation wurde am 30.10.2013 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, landnutzung und Umwelt am 31.01.2014 angenommen.

Publications

A. Elkelish, U. Kanter, J B Winkler, H Behrendt, J Durner & D Ernst. (2013). Effects of air pollution and drought on the growth and pollen production of *Ambrosia artemisiifolia.* (in press).

A. Elkelish, F. Zhao, U. Kanter, W. Heller, A. Holzinger, J B Winkler, H Behrendt, J Durner & D Ernst. (2013). Molecular and proteomic characterization of ragweed (*Ambrosia artemisiifolia* L.) pollen exposure to elevated CO_2. Manuscript in preparation

A. Elkelish, F. Zhao, U. Kanter, W. Heller, A. Holzinger, J B Winkler, H Behrendt, J Durner & D Ernst. (2013). Transcriptional and physiological changes in ragweed (*Ambrosia artemisiifolia* L.) pollen upon drought stress and impacts of its allergenic potential. Manuscript in preparation

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II. ABBREVIATIONS

Acronym	Definition
AAAAI	American Academy of Allergy Asthma and Immunology
ABA	Abscisic Acid
ABC	Ammoniumbicarbonate
ACN	Actetonitrile
ABTS	2;2'-Azinobis(3-Ethyl-Benzthiazoline-6-Sulfonic Acid)
ACP	Aacyl carrier protein
ADP	Adenosine Diphosphate
AllFam	Database for classifying allergens into protein families
ANOVA	Analysis Of Variation
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
BVA	Biological Variance Analysis
CAM	Crassulacean Acid Metabolism (Photosynthetic Pathway)
CBP	Calcium Binding Protein
cDNA	complementary DNA
CO2	Carbon Dioxide
CPI	Cystatin Proteinase Inhibitor
Cy2	Cyanine dyes 2
Cy3	Cyanine dyes 3
Cy5	Cyanine dyes 5
Cy-dye	Cyanine dyes
DGDG	Digalactosyldiacylglycerol
DIA	Differential In gel Analysis
DIGE	Difference gel electrophoresis
DGDG	Digalactosyldiacylglycerol
DPI	Dots per inch
DTT	Dithiothreitol
EcoP15I	Immunoglobulin E
EST	Expressed Sequence Tag
FACE	Forests Absorbing Carbon Dioxide Emission
FASEB	Federation Of American Societies For Experimental Biology
FAT	Fatty Acid Translocase
FC	Fold Change
GA	Gibberellins
GDB	Genome Database
GO	Gene Ontology
H_2O_2	Hydrogen Peroxide

HPLC	High-Performance Liquid Chromatography
IEF	Isoelectric Focusing
IPCC	Intergovernmental Panel On Climate Change
IPG	Immobilized Ph Gradient
LC-MS / MS	Liquid chromatography–Mass Spectrometry
MW	Molecular Weight
NAD	Nicotinamide Adenine Dinucleotide
NADH	Reduced Nicotinamide Adenine Dinucleotide
NADPH	Reduced Nicotinamide Adenine Dinucleotide phosphate
NBT	Nitrotetrazolium Blue chloride
NCBI	National Center For Biotechnology Information
NGS	Next generation sequencing
nsLTP	Non Specific Lipid Transferase Protein
OD	Optical Density
OSI	Oxidative Stress Index
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEs	Pectinesterases
PEP	Phosphoenolpyruvate
PEPC	Phosphoenolpyruvate carboxylase
PMEs	Pectin methyl esterases
PEIs	Pectinesterases inhibitors
PMEIs	Pectin methyl esterases inhibitors
PL	Pectate lyase
PR	Pathogenesis-Related gene
ROS	Reactive Oxygen Species
RT	Reverse Transcriptase
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SAGE	Serial Analysis Of Gene Expression
SBP	Sub- pollen particles
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SE	Standard Error
SEM	Scanning Electron Microscope
SNPs	Single Nucleotide Polymorphism
SOD	Superoxide Dismutase
TAS	Total Antioxident Status
TCA	Trichloroacetic Acid
TIGR	The Institute For Genomic Research
TOS	Total Oxidant Status
UniProt	Universal Protein Resource

III. SUMMARY

The massive increase in emissions of air pollutants due to economic and industrial growth in the last century reduced air quality, in a large number of European and North American countries. Furthermore, several air pollutants, in particular carbon dioxide (CO_2) is in the list of greenhouse gases which are involved in the global warming. This climatic change will increase heat waves, droughts, floods and these are a real and daunting problem. Generally climate change will alter plant growth and also influence the onset, period and intensity of pollen production. Common ragweed (*Ambrosia artemisiifolia* L.) is an important agronomic weed and it causes problems related to human health, as the ragweed pollen is known to carry one of the strongest pollen allergens and the main cause of seasonal allergic rhinitis and asthmatic symptoms in North America.

In this study, the results from large scale analyses of ragweed pollen performed by superSAGE technology and real-time RT-PCR were described. The data include transcripts that were differentially expressed under control, elevated CO_2, drought and elevated CO_2 plus drought. Two-dimensional difference gel electrophoresis (2D-DIGE) was carried out to analyses the changes and abundances in allergenic protein. Scanning electron microscopy (SEM) analyses, 100 seed weight, pollen viability test, phenolic metabolite profiles of treated ragweed pollen are been assessed. The oxidative stress marker enzymes including NADPH oxidase activity, total oxidant status, total antioxidant status, hydrogen peroxide content and oxidative stress index of the pollen are also presented. An additional focus of global DNA methylation of the first and second generation pollen are been evaluated to detect any epigenetic changes. The experiment, using realistic outdoor light, temperature and CO_2 fumigation conditions, allowed the investigation of the effects of these changes on ragweed, including pollen development, over the entire growing season. Elevated CO_2 and elevated CO_2 plus drought significantly increased pollen production in the first and second generation plants, while drought suppressed pollen production in both generations. Elevated CO_2, drought, and elevated CO_2 plus drought increased the 100 seed weight in the first generation. Elevated CO_2, drought, and elevated CO_2 plus drought significantly reduced pollen viability compared with control pollen; drought had the largest effect followed by elevated CO_2 plus drought and finally elevated CO_2. .

Elevated CO_2 and/or drought had no influence on the size, shape, and surface structure of pollen. However, elevated CO_2 plus drought significantly increased the content of a number of phenolic compounds such as quercetin and kaempferol derivatives in pollen. Additionally, oxidative stress marker enzymes, hydrogen peroxide levels, total oxidant status, and the oxidative stress index were significantly increased by drought and to a lesser extent by elevated CO_2 plus drought, and NADPH oxidase enzyme activity significantly increased under elevated CO_2 conditions only.

The qRT-PCR analysis of ragweed pollen showed that Amb a 1 is the most abundant ragweed pollen allergen, and that all of the tested allergens were highly induced by elevated CO_2, drought, and CO_2 plus drought compared to control pollen. Drought induced the highest relative expression of allergens followed by elevated CO_2 plus drought then elevated CO_2. Transcriptomic analyses of the superSAGE libraries generated 236,942 tags which were consolidated into 40,782, 8,555, and 15,914 unique tags in the elevated CO_2, drought, and elevated CO_2 plus drought pollen, respectively. Of the identified genes, 86% were upregulated by elevated CO_2, 70% by drought and 84% by elevated CO_2 plus drought, and 14%, 30%, and 16% were downregulated by elevated CO_2, drought, and elevated CO_2 plus drought, respectively. Most of the identified upregulated genes were found to be associated with enhanced fatty acid biosynthesis, or were pectinesterase inhibitors and cysteine proteinases, which enhance and modify the cell membrane and cell wall to withstand unfavorable conditions. On the pollen allergen level, the qRT-PCR data confirmed that Amb a 1 was the predominant and most notably induced allergen in ragweed pollen under different treatments, although the superSAGE analysis revealed that elevated CO_2 led to a higher log_2 fold change in ragweed pollen allergens than drought and elevated CO_2 plus drought.

Analysis of the global DNA methylation patterns in the first and second generation pollen showed that only the first generation drought-treated pollen had a significantly higher methylation rate than the corresponding second generation pollen, indicating that drought induced an epigenetic change between the first and second generation pollen.

Finally, two-dimensional difference gel electrophoresis and proteomic analysis showed that a high percentage of the accumulated spots (41%) were related to metabolism, followed by allergens (20%) and proteins related to protein fate (9%).

This confirmed the transcriptomic analysis which demonstrated that elevated CO_2, drought, and elevated CO_2 plus drought up-regulated the expression of genes encoding allergenic proteins in ragweed pollen.
Moreover, drought led to higher levels of allergen accumulation than elevated CO_2 and elevated CO_2 plus drought, although these changes were not highly significant for all of the spots investigated. Our study adds a new set of crucial data which will help to further understand the transcriptomic and proteomic changes which occur in ragweed pollen under different environmental conditions.

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1. Chapter – INTRODUCTION

1.1 Ragweed (*Ambrosia artemisiifolia L.*)

Within the last 60 years, common ragweed, *Ambrosia artemisiifolia L.*, has become considered one of the most invasive weed worldwide; (Brandes and Nitzsche 2006, Oswalt and Marshall 2008). More recently, this invasive plant has gained more attention in certain European countries including France and Italy (Kiss et al. 2007). Previous results can contribute to our comprehensive knowledge of the species, provide information on the theoretical basis of its efficient control, and help to avoid its future spread into surrounding countries (Kazinczi et al. 2008).

1.1.1 Morphology and taxonomy of common ragweed

Ragweed is an annual, perennial, shrub or sub-shrub with erect, hispid stems growing in large clumps to a height of 75-150 cm. The stems are basally branched and the plants form a slender taproot or creeping rhizome. The leaf morphology and size of the male flowers are highly variable, depending on the age of the plant (Kazinczi et al. 2008). Each individual plant hold 10-100 yellow male flowers on each head (Bassett and Crompton 1975) and the pollen size ranges between 10-25 microns in diameter (Kazinczi et al. 2008). Of the *Ambrosia* species, common ragweed (*Ambrosia artemisiifolia L.*) is the most important. It belongs to the branch Angiospermatophyta, class Dicotyledonopsida, subclass Asteroideae (Synandrae), order Asterales, family Asteraceae (Compositae), and subfamily Asteroideade (Tubuliflorae) (Kazinczi et al. 2008). Payne (1970) identified 11 scientific species, subspecies, varieties and form names as synonyms of *A. artemisiifolia L.*

1.1.2 Origin and distribution of common ragweed

Hegi (1906) described more than 19 ragweed species. Currently, there are over 40 known species worldwide, which are very similar in appearance. Despite their widespread distribution, palatability grazing experiments have shown that, many sheep refuse to graze on common ragweed when given the choice between these weeds and other grass weeds like yellow foxtail (Rosenbaum et al. 2011). Unfortunately no animal grazes or consumes ragweed. The Sonara desert in Arizona is considered to be the gene centre of *Ambrosia* species (Bohar 1996).

Ambrosia species are distributed throughout temperate regions of the northern hemisphere and South America, with the exception of *A. maritima* (*A. maritima* subsp. senegalensis), which is endemic in the area around the Mediterranean Sea and in Africa (Hegi 1906, Singh U. et al. 1983, Wiersema and Leon 1999). Ragweed favour dry, sunny plains and sandy soils; it grows along river banks, roadsides, disturbed soil, and rural sites.

Ragweed was first discovered in USA prior 1838 (Wagner and Beals 1958) and in 1860 in Canada (Bassett and Crompton 1975); and its pollen has been found in interglacial deposits more than 60,000 years old (Bassett and Teresmae 1962). Ragweed was less common in the eastern United States before dense European agriculture settlement in the late 1700s (Kazinczi et al. 2008); however ragweed is currently endemic in North-America. The importance of ragweed has increased in the last 250 years, due to soil disturbance and large scale deforestation (Bassett and Crompton 1975). In Asia, ragweed was founded in China and Japan (Siyu et al. 1985, Sugaya et al. 1997).

The first colonisation of ragweed in Western- Europe was reported in 1863 at Pfaffendorf, Germany (Kazinczi et al. 2008). Ragweed was subsequently identified in many other countries of Western- Europe; however, due to climatic factors it could not be naturalized in these area (Hegi 1906). After the First World War, the actual naturalization and propagation of ragweed in Europe began. *Ambrosia* seeds were transferred from America to Europe in purple clover seed shipments, grain and potato imports. The spread of ragweed probably started from the European ports: e.g. from Rijeka towards Croatia and Transdanubia, from Trieste and Geneva towards Northern Italy, and from Marseille towards the Rhone valley (Comtois 1998).

Later, after the Second World War, the spread and distribution of ragweed in Europe became more rapid and prominent. (Kazinczi et al. 2008). Ragweed spread widely throughout the South of Europe and Russia (Kott 1948); especially in Ukraine (Ivanov and Szavickij 1949) and the Balkan Peninsula (Kovacevic and Miller 1958). Currently, the Rhone (France), Northern Italy, and the Carpathian Basin are the three main regions invaded by ragweed in Europe (Rybnicek and Jager 2001).

Kazinczi and co-workers (2008) stated that in the last century ragweed populations became extensive and increased in Hungary, and France, and then rapidly moved towards Italy, Germany, Austria and Switzerland.

On the basis of the latest surveys, rapid distribution of ragweed pollen can be observed in Austria, Slovakia, Poland and Turkey (Kazinczi et al. 2008) (Fig. 1).

Over the last two decades, ragweed has become one of the most well recognized weeds in Eastern Europe (Csontos et al. 2010). This is due to the fact that many people suffer from allergies to its airborne pollen; therefore, national governments have had to develop public health initiatives programs to bring attention to this noxious weed.

The recent boom in the spread of ragweed may be linked to the political transitions that led to the formation of the young democracies in Eastern Europe. Several new roads, motorways, shopping centres, transportation routes etc. were formed, but little effort was put into landscape management to prevent the spread of the ragweed (Kazinczi et al. 2008).

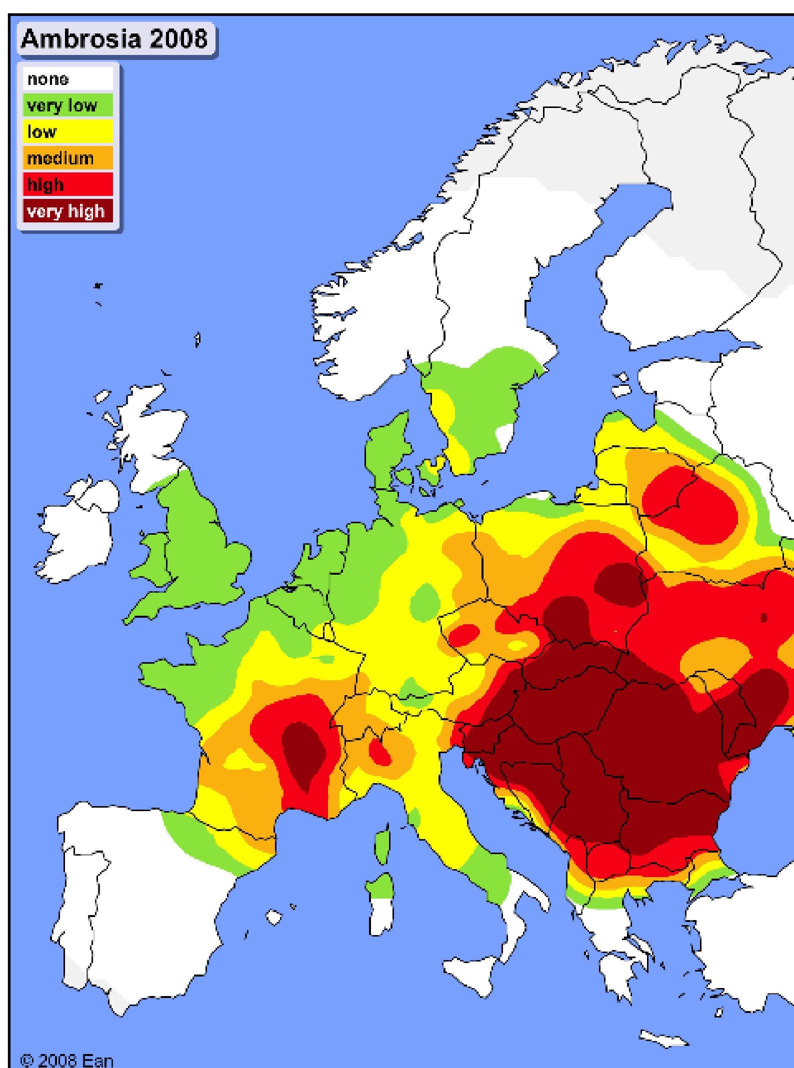

Figure 1: Distribution of ragweed pollen in Europe 2008. EAN (European Aeroallergen Network https://ean.polleninfo.eu/Ean) and epi (European Pollen Information http://www.polleninfo.org).

1.1.3 Life cycle

Seasonal changes affect ragweed, with field emergence beginning at the end of March (Beres and Hunyadi 1980). The peak period of germination occurs in April and May, with 60% of seeds germinating between April 10 and May 20 (Kazinczi et al. 2008).

During hot summer periods, secondary dormancy may be induced in ragweed seeds (Milanova and Nakova 2002). An absence of light can also lead to secondary dormancy formation in ragweed seeds (Baskin et al. 1980). More than 64 – 72 % of ragweed seed germination occurs in soil with a pH value between 6 and 9, and at a water potential below -0.4 MPa (Kazinczi et al. 2008); seed germination significantly decreases under drought stress (Hsu 2005). The emergence of ragweed plants and root growth are generally stimulated by phosphorus and potassium (Milanova and Nakova 2002).

Vegetative development and seed production by ragweed depend on the germination time. Plants which germinate in April grow to an average height of 170-180 cm, and will establish about 3000-4000 achenes (Beres 2004). Generally flowering depends on the average temperature and germination time in April, May or June (Milanova and Nakova 2002). The emergence of the first female flowers can be expected between July and August, and finally the first ripened seeds occurs in September (Kazinczi et al. 2008). The interval between flowering and complete seed ripening is relatively constant (58-71 days) (Kazinczi et al. 2008).

1.1.4 Reproduction strategy

Ragweed can only be propagated by seeds; 95% of the plants are monoecious (Gebben 1965). Ragweed is a wind pollinated species (Bassett and Crompton 1975), and self-pollination can develop viable seeds. Seed production by ragweed is dependent on plant size, competition, and ecological factors such as temperature and precipitations. Under average conditions, approximately 3000 seeds are produced per plant (Beres and Hunyadi 1980). Several authors have stated that freshly harvested ragweed seeds are always in a state of primary dormancy, and that this dormancy greatly depends on the balance of endogenous promoters and inhibitors (Black 1970, Kazinczi et al. 2008); dormancy is characterised by a low promoter (gibberellins) and high inhibitor (abscisic acid) content (Willemsen and Rice 1972).

1.2 Allergenic potential of ragweed pollen

1.2.1 Allergenic pollen

Pollen allergy has a distinct clinical impact all over Europe, and several lines of evidence suggest that the incidence of respiratory allergic reactions induced by pollens has increased in Europe over the last few decades (D'Amato et al. 2000). Exposure to pollen grains and air pollution represent key factors among the environmental determinants of asthma (Eder et al. 2006). The pollination period in Europe covers almost about half the year, from spring to autumn. The distribution of airborne pollen that have allergological interest are grouped into five vegetational areas arctic (birch), central (birch and grasses), eastern (grasses, mugwort and ragweed), mountains (grasses), and Mediterranean (olive trees, grasses and cypress) (D'Amato et al. 2007b).

1.2.2 Weed pollen

Within weed species, Compositae (Asteraceae) is one of the largest plant families with almost 20,000 species. Ragweed (*Ambrosia*) and mugwort (*Artemisia*) are the species most associated with pollenosis (D'Amato et al. 2007b). Mugwort is present in both urban and suburban areas. Its flowering occurs from late July to the end of August in northwest Europe. The genus *Ambrosia*, which includes both *Ambrosia artemisiifolia* (short or common ragweed) and *Ambrosia trifida* (giant ragweed), has long been recognized as the main contributor to allergic rhinitis (D'Amato et al. 2007a).

The pollen of ragweed is produced in massive amounts a millions of pollen grains may be produced from a single plant (D'Amato et al. 2007b). Due to the tiny size of the pollen grains, they can easily travel a long distance (Mandrioli et al. 1998).

Ragweed and mugwort have similar periods of seasonal flowering and a high degree of cross-reactivity (D'Amato et al. 2007b). Asero (2006) showed that patients with allergic rhinitis can be co-sensitized when they show immunoglobulin E (IgE) reactivity to both ragweed and mugwort pollen. The magnitude of pollen production may be related to allergenicity, as the development of sensitization is positively correlated to exposure (Roitt and Delves 2001). The concentration of pollen in the air does not always directly correlate with the development of the symptoms of allergic rhinitis, as several other factors also play a role in the dose response relationship (Zemmer et al. 2012).

These include the size and type of allergen, pollen cross-reactivity, air pollution, and human factors in the form of individual variation between patients, as some individual are more sensitive to pollen than others (Subiza 2001). However Frenz (2001) debated that once a concentration of pollen provokes an allergic reaction, higher concentrations do not induce an increase in the severity of the symptoms.

For atopic patients (clinical syndrome involving type I hypersensitivity (allergy) with a hereditary predisposition), a few grains of ragweed pollen might be enough to trigger symptoms (Zemmer et al. 2012). Oswalt and Marshall (2008) reported that the development of pollinosis could be predicted for daily pollen concentrations of 5 to 20 grains m^{-3}. When pollen is airborne, the atmosphere is known to hold pollen-fragments of a few microns in size (Solomon 2002). These fragments are known as subpollen particles, and can initiate significant inflammation of the lower airways (Bacsi et al. 2006). This provides further incentive for research into the allergenic potential of the ragweed pollen population.

1.2.3 Action of allergenic pollen

When pollen grains penetrate the upper respiratory tract and land on the mucosa, both allergenic and non-allergenic proteins are liberated when the pollen is rehydrated by mucosal fluids. Normally, the first in grass pollen allergens to reach the mucosa are microscopic sub-pollen particles which may be derived from the bursting of pollen grain during rainfall (Speranza and Scoccianti 2012). These particles can easily and directly reach the lower respiratory tract.

Mast cells are one of the most important mediators in the pathogenesis respiratory system allergies (Bloemen et al. 2007). Allergens stimulate IgE antibody production from B-lymphocytes, then IgE binds to corresponding receptors on the surface of mast cells (Speranza et al. 2012).

This triggers the release of exocytotic cytoplasmic granules, which contain a combination of preformed and newly synthesized compounds involved in the allergic inflammatory response (e.g., histamine, proteases, prostaglandins, leukotriene and cytokines) (Swindle and Metcalfe 2007)(Fig. 2).

Allergenicity is not only induced by IgE-mediated mast cell degranulation, but may also be stimulated by increased levels of oxidative stress in the microenvironment to which mast cells are exposed (Speranza and Scoccianti 2012).

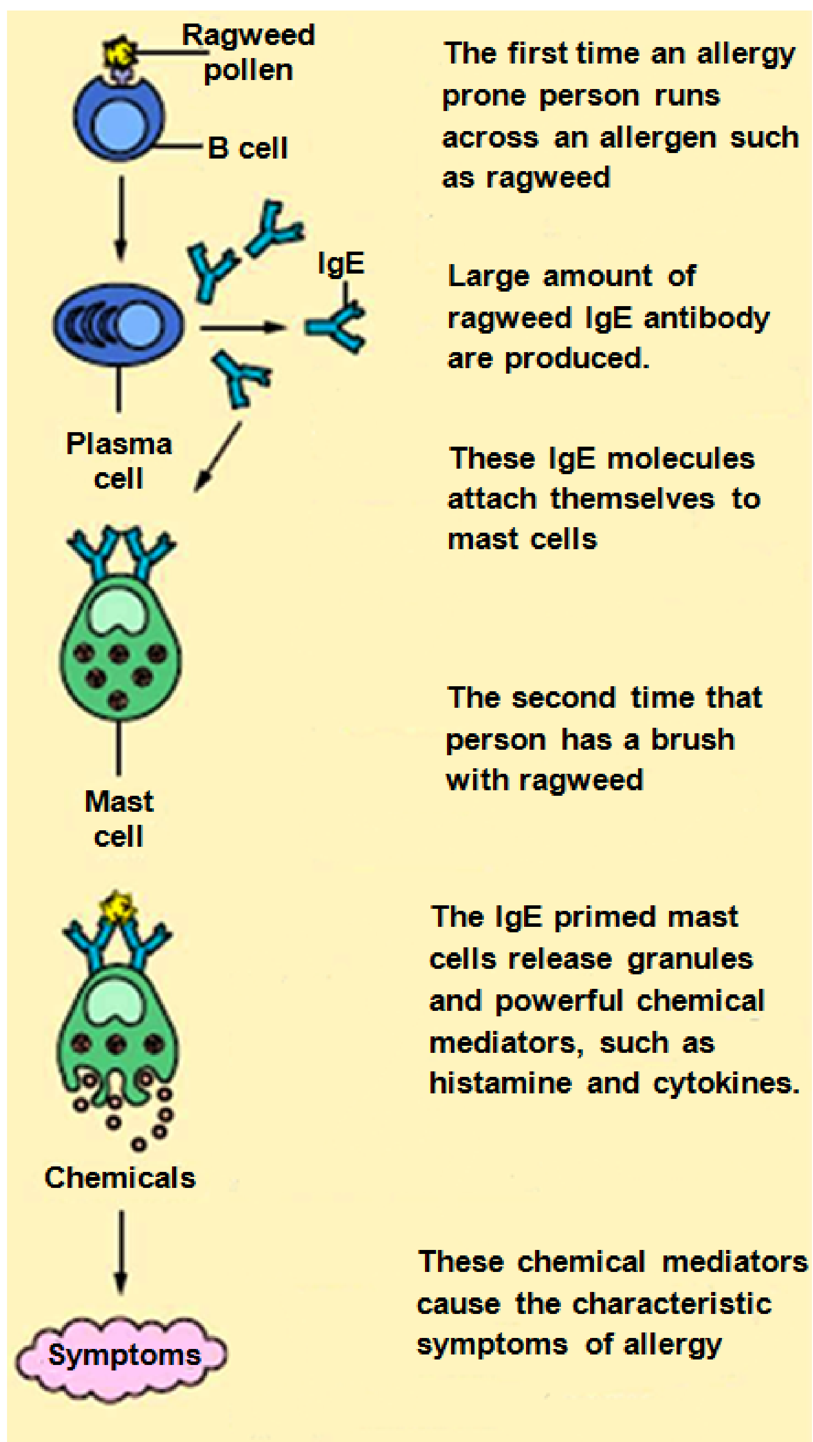

Figure 2: Schematic representation of the pollen allergens and inflammation response (Understanding the Immune System How It Works Aeroallergen, National Institute of Allergy and Infectious Diseases, National Cancer Institute, www.niaid.nih.gov www.nci.nih.gov).

Chodaczek et al. (2009) suggested an alternative hypothesis on the base of pollen experiments using ragweed as a species that indisputably causes severe allergy symptoms in people all over the world. Mast cells that come into contact with ragweed pollen extracts will suffer from mitochondrial dysfunction, which results in excess reactive oxygen species (ROS) production that in turn enhances the secretion of histamine and serotonin by mast cells (Endo et al. 2011).

In fact, ROS are well documented as being associated with the inflammatory effects of mast cells. Several reviews have described how intracellular ROS generation induced by different agents is known to prompt degranulation (Collaco et al. 2006). During acute or chronic inflammation, the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase system is considered to be the major source of oxidative stress in the cell.

The oxidative activity of NADPH oxidases produce superoxide ($^{\bullet}O_2^-$) ions, a major type of ROS, which are subsequently transformed into H_2O_2 by the action of superoxide dismutase (Swindle and Metcalfe 2007, van der Vliet 2011).

Ultimately, it is clear that the allergic responses induced by the allergenic proteins indicated in Tab.1 involve IgE-dependent or ROS-mediated mast cell activation, with the recipient host cells producing these molecules in response to the foreign pollen grains (Speranza and Scoccianti 2012).

Table 1: All the ragweed pollen allergen.

Allergen	Molecular Weight	Allergenicity	Isoforms	Common name	Family	Biological / biochemical aspect
Amb a 1	38	Major	Amb a 1.0101, Amb a 1.0201, Amb a 1.0202, Amb a 1.0301, Amb a 1.0302, Amb a 1.0303, Amb a 1.0304, Amb a 1.0305, Amb a 1.0401, Amb a 1.0402, Amb a 1.0501, Amb a 1.0502	a24, a789, AgE, Amb a 1-like, Antigen E, w230	Pectate lyase	Secreted, acidic, nonglycosylated, single-chain protein, proteolytic cleavage during chromatographic purification (α chain of 26 and β chain of 12 kDa); N-terminus is blocked
Amb a 3	11	Minor	Amb a 3.0101	Ra3	Plastocyanine	Secreted basic glycoprotein (8% carbohydrate); 3 cysteines (probable 1 S-S); proposed O-linked and/or N-linked carbohydrates
Amb a 4	30	Minor	Amb a 4.0101	Art v 1-like	Defensin-like protein	Contained hydroxyproline-linked arabinogalactan chains with one galactose and 5 to 20 and more α-arabinofuranosyl residues with some β-arabinoses in terminal positions
Amb a 5	5	Minor	Amb a 5.0101	Ra5, Ra5S	Group 5 ragweed allergen	Secreted basic protein; four S-S bonds; heat stable; enzymatically cleaved at the C-terminus; homologous to Amb t 5 and Amb p 5

Continued

Allergen	Molecular Weight	Allergenicity	Isoforms	Common name	Family	Biological / biochemical aspect
Amb a 6	10	Minor	Amb a 6.0101	LTP, Ra6	Prolamin superfamily	Secreted basic protein; putative glycosylation site; 4 S-S bridges; homology to nsLTPs
Amb a 7	12	Minor	Amb a 7.0101	Ra7	Plastocyanine	Basic protein; colourless and blue appearance; made up of 38 amino acids (aas) of both proteins were identified unequivocally by N-terminal aa sequencing, and showed homology with the 96 residue cucumber “cusacvanin”
Amb a 8	14	Minor	Amb a 8.0101, Amb a 8.0102	Actin-binding Proteins	Profilin	Binds to actin and affects the structure of the cytoskeleton. At high concentrations, profilin prevents the polymerization of actin, whereas it enhances it at low concentrations.
Amb a 9	10	Minor	Amb a 9.0101, Amb a 9.0102	Calcium binding protein, Polcalcin	EF hand domain	Calcium ion binding
Amb a 10	18	Minor	Amb a 10.0101	Calcium binding protein, Polcalcin-like protein	EF hand domain	Calcium ion binding
Amb a CIP	11	Minor	-	Cystatin Proteinase Inhibitor	Cystatin Proteinase Inhibitors	Absence of disulfide bonds in the predicted translation product suggests that it most resembles family-I members of the Cyt superfamily.

1.3 Global warming and its impacts on allergenic plants

Human activities have triggered global environmental changes which affect all of the ecosystems on earth and it is a major challenge to understand the consequences of these environmental changes on the organisms in communities (de Sassi and Tylianakis 2012). Many studies have studied the distribution of different taxa under the effects of climate change (Hickling et al. 2006, Walther et al. 2002).

The Intergovernmental Panel on Climate Change (IPCC 2001) stated that climate change will result in more extreme weather events, including an increase in the frequency of severe droughts and extreme rainfall. The average global surface temperature has increased over time, due to the increase in greenhouse gas concentrations (IPCC 2001).

Global warming and climate change can affect a wide range of environmental processes that have a direct effect on human health (Blando et al. 2012). These effects include increasing the ecological range of disease vectors and other pests (Beard et al. 2003). However, the impact of climate change on increasing ecological range is not limited to animals, but also affects plant species (Blando et al. 2012).

One of the major impacts of climate change on public health is the emergence of allergic respiratory diseases, including asthma and allergic rhinitis, due to changes in the distribution of aeroallergens such as pollen and mould spores (Ziska L. H. and Beggs 2012).

The IPCC (2007) reported clear increasing trends in the ranges of plant species that produce clinically relevant pollen. These shifts include alteration to the timing of the onset of allergenic pollen production, which is linked to pollen abundance and/or potency. The American Academy of Allergy, Asthma and Immunology (AAAAI 2008) stated that climate change is a very important factor which can increase the allergenic potential of pollen (Beggs P. J. and Bambrick 2005).

1.3.1 Elevated CO_2

Allergenic grasses are generally divided into two physiological groups: those that use either C3 or the C4 photosynthetic pathways for carbon fixation (Blando et al. 2012).

The rising atmospheric CO_2 concentration has two major direct physiological effects on C3 plants: it enhances the photosynthetic rate, which can lead to increased plant productivity; and reduces stomatal conductance, which can lead to reduced plant water use (Barton et al. 2012).

Several, arguments have developed regarding which type of plants will have the overall competitive edge in relation to climate change (Ziska L. H. and Beggs 2012). Elevated CO_2 levels may allow C3 plants to out-compete C4 plants due to their ability to flourish and develop in higher CO_2 concentrations. Subsequently, C4 plants may be displaced and their biodiversity limited in certain ecosystems. However, a counter debate suggests that C4 plants possess advantages linked to pre-adaptation, due to their ability to survive in hot and drier conditions (Wand et al. 1999).

Blando et al. (2012) recently reported that synchronous changes in precipitation, temperature, and CO_2 levels may minimise the reproductive efficiency of plants. Perhaps these effects will be most important on plants which have considerable value as a food source. Matsui et al. (1997) showed that high temperatures together with elevated CO_2 level, decreased the pollen fertility of the indica rice plant and could therefore minimize the yield of rice crops. Other research has shown that high temperatures due to climate change may result in reduced germination of pollen from peanut and grain sorghum (Prasad et al. 2011), cowpea (Singh S. K. et al. 2010).

Currently, there is debate and assessment regarding the increasing overall allergenic content of important food products due to climate change. Beggs (2009) and Beggs and Walczyk (2009) observed that the number of children suffering from peanut allergy doubled between 1997 and 2002, leading to speculation that climate change may have played a key role in this increase.

Several effects of global climate change, would result in an increased pollen concentration, prolongation of the pollen season, and an increase in pollen allergenicity (Ziska L. H. and Beggs 2012). For example, it has been proven that plants produce higher quantities of pollen under increased CO_2 concentrations (Wayne et al. 2002, Ziska L. H. and Caulfield 2000).

Moreover, a significant increase in ragweed pollen production was observed in plants cultivated at the current ambient atmospheric CO_2 concentration (Rogers et al. 2006, Wayne et al. 2002, Ziska L. H. and Caulfield 2000).

1.3.2 Drought

In response to climate change, prolonged periods of drought are one of the major stresses that can negatively affect plant growth, crop yield and the natural status of the environment in general (Gusmao et al. 2012). Plants respond to drought stress via a variety of physiological mechanisms, like leaf rolling, stomata closure, decreasing the photosynthetic rate, and increased respiration (Blödner et al. 2007, Shinozaki and Yamaguchi-Shinozaki 2007). Moreover drought may affect multiple pathways which stimulate the synthesis of increasing signaling molecules e.g. abscisic acid which is responsible for stomatal closure and activation of stress tolerance genes (Bahrun et al. 2002, Zhu 2002).

Global climatic change is likely to affect human health to a great extent by significantly changing in several biological systems (Parry et al. 2007). The effects relevant to allergic disease and asthma include changes in the distribution, quantity, and quality of aeroallergens, and further impairment to air quality in general (Breton et al. 2006).

1.4 Transcriptomics and proteomics as a tool to monitor molecular changes

Molecular approaches are currently being used and have helped to explain some of the mechanisms controlling the allergenic potential of ragweed pollen and its response to different abiotic stresses on ragweed pollen (Edlund et al. 2004).

With the increasing availability of DNA sequences (genomes) towards the end of the last century, the current challenge is to understand the environmental factors which directly affect gene expression, and therefore affect the biological functions of genes (Zulhendri 2012).

Fig. 3 illustrates the process by which DNA is transcribed and translated to functional proteins. The information contained in a gene (DNA) does not always explain the factors affecting its transcription to mRNA, translation to a protein, or its subsequent post-translational modifications and hence its functionality (Rabilloud and Humphery-

Smith 2000). Therefore, a range of experimental techniques are needed to examine the different levels of gene expression and regulation.

Thus, two branches of 'omics' have been developed, the first of which is focused on transcription (mRNA expression levels) and is known as transcriptomics. The second, which focuses on translation (protein expression levels), is called proteomics. Initially, the study of transcriptomics was considered to be a useful tool to elucidate gene functionality by measuring gene expression. However, it was subsequently found that the mRNA expression level of a gene frequently does not correlate with its proteins expression level, or even the translation of a functional protein (Anderson and Seilhamer 1997, Tew et al. 1996).

Gygi et al. (1999) reported that due to the low correlation between mRNA and protein expression levels, quantitative mRNA data cannot be used to predict protein expression levels in yeast.

In the sequence of events starting from the expression of genes to expression of the final active proteins, proteomics can be considered as a study of the end product of the genes (Rabilloud and Humphery-Smith 2000). The aim of proteomics is to understand how particular biological events affect aggregate protein expression patterns, and subsequently identify the proteins affected by the conditions under study (Unlü et al. 1997).

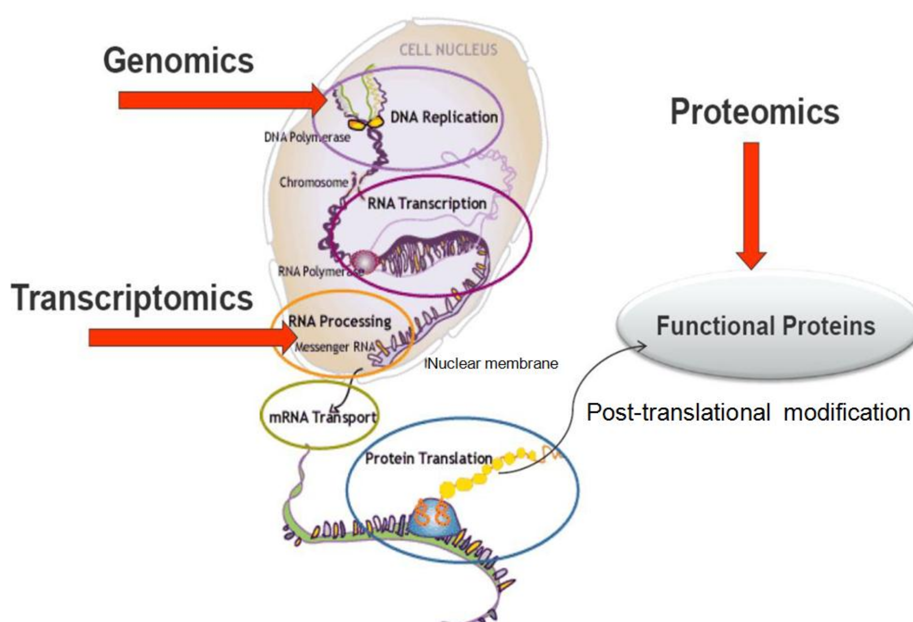

Figure 3: A cascade of events from DNA to mRNA to protein, associated fields of study genomics (DNA), transcriptomics (mRNA) and proteomics (proteins) (modified from www.nobelprize.org).

1.4.1 Serial analysis of gene expression (SAGE)

Technologies for gene expression analysis have dramatically improved in recent years. Northern blot analysis and reverse transcription in combination with quantitative polymerase chain reaction (qRT-PCR) are still, to some extent, standard tools for the expression analysis of individual genes (Matsumura et al. 2012). Nevertheless, despite their virtues, this technique cannot be extended to measure gene expression of the whole genome. The advent of data from genome and transcriptome sequencing projects, has promoted the analysis of a large number, preferably all, genes at the same time (Matsumura et al. 2012).

Serial analysis of gene expression (SAGE) is an extremely powerful, efficient, and comprehensive approach for analysing gene expression profiles. Since its invention, SAGE has become one of the leading functional genomics methodologies, and is used by various groups in academia and industry (Matsumura et al. 2006, Matsumura et al. 2011, Matsumura et al. 2005).

1.4.1.1 Advantages and principals of the SAGE methodology

A statistical description of the mRNA population present in a cell can be provided by SAGE without previous knowledge or selection of the genes to be studied. This represents the major advantage of SAGE compared to gene expression chip-based assays (Matsumura et al. 2011); furthermore, microarrays can only study the genes represented in the chip (Aldaz et al. 2002).

Aldaz et al. (2002) summarized the major advantages of the SAGE method. Firstly, the information is generated in digital format. Secondly, the data obtained can be directly compared with data generated from any other laboratory or with data available in public databases. Thirdly, the information generated is virtually "immortal," and has the advantage of being constantly updated and subject to reinterpretation, since the more we learn on the identification of new transcripts, the more complete and accurate the SAGE datasets become.

1.4.1.2 Overview of SAGE protocols

When cDNA library construction took place by SAGE, double-stranded cDNA is synthesized from mRNA isolated from the tissue/material of interest using oligo.dT primers. The resulting cDNA fragments are digested with a 4 bp anchoring restriction enzyme *NlaIII*, which is expected to cleave most transcripts at least once (Fig. 4) (Velculescu et al. 1995). The cDNA is divided into two pools and each is ligated to a different adapter containing a type IIS restriction enzyme priming site: *MmeI* for SAGE and *EcoP15I* for SuperSAGE.

Type IIS restriction enzymes (tagging enzymes) are used to cleave the fragments at a defined distance downstream of the recognition site (Tucholski et al. 1995). This digestion leaves the adapter attached to a small sequence tag of the original transcript. The two pools are combined at this stage and ligated to form ditags (Velculescu et al. 1995). Production of uniform ditags allows amplification of the sample without introducing PCR bias. Cleavage of the PCR product with the original anchoring enzyme allows the ditags to be isolated and the adapter molecules to be removed (Velculescu et al. 1995). The resulting ditags are ligated to form concatemers which are cloned and sequenced by 454 pyrosequencing analysis or Illumina genome analyser data analysis (Fig. 4).

Since SAGE was first described, several modifications have been suggested to improve the yield and transcript identification in SAGE libraries (Zhang and Gilles 2003). A major theme among these modifications has been to improve the accuracy of tag identification. Different restriction endonucleases have been used to produce longer tags of 21 bp in LongSAGE (Saha et al. 2002, Zhang and Gilles 2003) and 26 bp in SuperSAGE methods (Matsumura et al. 2003).

1.4.1.3 SuperSAGE

SuperSAGE is an amended version of the SAGE technology, whereby 26-bp tags are extracted from cDNA using the type III enzyme *EcoP15I* (Meisel et al. 1992, Möncke-Buchner et al. 2009). The advantage of using *EcoP15I* is that the distance between the recognition and cleavage sites is the largest of all known restriction enzymes; *EcoP15I* cuts 25/27 bp away from its recognition site (Matsumura et al. 2005).

The introduction of Next Generation Sequencing (NGS) platforms has succeeded in changing a number of research strategies, especially SAGE protocols (Matsumura et al. 2012). The output of NGS DNA sequencers is a huge number of short sequences, known as reads. This merit of NGS is highly optimized for sequencing 26 bp SuperSAGE tags. Therefore, the combination of SuperSAGE and NGS technology has resulted in DeepSuperSAGE, with greatly supports traditional SuperSAGE technology (Matsumura et al. 2012) (Fig. 4). The biological response of the transcriptome to abiotic stimuli has been analysed by SAGE (Jung et al. 2003). Cold-treated Arabidopsis leaves identified 272 differentially expressed genes compared to normal leaves (Jung et al., 2003). Moreover, the total number of expressed genes reduced after cold stress, but the average copy number per gene was increased (Jung et al., 2003). These data are being confirmed by further analysed by Northern blots (White et al. 2006).

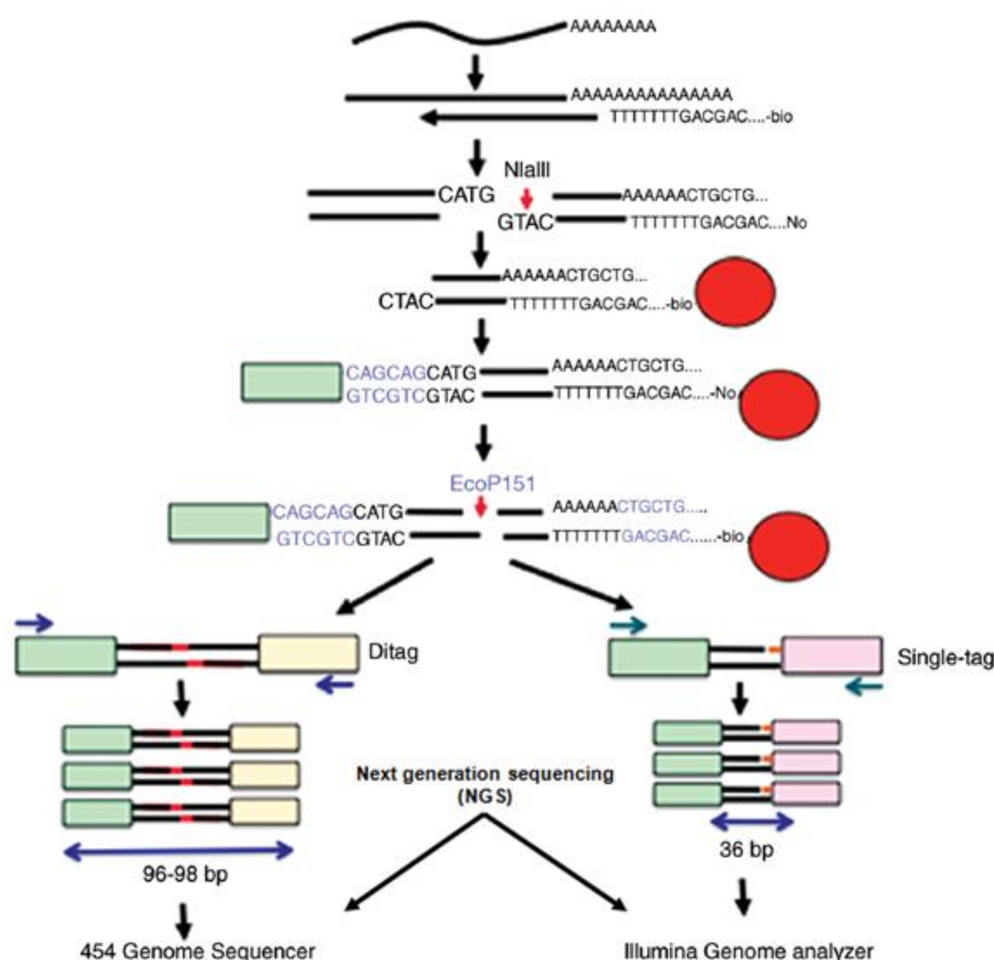

Figure 4: Schematic for DeepSuperSAGE. After EcoP15I digestion of linker (adapter)-ligated cDNA fragments immobilized on paramagnetic beads, ditags were formed for 454 pyrosequencing analysis (left) or another adapter was immediately ligated to the EcoP15I diges (Matsumura et al. 2012).

1.4.2 Two-dimensional difference gel electrophoresis (2D-DIGE)

Two dimensional difference gel electrophoresis is a powerful technique for studying protein expression which enables the simultaneous resolution of thousands of proteins (O'Farrell 1975). The concept of 2D-electrophoresis is based on the separation of proteins in the first dimension by their charge using isoelectric focusing (IEF) and in the second dimension by their size using define SDS-PAGE (Marouga et al. 2005).

The 2D-DIGE technique has been employed to identify proteins that are consistently differentially expressed between different treatments (Unlü et al. 1997). The method involves labelling the lysine residues of the proteins in different homogenates using one of three different spectrally resolvable cyanine florescent dyes, called Cy2, Cy3 and Cy5. The use of these dyes allows the multiplex analysis of three differentially labelled protein samples on the same gel (Fig. 5). A pooled mixture containing an equal amount of each sample being examined is labelled in bulk with Cy2 and used as an internal standard to match between multiple DIGE gels (Cheng 2010). In addition to the internal standard, the DIGE gels each contain two different samples individually labelled with Cy3 and Cy5, respectively. Quantitative comparison of proteomic differences between each sample is possible by analysing the replicate samples relative to the same internal standard (Fig. 5).

Proteomic studies complemented by transcriptomic analysis using traditional molecular biology techniques can significantly contribute to building complete proteome maps of cells under both normal and altered conditions (Díez 2010). The valuable information provided by qualitative and quantitative proteome maps will enable further identification of mechanisms involved in various stress tolerance.

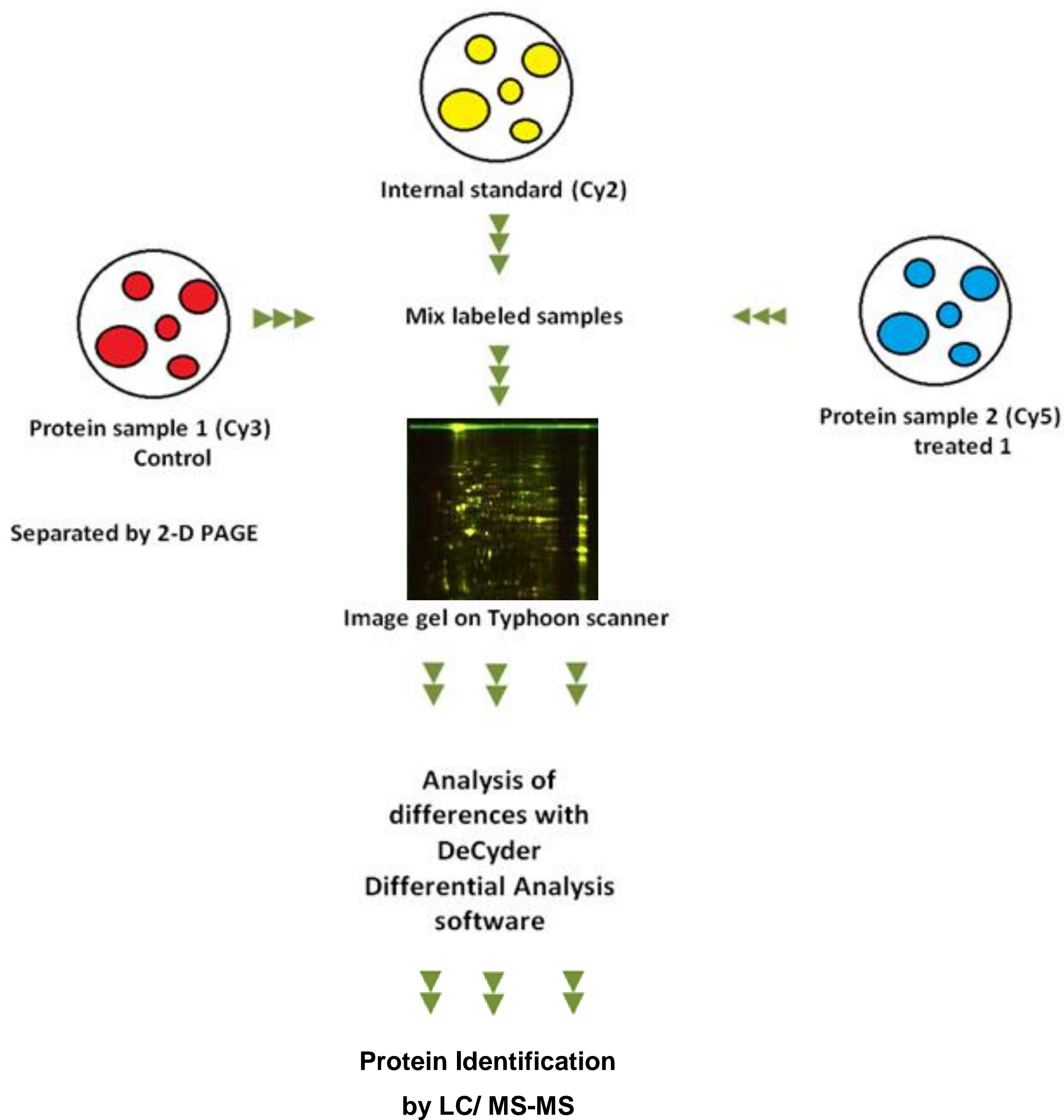

Figure 5: The principle of 2-DIGE from protein mixture labeling for identification of protein spots of interest (modified from Tonge et al., 2001).

1.5 Specific objectives of the present work

The pollen of the common ragweed (*Ambrosia artemisiifolia L.*) is known to be a main cause of allergenic diseases in Northern America (Gadermaier et al. 2008, Ziska Lewis et al. 2011), and the weed has become a spreading neophyte in Europe and represents a serious health problem in sensitized populations. Climate change and air pollution may affect the allergenic potential of pollen, either by altering the length of the pollen season, changing the amount of pollen, changing of the surface exine, of pollen, or by directly increasing the allergenicity of pollen by altering the levels of transcripts and proteins and the interactions of these proteins with biological ligands. Several studies suggest that global climate change will affect the allergenic potential of pollen and may increase the incidence of related human diseases, such as allergic rhinitis and asthma. From this perspective a transcriptome-wide analysis of the highly allergic pollen of ragweed would not only aid in understanding the impact of climate change on the transcripts expressed by ragweed pollen, but also enable a deeper insight into the expected changes to pollen allergens in response to climate change.

The overall goal of the present study was to analyse the impact of different climatic parameters (elevated CO_2, drought and elevated CO_2+ drought) on:

1- The physiological and morphological levels
2- The transcriptome of ragweed pollen using quantitative qRT-PCR and superSAGE libraries
3- The proteome of ragweed pollen using 2D-DIGE

In order to investigate the induction of potential allergenic components in ragweed pollen in response to different parameters of climate change.

2. Chapter – MATERIAL AND METHODS

2.1 Plant growth conditions

Ragweed seeds were collected from a single plant from an outdoor stand, in order to avoid parental environmental effects on the growth and development of the next generation (Elwell et al. 2011). Seeds were sown in standard soil (Floradur®; Bayerische Gärtnerei Genossenschaft, Munich, Germany) in small multiflor palettes (6 x 6 cm) and transferred into two fully air-conditioned greenhouse cabinets (each 36 m^2; http://www.helmholtz-muenchen.de/en/eus/environmental-simulation facilities/greenhouse/index.html) on March 29, 2010. One cabinet was fumigated with 350 ppm CO_2 and the second with 700 ppm CO_2. Relative humidities and temperatures and were set according to the outside site conditions and are shown in Fig. 6. Fifteen days after germination, the seedlings were transferred into single pots (Ø 17 cm) and cultivated further in the greenhouse. Watering of the plants was carried out automatically by a tube system applying 100-200 ml water per pot each day. The drought stress started on May 21, when the watering was reduced (100 ml per 24-36 h); however, care was taken to ensure that leaves did not become withered.

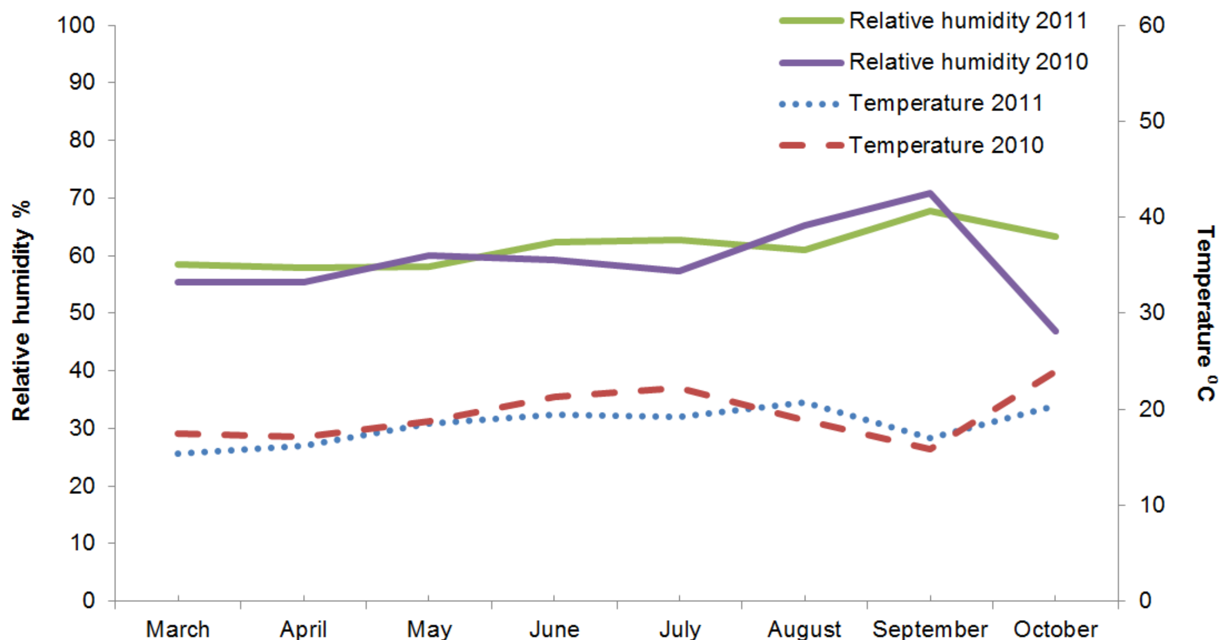

Figure 6: Overview of the monthly mean temperature and relative humidity of the greenhouses from March-October of 2010 and 2011.

Plants and inflorescences were sampled and stored at -80°C until analysis. Length was measured at the end of the experiment, and pollen was sampled on August 9 using a modified ARACON system (BETATECH, Ghent, Belgium) as shown in Fig. 7 that covered the inflorescenses; pollen. To allow development of seeds, the plants were cultivated further until November 22, 2010.

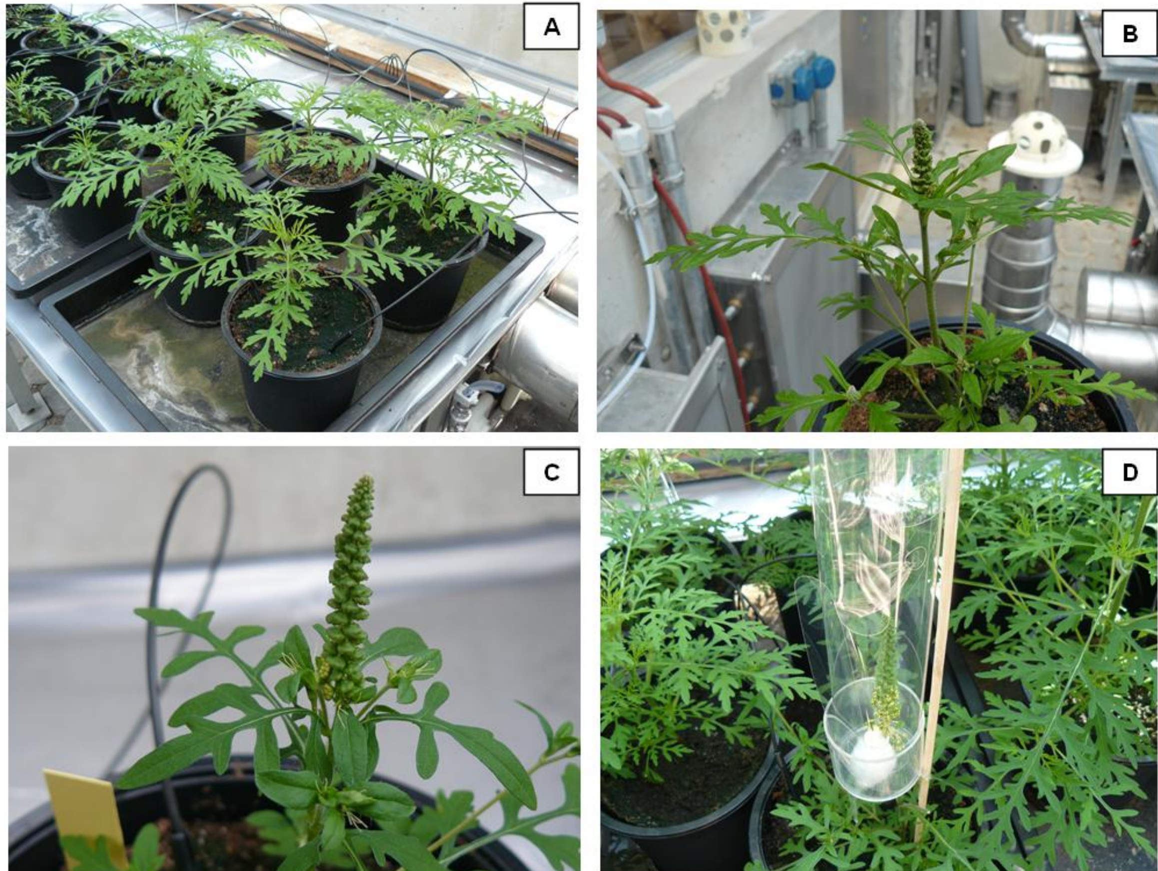

Figure 7: Ragweed was grown in the greenhouse. A) A ragweed seedling at the beginning of the experiment showing the irrigation system. B) Later stages of growth and appearance of the main inflorescence. C) Mature inflorescence and the start of pollen liberation in acropetal succession. D) The modified ARACON system used to collect the pollen.

To collect more pollen, ragweed seeds that were used in 2010 were sown on March 29, 2011 (Fig. 8). Additionally, the seeds that were collected from the plants grown in 2010 were also cultivated; the seeds from each treatment group (Control, Elevated CO_2, Drought and Elevated CO_2+Drought) formed the second generation plants (Fig. 9).

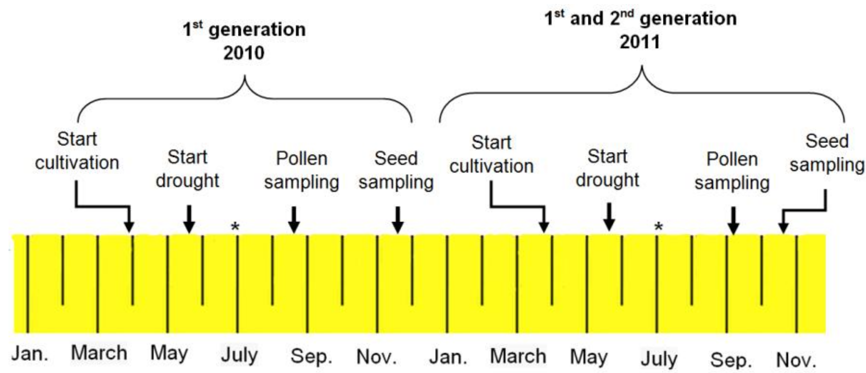

Figure 8: Schematic representation of the time line of the greenhouse experiment. Ragweed plants were cultivated for two years; in 2010 and 2011 for the first generation, and only in 2011 for the second generation.* indicates to the start of pollen liberation from the flower and pollen collection using the ARACON system.

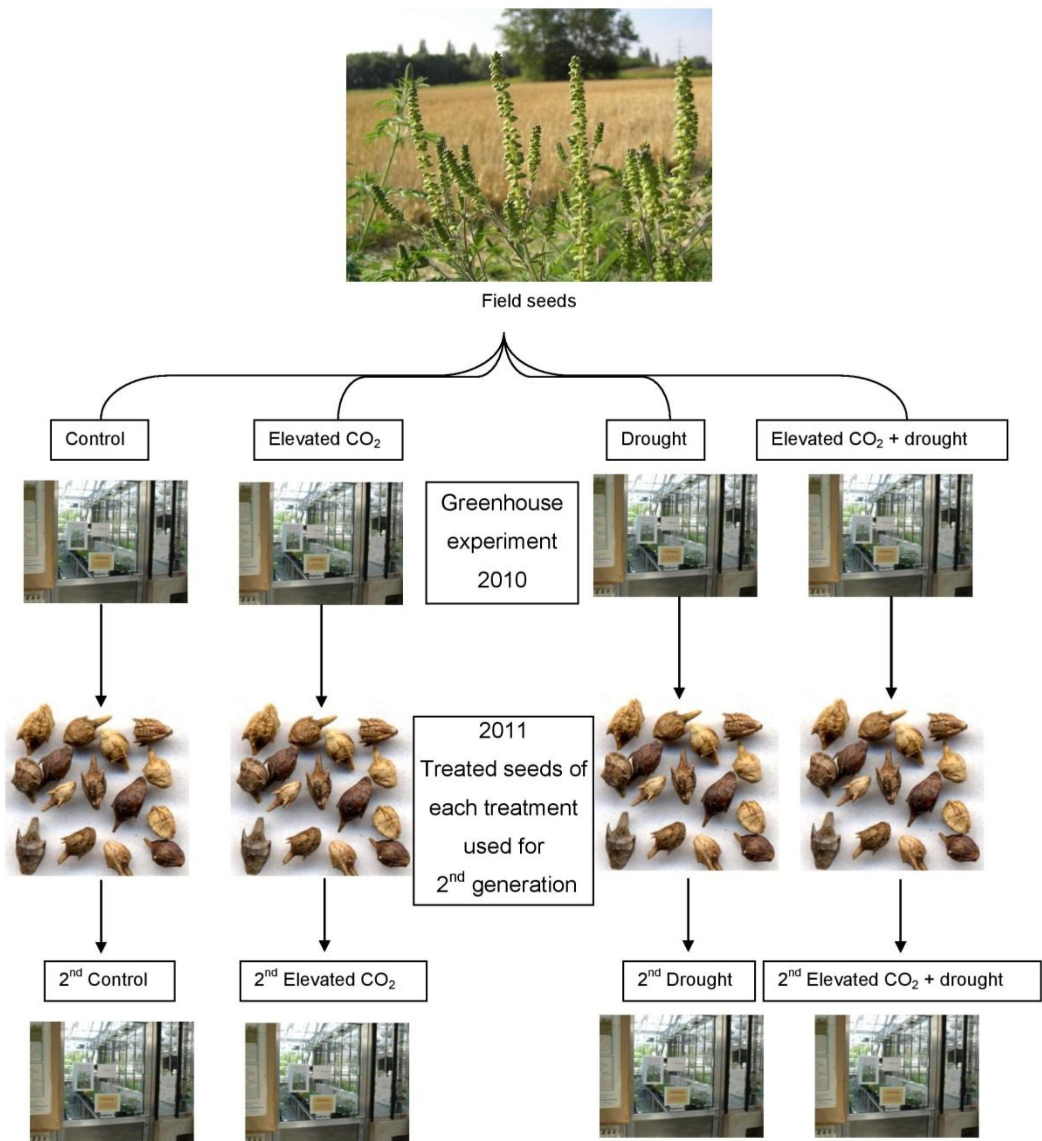

Figure 9: A diagram illustrating the ragweed seed that used in 2010 for first generation and 2011 for the second generation.

2.2 Morphological and enzymatic analysis

2.2.1 100 seed weight (g)

Ten samples of 100 clean seeds (broken grains were removed) were randomly sampled, counted and weighed.

2.2.2 Test of pollen viability

Pollen viability was estimated using the *p*-phenylenediamine test to detect the presence of myeloperoxidase, as described by (Tomas Rodriguez-Riano 2000). The test solution consisted of one vial peroxidase indicator reagent (Sigma #390-1; St. Louis, MO, USA), and 200 µl of 3% hydrogen peroxide (1:9 dilution of 30% hydrogen peroxide in phosphate buffered saline solution, pH 7.4) added to 50 ml of Trizmal 6.3 prewarmed dilution buffer (Trizmal 6.3 buffer concentrate [Sigma 90-3 C] diluted 1:9 in deionized water). The solution could be kept in the refrigerator for about 15–20 days without loss of potential activity. The solution was discarded if it turned from light brown to very dark brown or black during this time. The fresh solution was always prepared, stored and used in the dark.
To stain pollen grains, a small amount of the solution was prewarmed to 37°C for about 10–15 min. The pollen grains were considered viable if they turned totally black.

2.2.3 Scanning electron microscopy

Scanning electron microscopy (SEM) was kindly performed by A. Holzinger (University of Innsbruck). Ragweed pollen grains from the four treatment groups were studied using SEM. Three pollen grain samples from three individual plants from each treatment group were combined. SEM was performed following the standard method described by (Holzinger et al. 2008). Instead of the dehydration procedure, the air-dried pollen was surface coated with gold and examined using Philips XL20 scanning electron microscope (Philips Electronics, Eindhoven, The Netherlands) at 10 kV. Equatorial diameters of spheroidal pollen were measured from the photomicrographs (10 photos per treatment; each photo contained 2 to 20 pollen grains). The measurements included the central sphere and peripheral structures of each grain.

2.2.4 Analysis of phenolic metabolites by reverse-phase HPLC

Reverse-phase HPLC (RP-HPLC) was kindly performed by S. Stich (Helmholtz Zentrum Muenchen). The phenolic metabolites were extracted from frozen pollen phosphate buffer saline (PBS) and the residue was then extracted with methanol. The RP-HPLC separation protocol for water and methanolic extracts was as described by Ghirardo et al (2012) and proceeded as follows:

Sample preparation:

1) Extraction with PBS-buffer: 10 – 15 mg pollen was extracted with 1.2 ml PBS-buffer for 1 hour at room temperature on a shaker (350 rpm; Bühler Swip, Edmund Bühler, Hechingen, Germany) in the dark. After centrifugation for 5 min at 9,000 *g*, the clear supernatant was transferred into a new tube and stored at -80°C until HPLC analysis.

2) Extraction with methanol: 1.2 ml methanol was added to the residue from the PBS extraction and incubated for 1h at room temperature on a shaker (Bühler Swip) at 350 rpm in the dark. After centrifugation for 5 min at 9,000 *g*, the methanolic supernatant was transferred into a new tube and stored at -80°C until HPLC analysis.

3) Preparation for HPLC-Analysis:

A- PBS extracts: After reaching room temperature (RT), the extracts were mixed well and centrifuged for 5 min at 9,000 *g*. An aliquot was transferred into an HPLC-vial.

B- Methanol extracts: After reaching RT, the extracts were mixed well, and an aliquot of the extract was diluted to 75% methanol using double-distilled water and centrifuged for 5 min at 9,000 *g*. An aliquot of the clear supernatant was transferred into an HPLC-vial.

HPLC components :

Software: Gold 7.11, Beckmann Coulter (Duesseldorf, Germany)
Pumpe: 114 Mpa
Autosampler: LC-507e
UV-Detector: Beckman Diodenarray-Detector 168
Channel A: 280 nm
Wavelength:Scan Mode 2, from 250 to 450 nm

Solvents:

Solvent A: 980 ml double-distilled water
+ 20 ml of 5% ammonium formate in formic acid

Solvent B: 882 ml methanol (Merck, Darmstadt, Germany)
+ 98 ml H_2O double-distilled water
+ 20 ml of 5% ammonium formate in formic acid.

Gradient program used for pollen extracts:

100% solvent A for 5 min
100% solvent B (gradient) for 40 min
100% solvent B for 5 min
100% solvent A (gradient) for 5 min

2.2.5 Preparation of pollen extracts for enzymatic activity analysis

Pollen grains (100 mg) were hydrated in 1 ml PBS for 25 min at RT with gentle shaking. The pollen suspensions were then centrifuged at 14,000 *g* for 10 min and the supernatants were recovered (Pasqualini et al. 2011). The soluble protein concentrations of the supernatants were measured using the dye-binding method of Bradford (1976), using bovine serum albumin as a standard.

2.2.6 NAD(P)H oxidase enzymatic activity

The NAD(P)H oxidase activity of the pollen extracts was measured using the nitroblue tetrazolium (NBT) assay (Bacsi et al. 2005). Briefly, pollen extract containing 25 mg protein was used for each assay, and mixed with 2 mM NBT without or with 100 mM reduced NAD phosphate (NADPH) in the presence of absence of the $^{\bullet}O_2^-$ scavenging enzyme superoxide dismutase (SOD; 100 U ml^{-1}). The mixtures were incubated at 37°C for 15 min, NBT was completely removed by repeated washing with fresh PBS, and the formazan precipitate was dissolved in 100% methanol. The absorbance values of the solutions were determined at 530 nm by spectrophotometry.

2.2.7 Hydrogen peroxide content

Hydrogen peroxide content was measured using the Pierce® Quantitative Peroxide assay kit (aqueous-compatible formulation; Thermo-Fisher Scientific, Schwerte, Germany). Briefly, 20 µL aliquots of 1:100 dilutions of pollen extract and 200 µL of working reagent (provided in the kit) were added to each micro-plate well and incubated at RT for 20 min to allow the reaction to reach the endpoint. Once formed, the complex is relatively stable, and the absorbance values the wells were measured at 595 nm. The concentration of peroxide in the pollen extract was calculated by reference to a standard curve, which was prepared by serial dilution of a 30% (8.8M) hydrogen peroxide stock solution to achieve 8-10 standards in the concentration range of 1-1000 µM. Each measurement was performed three times. The hydrogen peroxide content of the pollen grains from the different groups was analyzed by t-test.

2.2.8 Determination of total oxidant status

The total oxidant status (TOS) was determined as described by Erel (2005). Oxidants present in the sample oxidize the ferrous ion–*O*-dianisidine complex to ferric ion. Ferric ion reacts with xylenol orange in acidic medium to form a coloured complex. The colour intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay was calibrated by reference to a standard curve created using hydrogen peroxide and the results were expressed in terms of nmol H_2O_2 equivalent/mg protein.

- Reagent 1: 0.15 mM xylenol orange, 140 mM NaCl and 1350 mM glycerol in 25 mM H_2SO_4, pH 1.75.
- Reagent 2: 5 mM ferrous ammonium sulphate and 10 mM *O*-dianisidine dihydrochloride in 25 mM H_2SO_4 solution.

The ragweed pollen extracts were centrifuged at 10,000 *g* for 30 min, and then the supernatants were used for analysis. An aliquot of 225 µl of reagent 1 was added to 35 µl of pollen homogenate, mixed well, and the absorbance values were determined at 560 and 800 nm as the sample blank. Then, 11 µl of reagent 2 was added, the mixture was incubated for 3-4 min, and the absorbance values were determined again at 560 and 800 nm.

$$\text{Absorbance} = A_2 - A_1$$

Where A_1 = 560 nm/800 nm before addition of reagent 2 and A_2 = 560 nm/800 nm after addition of reagent 2

Hydrogen peroxide solutions (10-200 nmol/ml) were prepared in deionised water and treated similarly as the pollen extracts to prepare a standard curve.

2.2.9 Determination of total antioxidant status

The total antioxidant status (TAS) was determined using the 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS)-based colorimetric method, as described by Erel (2004) with minor modifications. The reduced ABTS molecule can be oxidized to $ABTS^+$ (deep green colour) by hydrogen peroxide in acidic medium. Antioxidants present in the sample accelerate the bleaching rate by a degree proportional to their concentration. This reaction can be monitored spectrophotometrically and the bleaching rate is inversely related to the TAS of the sample. The reaction rate was calibrated using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and the assay results are expressed as nmol Trolox equivalent/mg protein.

- Reagent 1: 400 mM acetate buffer solution, pH 5.8
- Reagent 2: consists of 10 mM ABTS and 1 mM H_2O_2 in 30 mM acetate buffer solution, pH 3.8

The ragweed pollen extracts were centrifuged at 10,000 g for 30 min, and the supernatant was used for analysis. A volume of 200 µl of reagent 1 was added to 5 µl of sample, mixed well and the absorbance value was determined at 660 nm as a sample blank, then 20 µl of reagent 2 was added, the mixture was incubated for 5 min, and the absorbance value was determined again at 660 nm. Trolox solutions (serial dilutions; concentration range 0.01-0.2 nmol/ml) were prepared in 50 mM phosphate buffer pH 7.4 and treated similarly as the pollen extracts to establish standard curve.

The percentage inhibition of ABTS bleaching by the samples and standards was obtained using the following equation:

$$\%\ \text{inhibition} = \frac{A_B - A_S}{A_B} \times 100$$

Where A_S is the difference in the absorbance values of the sample before and after adding reagent 2, and A_B is the blank absorbance value.

2.2.10 Determination of oxidative stress index

The percentage ratio of TOS to TAS provides the oxidative stress index (OSI), which is an indicator of the degree of oxidative stress. To perform the calculation, the OSI value was calculated as described by Altindag et al. (2008) using the following equation:

$$OSI = \frac{\text{TOS (nmol } H_2O_2 \text{ equivalent/mg protein)}}{\text{TAS (nmol Trolox equivalent/mg protein)}} \text{ X } 100$$

2.3 Genomic and transcriptomic analysis

2.3.1 Isolation of pollen RNA and transcription of cDNA

Total RNA was isolated from 10 mg pollen samples from both generations of plants using a modified Qiagen RNeasy Mini Kit protocol (Kanter et al. 2013). The pollen, together with 150 ml of RLT buffer, was transferred into 2 ml tubes containing ceramic spheres (Ø 1.4 mm), silica spheres (Ø 0.1 mm), and a single glass sphere (Ø 4 mm). The pollen was homogenised ten times at 6.5 ms^{-1} for one min on dry ice using FastPrep 24 machine (MP Biomedicals). Then, another 600 ml of RTL buffer was added, the tubes were mixed, one volume of chloroform was added and incubated for 10 min on a shaker. After centrifugation, the supernatant was transferred to a new reaction tube, mixed with 0.5 volumes of ethanol by gentle inversion, and the solution was transferred to an RNeasy column (RNeasy Mini Kit, Qiagen) and centrifuged for 15 s at 10,000 *g*. The column was washed with 450 ml of RW1 buffer for 5 min, centrifuged the flow-through was discarded, and then DNase digestion was performed using the RNase-Free DNase Set (Qiagen) following the manufacturer's instructions.

Subsequently, the column was incubated twice with 500 ml of RPE buffer for 2–3 min each. Drying and elution of the RNA were performed according to the user manual of the RNeasy Mini Kit (Qiagen). DNA digestion was carried out using RQ1 RNase-Free DNAse (Cat.# M6101; Promega, Mannheim, Germany) according to the manufacturer’s instructions. Total RNA was quantified photometrically using the NanoDrop System at 230, 260 and 280 nm. Only RNA samples with acceptable ratios of 260/280 (> 1.8) and 260/230 (> 2.3) were used and reversed transcribed. For cDNA synthesis, 2 µg of total RNA was subjected to first-strand cDNA synthesis using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, US) according to the manufacturer’s procedure.

2.3.2 Quantitative real-time RT–PCR of ragweed pollen grains

The cDNA samples were diluted 1:20, and Quantitative real-time RT–PCR (qRT–PCR) was performed in 20 µl reactions containing 12.5 µl SYBR Green ROX mix (Thermo Scientific QPCR, Surrey, UK), 5 µl of diluted cDNA, and 1.25 µl each of the forward and reverse primers on an ABIPrism 7500 fast real time PCR system (Applied Biosystems, Darmstadt, Germany). Amplification of the PCR products was monitored via intercalation of the fluorescent dye SYBR® Green. The following program was applied: initial polymerase activation at 50°C for 2 min and 95°C for 10 min (1 cycle); followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The internal control genes α-tubulin and 18S rRNA were used. Three biological replicates were carried out for each group, and each transcript was quantified in triplicate for each sample. This resulted in nine independent values for calculation of the relative expression levels, according to Pfaffl et al. (2002), using the REST© software tool (QIAGEN GmbH,). The gene-specific primers for standard α-tubuline, 18S rRNA and the ragweed allergens are listed in Tab. 2.

Table 2: Primer sequences used for semi-quantitative RT-PCR analysis and quantitative real-time RT–PCR analysis.

Gene	Forward & reverse primers (5'-3')	Gene Bank Accession No.	Annealing temp. °C	RT-PCR product size/bp
Amb a 1.1	**F** ggggctggtgacgaaaatattg **R** caccatgccttcctaggacatt	M80558	56.3	250
Amb a 1.2	**F** taacatcgttaacgccggtctcac **R** tgatatcgagcagcccatcggaa	M62981	59.5	230
Amb a 1.3	**F** ggtcggggaaatcttaccttcagt **R** tgaccgtgtagacatcaccccatt	M80560	59	188
Amb a 1.4	**F** tttgacgagcgaggcatgctat **R** ctctgacatggcggattcaccata	M80562	59	243
Amb a 1.5	**F** ggagccagaatggatgacttggaa **R** tgtggaaccatatctcccggttca	M80561	59	150
Amb a 5	**F** aggatccacagatgaagtcgatga **R** aaaccacttgccaaggacagtacc	M84987	58	125
Amb a 6	**F** gtttcatggaggccaacgatgttc **R** gccacacgatcagctttggttt	U89793	58.7	167
Amba 8.0101	**F** acgtgtgggccaaaagctct **R** aataacagctccaggctcaccttg	AY894660	59.1	158
Amba 8.0102	**F** gaattcgatgcagctggtaccctt **R** gggctcttcgtaaatgccaaacac	AY894661	59.2	171
Amba 8	**F** aacctgaggagatgaaaggca **R** gcttggcctgttttcttgatgc	AY268427	56.5	172
Amba 9	**F** aagaatctcggctcggtgtca **R** cttgccaacgtccttcattaagcc	AY894657	58.5	141
Amba 9.0102	**F** aagaatctcggctcggtgtca **R** gaagaatctcgggtcggtatcac	AY894658	57.5	142
Amba 10	**F** atgtcaaggaagggaggtgatggt **R** cgcgacgcgttattccagtgatt	AY894659	59.8	460
Amba CPI	**F** gctaaattcgccatcgctgaacac **R** ccgtccatatggagttaaggtgaggt	L16624	59.6	246
α-Tubulin	**F** tgcagagggctgtttgcatga **R** acccacgtaccagtgaacaaaag	GW917730	58	119
18S rRNA	**F** gaggccttgtcgttgtgtgtctat **R** gcaagacaatgcgtcagggtact	EF065545	59	554

2.3.3 SuperSAGE libraries

Four SuperSAGE libraries were constructed and bioinformatic analysis was performed by GenXPro GmbH (Frankfurt am Main, Germany) for ragweed pollen: control (350 ppm CO_2); elevated CO_2 (700 ppm CO_2); drought (350 ppm CO_2 with low water); and elevated CO_2 with drought (700 ppm CO_2). To minimize gene expression differences due to biological subject-to-subject variation, pollen from three independently grown plants was pooled for each of the four treatment groups prior to the SuperSAGE analysis. Pooling of samples is especially important when biological variability is large in relation to the technical variability (Kendziorski et al. 2003). The procedures for SuperSAGE library generation followed the protocol described by Matsumura et al. 2008, and included the attachment of library-specific adaptors allowing the identification of library-specific reads after SOLEXA sequencing.

Briefly, the technique involves the following steps:

(1) mRNA extraction.

(2) cDNA synthesis using a biotinylated oligo-d(T) primer and conversion of single stranded cDNA into double-stranded cDNA.

(3) Digestion of the cDNAs with a four-base cutter NlaIII, and collection of the 3´-end cDNA fragments using streptavidin-coated magnetic beads.

(4) Division of the collected cDNAs into two tubes and ligation of different linker fragments to the 5´-ends of the cDNAs in each tube.

(5) Mixing the contents of the two tubes, and digestion of the linker-cDNA fragments with EcoP15I and release of linker-tag fragments.

(6) Ligation of two linker-tag fragments in head-to-head orientation to generate linker-ditag-linker fragments.

(7) PCR amplification of linker-ditag-linker fragments.

(8) Removal of the linker fragments by digestion with NlaIII to generate ditags.

(9) Concatenation of ditags.

(10) Cloning of ditag concatemers into a plasmid vector.

(11) Sequencing of the plasmid insert.

(12) Extraction of tag sequence and compilation of the data.

2.3.3.1 Statistical analysis and tag-gene annotation

The 26 bp tags were extracted from each library. Statistical tests were applied to the tags (Audic Test, Claverie; $P \leq 0.05$). Each 26 bp tag is highly specific and can be exactly annotated to the corresponding gene in different EST databases:

1- GDB Plant all EST 2- GDB Plant all mRNA (http://www.plantgdb.org/)

3- All TIGR Plant.fa 4-Asteraceae TIGR (http://plantta.jcvi.org/)

5- Asteraceae Entrez mRNA.fasta (http://www.ncbi.nlm.nih.gov/)

2.3.3.2 Gene ontology analysis of SuperSAGE Hits

ESTs matching to the analyzed tags were categorized via gene ontology (GO) analysis using the web based toolkit STDGE2GO (http//genxpro.ath.cx/index.php). GO analysis provides an interpretation and visualisation of biological relationships for high-throughput experimental results. STDGE2GO identifies significantly enriched GO categories from the transcription profiles using statistical methods such as Fisher's exact test and provides the data in a conveniently analysable form (Fig. 10). ESTs related to the GO subcategories concerning response to water deprivation and oxidative stress were identified, and only the individual up-regulated tags related to these classes were concerned. Sets of up-regulated tags related to the different experimental conitions (control, elevated CO_2, drought, and elevated CO_2 + drought) were annotated, generating Venn diagrams, aiming the visualization of specific or shared tags regulated by the different treatments.

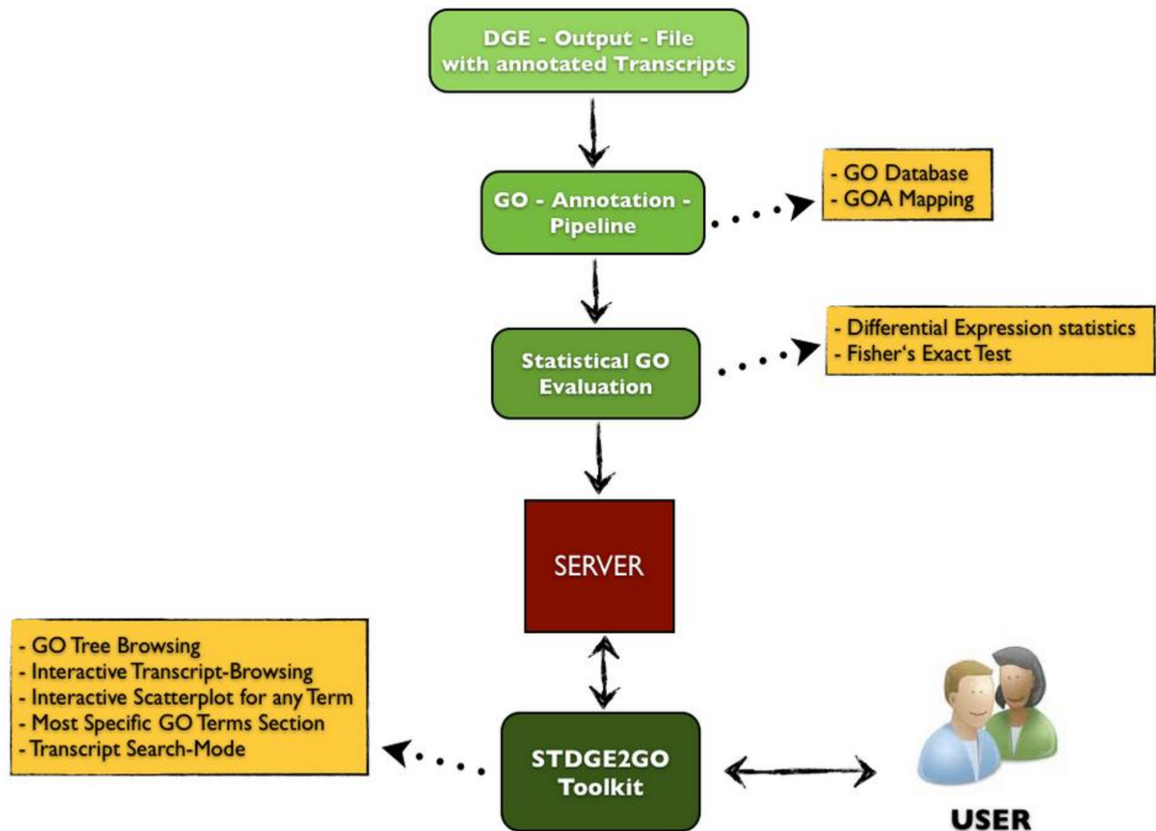

Figure 10: Workflow for a complete functional analysis of superSAGE data processing up to reaching the STDGE2GO toolkit (http://genxpro.ath.cx/).

2.3.4 Isolation of DNA and global DNA methylation

Total DNA was isolated from 50 mg pollen samples using 200 µl AP1 buffer and 4 µl RNase from the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The solution was transferred to 2 ml tubes containing ceramic spheres (Ø 1.4 mm), silica spheres (Ø 0.1 mm), and a single glass sphere (Ø 4 mm). The pollen was homogenised ten times at 6.5 ms^{-1} for one min on dry ice using a FastPrep 24 machine (MP Biomedicals, Eschwege, Germany). Another 200 µl AP1 buffer was added on top and the samples were vigorously vortexed, incubated for 10 min at 65°C, then130 µl of AP2 buffer was added, mixed and incubated for 5 min on ice, and then the samples were centrifuged for 5 min at 13000 rpm at 4°C. The lysate was transferred into a QIA-shredder Mini spin column, centrifuged for 2 min at 13000 rpm, and the flow-through fraction was transferred into a new tube without disturbing the pellet.

Then 1.5 volumes of AP33/E-buffer were added to the flow-through, mixed by pipetting, and 650 µl of the mixture was transferred into a DNeasyMini spin column, centrifuged for 1 min at 8000 rpm and the flow-through was discarded; this step was repeated with the remaining sample volumes. The spin column was placed into a new 2 ml collection tube, 500 µl AW buffer was added, centrifuged for 1 min at 8000 rpm and the flow-through was discarded. Another 500 µl of AW buffer was added, centrifuged for 2 min at 13000 rpm, the spin column was placed into a new 1.5 ml microcentrifuge tube, and 100 µl AE buffer was added to elute the DNA by centrifugation for 1 min at 8000 rpm. The eluted DNA was stored at -80°C. The DNA concentration and quality were determined by measuring the absorbance values at 260 nm and 280 nm using a spectrophotometer (NanoDrop system, Kisker, Steinfurt, Germany). Only DNA samples with acceptable 260/280 ratios (<1.8 and >2.0) were used for global DNA methylation analysis.

Analysis of global DNA methylation

To investigate the global DNA methylation levels of the first generation and second generation pollen, the Imprint® Methylated DNA Quantification Kit (Sigma, Cat. No. MDQ1–96) was used following the supplier's protocol; 100 ng pollen DNA was analyzed for each sample. In this assay, 5-methylcytosine-modified genomic DNA is recognized by a 5-methylcytosine antibody, and the bound DNA is quantified using a colorimetric reaction.

Positive (cytosine methylated) and negative (unmethylated) control DNA was supplied with the kit. The relative global DNA methylation level was calculated as percentage relative to the methylated control DNA. For global methylation analysis three samples from each group in triplicate were used; the results were analyzed using the t-test.

2.4 Proteomic analysis

2.4.1 Protein extraction:

Pollen samples (10 mg) were mixed with 300 µl acetone containing 10% TCA (w/v) and 1% DTT (*w/v*) as previously described (Sheoran et al. 2009), and transferred to 2 ml tubes containing ceramic spheres (Ø 1.4 mm), silica spheres (Ø 0.1 mm), and a single glass sphere (Ø 4 mm). The pollen was homogenised ten times at 6.5 ms $^{-1}$ for one min on dry ice using a FastPrep 24 machine (MP Biomedicals).

The samples were stored at -20°C overnight centrifuged at 25,000 *g* for 20 min at 4°C, then the pellet was washed twice with acetone containing 1% DTT, incubated at -20°C for 1 hour, and centrifuged. The vacuum dried pellet was dissolved in direct iso-electric focusing (IEF) buffer comprising 8 M urea, 20 mM DTT, 4% CHAPS and 2% ampholyte (pH 4–7) by vortexing for 1 h at 20°C.

This solution was centrifuged at 20°C for 20 min at 25,000 *g*, the supernatant was collected and the residue was re-extracted with IEF buffer. The combined supernatants were centrifuged and used for protein estimation and electrophoresis analysis. Protein concentrations were measured according to the Bradford assay (Bradford 1976) using BSA as a standard. Fifty micrograms of each protein sample were precipitated and purified using the 2D-Clean up Kit (GE Healthcare, Uppsala, Sweden). The proteins were re-suspended by incubation for 1 h in labelling buffer (7 M urea, 2 M thiourea, 30 mM Tris-HCl, pH 8.5, 4% [*w/v*] CHAPS) and adjusted to pH 8.5 using NaOH.

2.4.2 Gel electrophoresis

2.4.2.1 One-dimensional polyacrylamide gel electrophoresis

One-dimensional polyacrylamide gel electrophoresis (1-DE) was carried out according to the protocol of Laemmli (1970). Protein samples were combined with 2x sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% [*w/v*] SDS, 10% [*v/v*] glycerol, 5% [*v/v*] [β-mercaptoethanol], 0.001% [*v/v*] bromophenol blue) and incubated in a water bath at 100°C for 3 min prior to loading.

The proteins were resolved by SDS-PAGE using a 4% (*w/v*) stacking gel and a 12% (*w/v*) separating gel on a Mini-protean II (Bio-rad, Munich, Germany) vertical electrophoresis system for approximately 70 min at 30 mW in running buffer consisting of 25 mM Tris, 192 mM glycine and 0.05% (*w/v*) SDS (Laemmli, 1970).

Pre stained molecular mass standards (PageRuler®-Prestained Protein Ladder; Thermo-Fisher Scientific) were utilized to monitor migration and assess molecular mass. Once separation was completed, the gels were stained with 0.1% (*w/v*) Coomassie brilliant blue in 50% (*v/v*) methanol and 7% (*v/v*) glacial acetic acid for a minimum of 1.5 h followed by destaining in 20% (*v/v*) methanol and 7% (*v/v*) glacial acetic acid overnight.

2.4.2.2 2D-difference-in-gel-electrophoresis (2D-DIGE)

For the first and second generation plants, pooled pollen was sampled from 10 individual plants, and each group of five samples was pooled to produce two biological samples per group. Each biological sample was analyzed in triplicate, as showed in Fig. 11.

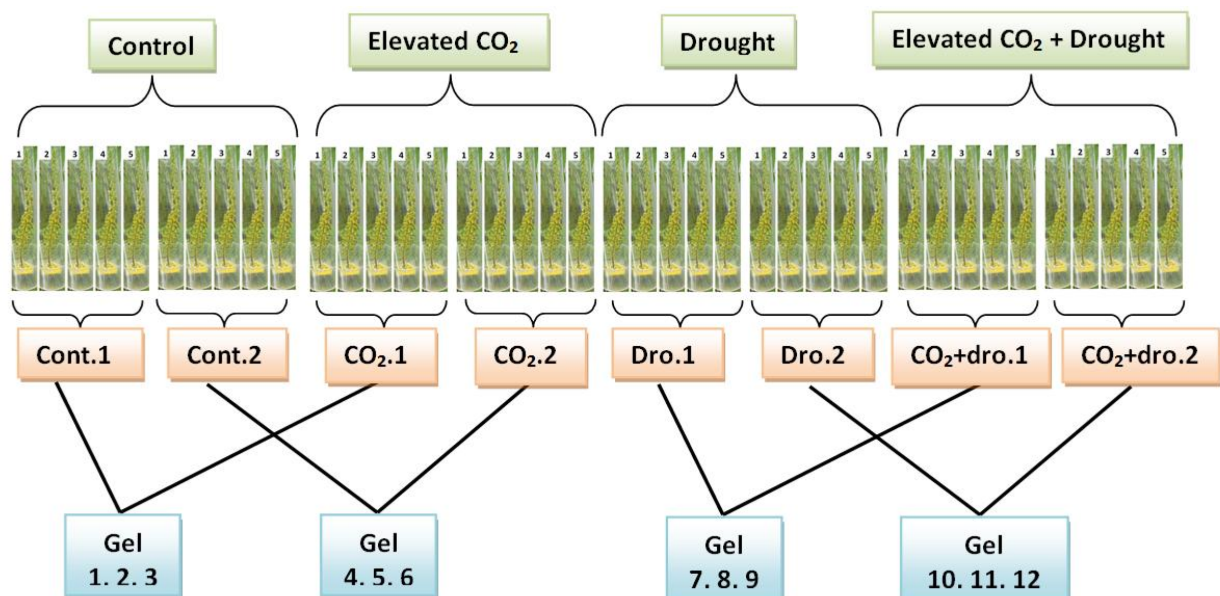

Figure 11: Workflow for analysis of the extracted protein samples by 2D-difference-in-gel-electrophoresis (2D-DIGE).

2.4.2.2.1 Fluorescent labelling of proteins

Protein labelling was performed using CyDye fluors for fluorescence 2-D DIGE technology (GE Healthcare) according to the manufacturer's minimal labelling protocol. Stock solutions of the cyanine dyes Cy2, Cy3, and Cy5 (1 nmol/ml in dimethylformamide) were diluted to 400 pmol/ml.

Fifty micrograms of protein from each control and treatment sample were labelled with 400 pmol of Cy3 or Cy5 dye as shown in Tab. 3, while 50 mg of a pooled internal standard containing equal amounts of each protein sample was labelled with Cy2 dye. After incubation on ice for 30 min in the dark, the labelling reaction was terminated by the addition of 1 ml of 10 mM lysine and incubated for 15 min on ice. For analytical 2-D DIGE gels, the differentially labelled samples were immediately combined in a 1:1:1 ratio and adjusted to final volume of 150 µl by adding equal amounts of lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% IPG-buffer, pH 3–11, 2% DTT, 0.04% bromophenol blue).

Table 3: CyDye fluorophore labelling of the samples from different treatment groups for 2-DIGE.

Gel / Dye	Cy2	Cy3	Cy5
Gel 1,2,3,4,5,6	Internal standard	Control	Elevated CO_2
Gel 7,8,9,10,11,12	Internal standard	Drought	Elevated CO_2 + Drought

2.4.2.2.2 Two-dimensional gel electrophoresis

The CyDye-labelled samples were loaded onto immobilized non-linear pH gradient strips (pH 4–7, 24 cm; GE Healthcare), which had been rehydrated overnight at RT in rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG buffer, pH 4–7, 0.8% DTT, 0.002% bromophenol blue). The samples were applied to the strips by cup-loading and separated at 20°C with a maximum current setting of 50 mA per strip using an Ettan IPGphor3 Unit (GE Healthcare) with the following settings: 150 V for 3 h, 300 V for 3 gradient from 300 to 1000 V for 6 h, gradient from 1000 to 10000V for 3 h, and 10000 V for 3 h for a total of 51750 Vh.

Afterwards, the stripes were equilibrated in 10 ml equilibration buffer (6 M urea, 2% SDS, 50 mM Tris-HCl, pH 8.8, 30% glycerol, 0.002% bromophenol blue) containing 1% DTT for 15 min and containing 2.5% iodoacetamide for 15 min, and then transferred to 12.5% SDS-PAGE polyacrylamide gels. Second dimensional SDS-PAGE was performed in running buffer (250 mM Tris, 1.92 M glycine, 1% SDS) at 12°C at 15 mA per gel for 16–18 h using the Ettan DALT six systems (GE Healthcare).

2.4.2.2.3 Image acquisition and data analysis

After electrophoresis, the DIGE gels were visualized and scanned using the Typhoon 9400 variable mode imager (GE Healthcare) according to the manufacturer's user guide. All gel images were scanned at 100 nm resolution and the photo-multiplier tube was set to ensure maximum pixel intensity without spot saturation on each image. Images were curated and analysed using Decyder software (GE Healthcare). Via two processing modules: firstly, the differential in-gel analysis (DIA) module and then the biological variance analysis (BVA) module. DIA performs spot detection and calculates spot volumes/abundances for the three images (Cy2, 3 and 5) from a single gel and then performs normalisation. During BVA, the spots are manually matched between multiple samples across all the gel images in an experiment. This allows statistical analysis of the changes in the abundance of the spots across samples. The internal standard was employed to facilitate spot matching and allow a spot-by-spot standardisation for improved reproducibility and accurate quantification of protein abundance changes across the samples. The one-way Anova was performed to compare the average standardised abundances of the replicate samples across the different conditions being tested.

2.4.2.2.4 Preparative gels and in-gel digestion

For spot picking, preparative gels were prepared on which 300 mg of the unlabelled proteins from the control and treated samples were separated by 2-DE and then silver stained. The protein spots of interest were manually excised and washed with water, then sample digestion and mass electroscopy (MS) analysis were carried out by the proteomic facilities (Helmholtz Zentrum Muenchen).

For destaining, the gel pieces were washed for 10 min with 200 µl of 60% actetonitrile (ACN), followed by a 10 min wash in 200 µl H_2O. For subsequent dehydration, the gel pieces were incubated for 10 min with 200 µl of 100% ACN, then 100 µl of 5 mM DTT was added to the gel pieces and incubated for 15 min at 60°C to reduce the proteins. After removal of the DTT and an additional dehydration step in 100% ACN, 100 µl of freshly prepared 25 mM iodacetamide (IAA) solution was added and incubated for 15 min at RT in the dark. The gel cubes were washed for 5 min with 100 µl H_2O and dehydrated again by incubation in 100% ACN for 10 min in order to remove the residual DTT and IAA.

After three 10 min wash steps in 50 mM ammoniumbicarbonate (ABC), 60% ACN and 100% ACN, respectively, the gel cubes were air-dried for 15 min at 37°C, then 100 µl of 0.01 µg/µl trypsin (Sigma) in 50 mM ABC was added. After incubation for 10 min, 25 mM ABC was added to cover the gel pieces completely during the overnight digestion at 37°C. For elution, 100 µl of 60% ACN/0.1% trifluoroacetic acid (TFA) was added to the gel cubes, incubated for 15 min with shaking, the supernatant was transferred to a new tube, and 100 µl of 99.9% ACN/0.1% TFA was added to the gel pieces, incubated for 30 min, then the supernatant was pooled with the first supernatant. The supernatants containing the eluted peptides were dried using a speedvac (UniEquip, San Diego, USA) and stored at -20°C until analysis.

2.4.2.2.5 MS analysis

Dried samples were thawed, dissolved in 60 µl of 2% ACN/0.5% trifluoroacetic acid by incubation for 30 min at RT with shaking, and the samples were centrifuged for 5 min at 4°C before loading. LC-MS/MS analysis was performed as described previously (Hauck et al. 2010). Each sample was automatically injected and loaded onto the trap column at a flow rate of 30 µl/min in 95% buffer A (2% ACN/0.1% formic acid [FA] in HPLC-grade water) and 5% buffer B (98% ACN/0.1% FA in HPLC-grade water). After 5 min, the peptides were eluted from the trap column and separated on the analytical column using a 120 min gradient which increased from 5% to 31% buffer B at a flow rate of 300 nl/min, followed by a short gradient from 31% to 95% buffer B over 5 min. Between each sample, the column was restored to 5% buffer B and allowed to equilibrate for 20 min.

From the MS pre-scan, the 10 most abundant peptide ions were selected for fragmentation in the linear ion trap (if they exceeded an intensity of at least 200 counts and were at least doubly charged). During fragment analysis, a high-resolution (60,000 full-width half maximum) MS spectrum was acquired from the Orbitrap over a mass range from 300 to 1500 Da.

2.4.2.2.6 Data processing and identification criteria

Mascot (version 2.3; Matrix Science, Boston, USA) was used for peptide identification with the following parameters: one missed cleavage allowed, a fragment ion mass tolerance of 0.6 Da, and a parent ion tolerance of 10 ppm. Carbamidomethylation was set as a fixed modification; methionine oxidation and asparagine or glutamine deamidation were allowed as variable modifications. The spectra were compared against the SwissPort database, and a Mascot-integrated decoy database search calculated an average peptide false discovery rate of < 2% when the searches were performed with an ion score cut-off of 30 and a significance threshold of $p < 0.01$.

Scaffold software (version 3_00_03; Proteome Software Inc., Oregon, USA) was used to validate the MS/MS-based peptide identifications and spectra. Peptide identifications were accepted if they could be established at greater than 80 % probability, as specified by the Peptide Prophet algorithm (Keller et al. 2002).

Detailed information of all of the identified proteins was collected and is made available in the Supporting Information. Additionally, in most cases, the theoretical isoelectric point (pI) and molecular weight (Mr) of the search results correlated well with the 2-DE position of the corresponding spot. Proteins that contained similar peptides but could not be differentiated based on the MS/MS analysis alone were grouped to satisfy the principles of parsimony.

3. Chapter – RESULTS

3.1 Morphological and enzymatic analysis

3.1.1 Morphological parameters

The impact of variability in the onset of spring under scenarios of ambient CO_2, elevated CO_2, drought, and a combination of elevated CO_2 plus drought was assessed in consecutive years 2010 and 2011 through bio-mass (plant height), and reproductive measures (inflorescence length, pollen production).

For the first generation (2010, 2011) ragweed plants, elevated CO_2 clearly enhanced the growth of the plants. The stem length increased in 2011 plants by around 42% and the length of inflorescence by around 20% and 34% for 2010 and 2011 plants respectively (Tab. 4 and Tab. 5). By contrast, drought reduced stem growth of 2011 plants by around 23%, as expected This effect could be partly mitigated by elevated CO_2, with a slight increase of around 17% (Tab. 5). Similarly inflorescence length was reduced under drought conditions by 22% and 7 % in 2011 and 2010 plants respectively (Tab. 4 and Tab. 5). Elevated CO_2 with drought in 2011 mitigated this reduction effect and resulted in approximately the same inflorescence length as found under normal CO_2 concentrations (Tab. 5). Pollen production was higher in ragweed growing under elevated CO_2 (Tab. 4 and Tab. 5) whereas drought reduced the amount, albeit not significantly ($P \leq 0.05$) (Tab. 4 and Tab. 5). This reduction effect of drought treatment was entirely neutralized and mitigated in elevated CO_2 plus drought, given that the pollen amount significantly increased through elevated CO_2 (Tab. 4 and Tab. 5).

On the other hand, in the second generation of ragweed plants from treated seeds, elevated CO_2 showed almost the same enhancement effect of plant length and increased stem length around 53% (Tab. 6). In contrast to the first generation, the drought-treated seeds plants showed a significance increase in stem length of around 28% and finally the elevated CO_2 plus drought increased the stem length by 62% (Tab. 6). Moreover, the infloresence length showed no significant change within the treatments (Tab. 6). Pollen production in elevated CO_2 did not significantly change, while there were a significant reduction in the drought-treated plants of around 47%. Finally, the elevated CO_2 plus drought showed no significant change in pollen production compared to the control (Tab.6).

Table 4: Ragweed plants of first generation 2010. Mean length of the inflorescences and the amount of pollen (number of plants N = 15-21; t-test).

CO_2	Stem length ± SD	Main inflorescence ± SD	Pollen/3 inflorescences ± SD
350 ppm (1)	-	31.2 ± 1.42 cm	0.18 ± 0.060 g
700 ppm (2)	-	37.2 ± 2.30 cm	0.24 ± 0.074 g
350 ppm + drought (3)	-	29.3 ± 1.15 cm	0.17 ± 0.050 g
700 ppm + drought (4)	-	28.4 ± 1.44 cm	0.25 ± 0.086 g
p-value (2 vs 1)	-	< 0.008	0.0000
p-value (3 vs 1)	-	0.1536	0.3725
p-value (4 vs 1)	-	0.0714	0.0647
p-value (4 vs 3)	-	0.335	0.0122

Table 5: Ragweed plants of first generation 2011. Mean length of the stem and inflorescences and the amount of pollen (number of plants N = 9-16; t-test).

CO_2	Stem length ± SD	Main inflorescence ± SD	Pollen/3 inflorescences ± SD
350 ppm (1)	64.9 ± 16.82 cm	41.09 ± 7.98 cm	0.27 ± 0,13 g
700 ppm (2)	92.4 ± 15.66 cm	54.83 ± 8.10 cm	0.38 ± 0.23 g
350 ppm + drought (3)	50.0 ± 9.98 cm	32.46 ± 4.80 cm	0.15 ± 0.23 g
700 ppm + drought (4)	76.1 ± 16.03 cm	39.78 ± 8.35 cm	0.29 ± 0.09 g
p-value (2 vs 1)	< 0.001	< 0.001	0.0004
p-value (3 vs 1)	0.007	0.006	0.001
p-value (4 vs 1)	0.108	0.621	0.201
p-value (4 vs 3)	< 0.001	0.024	0.0008

Table 6: Ragweed plants of second generation 2011. Mean length of the stem and inflorescences and the amount of pollen (number of plants N = 9-14; t-test).

CO_2	Stem length ± SD	Main inflorescence ± SD	Pollen/3 inflorescences ± SD
350 ppm (1)	45.33 ± 9.20 cm	30.77 ± 3.92 cm	0.23 ± 0.16 g
700 ppm (2)	69.00 ± 9.06 cm	31.36 ± 7.08 cm	0.24 ± 0.14 g
350 ppm + drought (3)	57.62 ± 7.04 cm	31.33 ± 5.03 cm	0.11 ± 0.86 g
700 ppm + drought (4)	73.33 ± 11.78 cm	34.66 ± 4.99 cm	0.26 ± 0.18 g
p-value (2 vs 1)	< 0.000	< 0.210	0.241
p-value (3 vs 1)	0.000	0.362	0.000
p-value (4 vs 1)	0.001	0.062	0.267
p-value (4 vs 3)	< 0.200	0.097	0.000

To assess the results obtained from the ragweed measurments of the first and second generation, each treatment in the first generation (2011) was compared with its equivalent in the second generation (2011).

The first generation showed a higher stem length, infloresence length and pollen amount than the second generation in almost all of the ragweed morphological parameter measurements (Fig.12, Fig. 13 and Fig. 14).

The control and elevated CO_2 plants; in the second generation had a significantly greater reduction in stem length than the first generation, by 30% (Fig.12). Interesitingly the stem length of the second generation ragweed drought-treated plants increased significantly more than the first generation plants by 35% (Fig.12). The elevated CO_2 plus drought displayed the same stem length in the first and second generation (2011).

The infloresecence length of ragweed in the first and second generation (2011) is shown in Fig. 13. The control of the second generation non-significantly decreased in the infloresence length; however, the second generation of elevated CO_2 significantly reduced the infloresecence length by 40%. Finally, the drought and elevated CO_2 plus drought showed no changes in the infloresence length between the first and second generation (Fig. 13).

Pollen production of the second generation in the control non-significantly reduced from that of the first generation (Fig. 14). On the other hand the pollen production in the second generation of both the elevated CO_2 and drought plants significantly decreased from the first generation, by 36% and 27%, respectively (Fig. 14). Finally, the elevated CO_2 plus drought showed no changes in the amount of pollen between the first and second generation (Fig. 13).

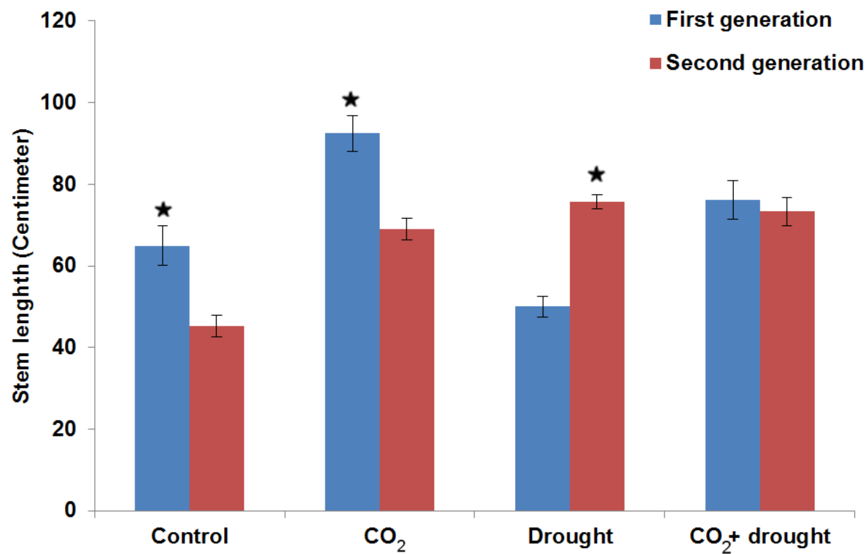

Figure 12: Ragweed plants of first and second generation 2011. Mean length of the stem (number of plants N = 9-21; t-test). Stars refer to significant difference between each treatments in the first and second generation ($P \leq 0.05$).

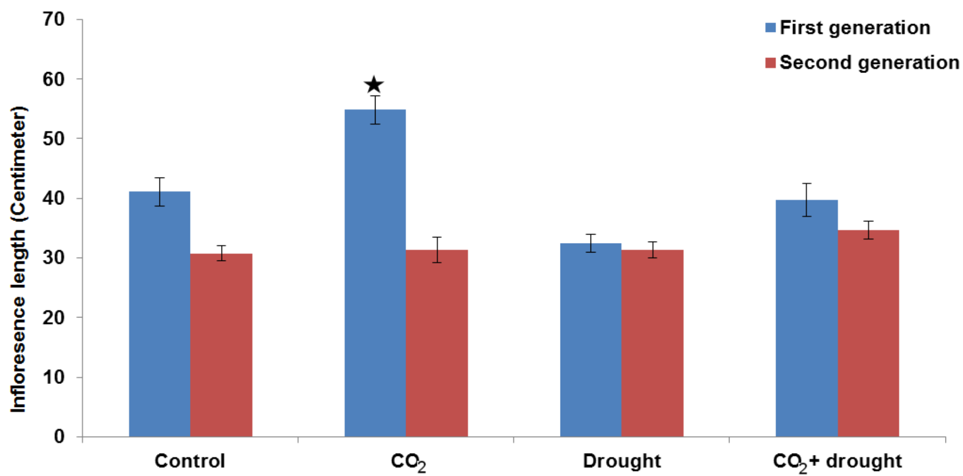

Figure 13: Ragweed plants of first and second generation 2011. Mean length of the inflorescences (number of plants N = 9-21; t-test). Stars refer to significant difference between each treatments in the first and second generation ($P \leq 0.05$).

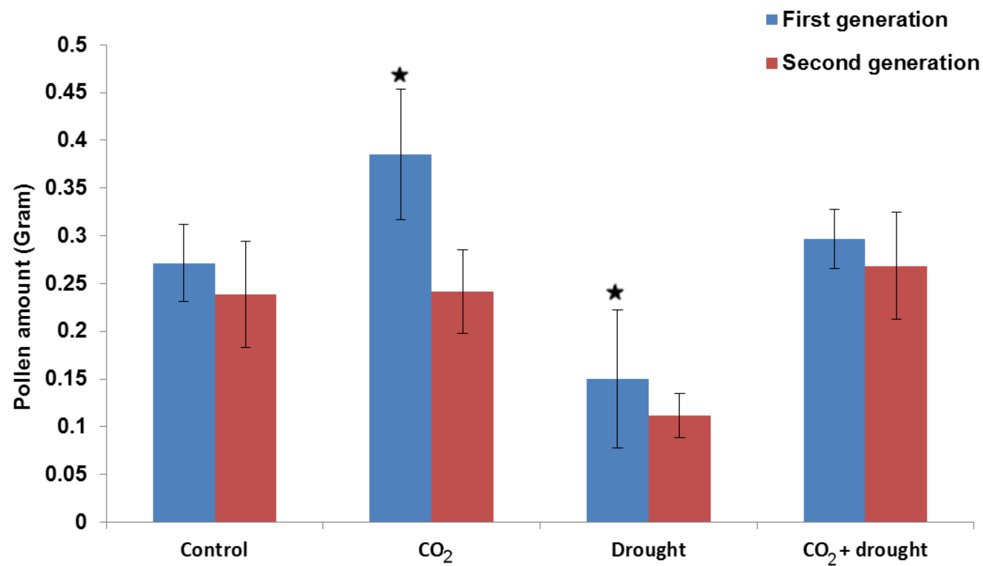

Figure 14: Ragweed plants of first and second generation 2011. Mean weight of the pollen grains (number of plants N = 9-21; t-test). Stars refer to significant difference between each treatments in the first and second generation ($P \leq 0.05$).

3.1.2 100 seed weight

Given that the plant has a fixed amount of resources to allocate reproduction, the plant decision must be made concerning both the number and size of their off-spring seeds (Smith 1974). Individuals producing seeds either smaller or larger than the optimum suffer from fitness reduction (Geritz 1995). The first generation of ragweed seeds for each treatment were collected at the end of the experiments, with 10 times of 100 seeds weighted; indeed, significant variation between treatments was observed ($P \leq 0.005$) (Tab. 7). The elevated CO_2 evidently increased the seed weight by around 10%. Moreover, the drought unexpectedly increased the weight by around 7%. Finally, the elevated CO_2 plus drought showed no significant change in the seed mass compared to the control seeds (Tab. 7).

Table 7: Descriptive statistics of first generation (2010) of 100 seed weight (gram) of ragweed. Mean weight (Number of samples 10 each 100 seed; t-test).

CO_2	Min./gram 100 seed weight	Max./gram 100 seed weight	Mean ± SD	Standard error
350 ppm (1)	0.51	0.63	0.54 ± 0.035	0.009
700 ppm (2)	0.5	0.63	0.59 ± 0.046	0.012
350 ppm + drought (3)	0.53	0.65	0.58 ± 0.033	0.009
700 ppm + drought (4)	0.48	0.60	0.56 ± 0.031	0.008
p-value (2 vs. 1)			0.002	
p-value (3 vs. 1)			0.001	
p-value (4 vs. 1)			0.058	
p-value (4 vs. 3)			0.100	

3.1.3 Test for pollen viability

The use of p-phenylenediamine represented a more reliable method than fluorescein diacetate and tetrazolium dyes to distinguish between viable and non-viable pollen (Fig. 15). Non-viable pollen turned almost greyish-brown, which strongly contrasted with the colour of fresh pollen; more importantly, it never stained non-viable (aborted) pollen. The data concerning the ragweed pollen (2010) viability under elevated CO_2, drought and CO_2 plus drought is displayed in Tab. 8. The mean of pollen viability was 46% in the control and 41% in the elevated CO_2.

The pollen of drought treatment significantly reduced the viability, reporting 24% ($P \leq 0.000$) (Tab. 8), while the reduction of pollen viability under drought was mitigated by elevated CO_2, recorded at 30% ($P \leq 0.000$) (Tab. 8).

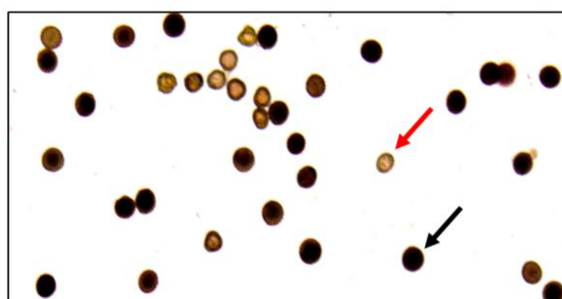

Figure 15: Ragweed pollen stained by p-phenylenediamine, black arrow refers to viable pollen while red arrow refers to dead pollen.

Table 8: Summary statistics for the percentage of viable pollen of the control, elevated CO_2, drought and elevated CO_2 plus drought of ragweed pollen (number of samples 100; t-test).

CO_2	Min. %	Max. %	Mean % ± SD	Standard error
350 ppm	35	57	46 ± 5.5	1.2
700 ppm	19	68	41 ± 17.8	4.0
350 ppm + drought	12	50	24 ± 13.1	2.9
700 ppm + drought	22	41	30 ± 4.8	1.1
p-value (2 vs. 1)			0.166	
p-value (3 vs. 1)			0.000	
p-value (4 vs. 1)			0.000	
p-value (4 vs. 3)			0.144	

3.1.4 Scanning electron microscopy (SEM)

The first generation (2010) ragweed pollen of control, elevated CO_2, drought and elevated CO_2 plus drought were studied for pollen morphology, with no morphological differences in the grown pollen found (Fig. 16). According to SEM photographs, the pollen is uniformly spinulose and the spinules are sharply pointed. The mean size of the roundish pollen was in the range of 17.5-21.9 µm, while no significant differences between the control, elevated CO_2, drought and elevated CO_2 plus drought plants could be detected in term of pollen size (control: 17.5±1.06 µm, n=80; elevated CO_2: 17.7±1.42 µm, n=80; drought: 21.9±1.30 µm, n=80; elevated CO_2 plus drought: 18.2±2.10 µm, n= 80).

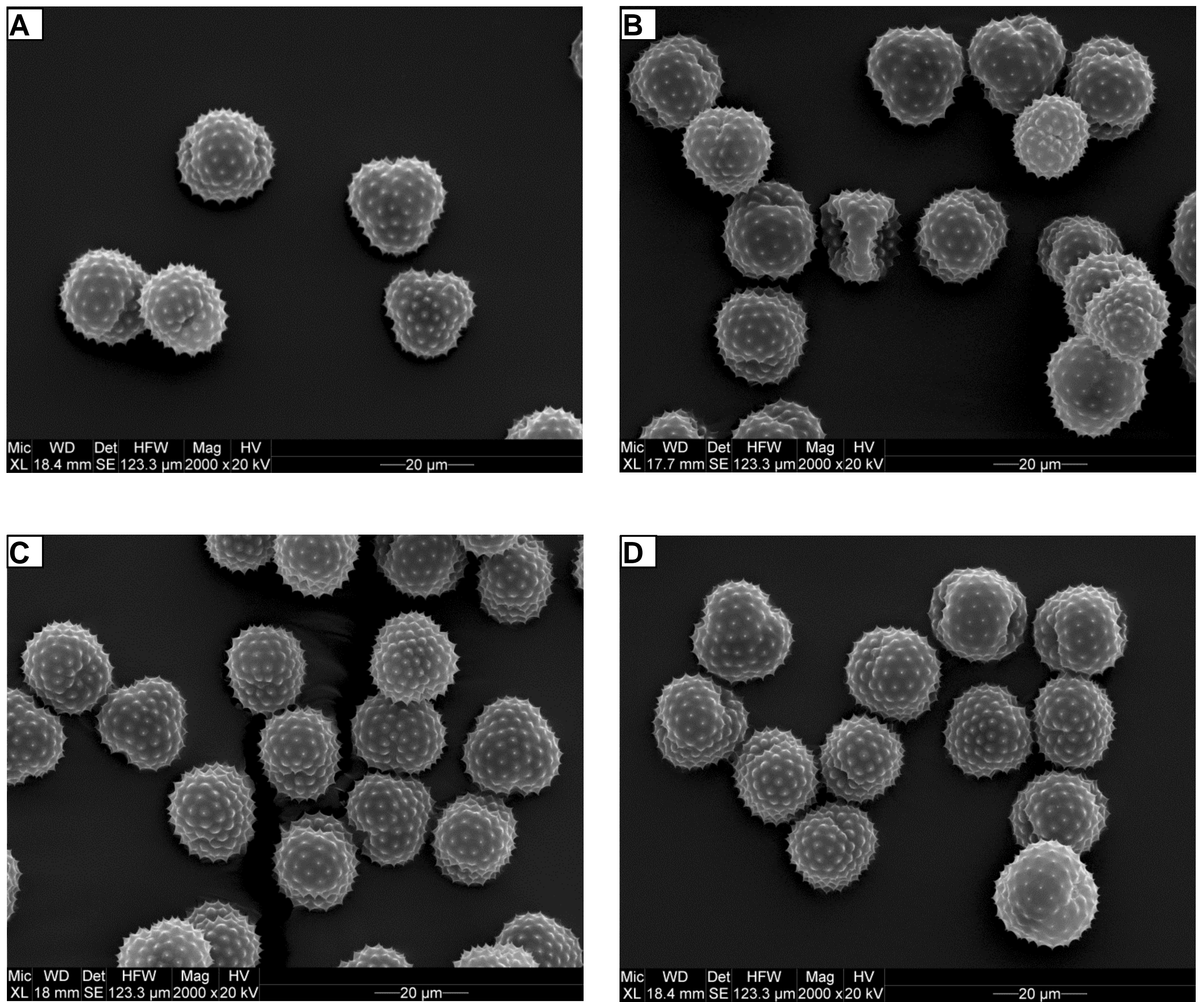

Figure 16: Scanning electron microscopy of ragweed pollen (A) Control. (B) Elevated CO_2. (C) Drought. (D) Elevated CO_2 + drought. Bars 20 µm.

3.1.5 Analysis of phenolic metabolites by reverse-phase HPLC (RP-HPLC)

The RP-HPLC is sufficiently simple and effective for the identification and quantification of major phenolic compounds in ragweed pollen. Extraction was performed with two solvents: first PBS to separate water-soluble compounds, followed with methanol.

17 prominent compounds were detected in PBS-soluble extracts (Fig. 17). The highest amounts were found for quercetin derivatives (Tab. 9). Two individual metabolites of the PBS buffer extract were shown to significant increase in CO_2 (Fig. 18; DA 5 and DA 16), while the drought showed higher amounts in five metabolites (Fig. 18; DA 3, DA 5, DA 8+9, DA 10 and DA 16). Furthermore, quercetin derivatives showed a higher amount in elevated CO_2 plus drought than other treatments (Fig 18; DA 13). Methanol-extractable phenolics showed 12 prominent compounds, characterised as hydroxycinnamic amides according to their typical diode-array spectra (Fig. 19). Two peaks representing quercetin derivatives in PBS-soluble extract (Tab. 9, Nr. 11 and 12) are also apperared in the methanol-extracte (Tab.10, Nr. 3 and 4). One individual peak for the elevated CO_2 (Fig 20; DA 1) and two peaks for the drought pollen showed a significant increase than control (Fig 20; DA 6 and DA 7+8). Finally, the HPLC pattern of the remaing ragweed pollen in methanolic extracts did not show any significant difference across the treatments.

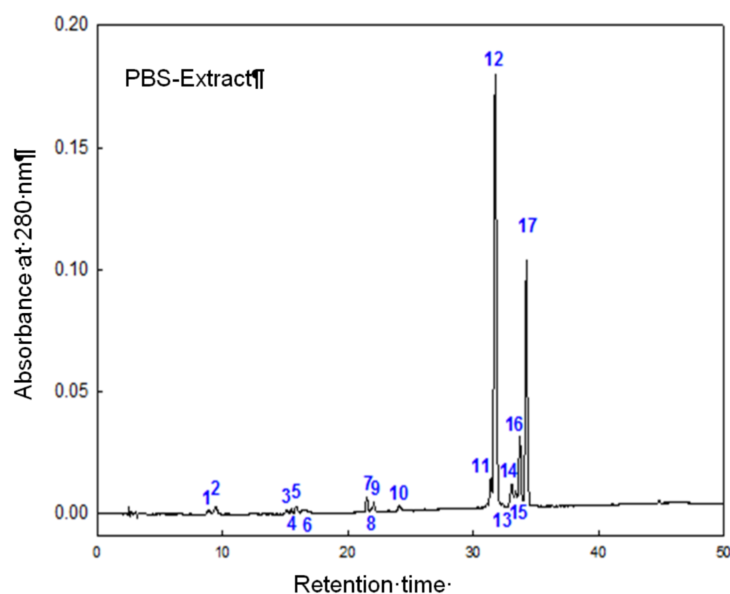

Figure 17: RP-HPLC analysis of soluble extracts (PBS-extract) of ragweed pollen. Graph spectrum shows the 17 peaks observed.

Table 9: RP-HPLC analysis of soluble extracts (PBS-extract) of ragweed pollen. Retention time of each peak with the maximum absorbance.

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Nr.	Time/min.	Maximum	Comment
1	8.8	285	-
2	9.3	263	-
3	14.8	274	-
4	15.1	255-277	-
5	15.4	258	-
6	16.2	264	-
7	21.4	263	-
8	21.7	206-266	-
9	22	263	-
10	24	272-278-289	-
11	31.4	256-356	Quercetin derivatives
12	31.7	257-356	Quercetin derivatives
13	32.3	261-264-356	Quercetin derivatives
14	33.2	267	-
15	33.4	252-266-354	Quercetin derivatives
16	33.8	265-348	Kaempferol derivatives
17	34.3	256-356	Quercetin derivatives

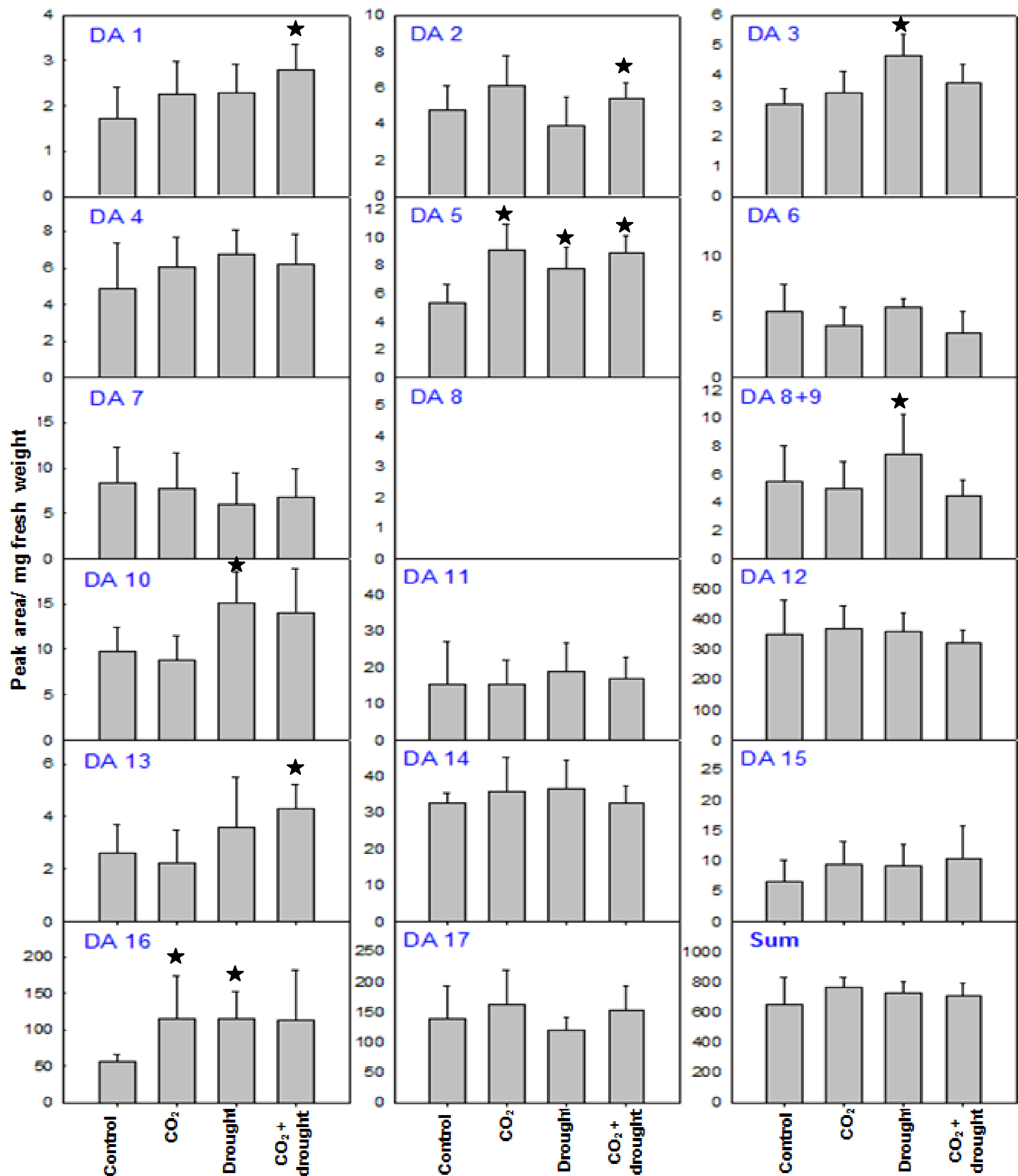

Figure 18: RP-HPLC analysis of soluble extracts (PBS-extract) of ragweed pollen. Bars indicate ± SD; n=5, t-test. Stars refer to significant difference between treatment and control ($P \leq 0.05$).

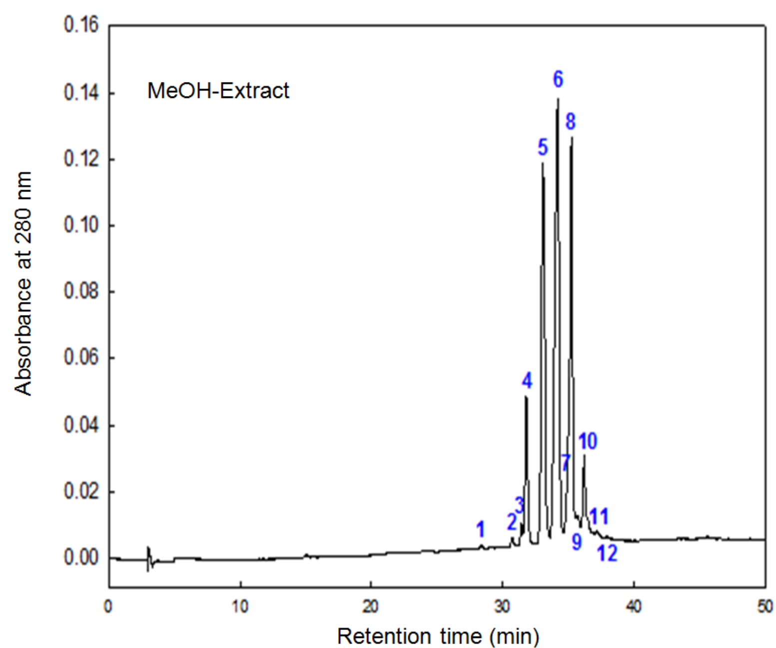

Figure 19: RP-HPLC analysis of methanolic extracts of different ragweed pollen. Graph spectrum shows the 12 peaks observed.

Table 10: RP-HPLC analysis of methanolic extracts from different ragweed pollen. Retention time of each peak with the maximum absorbance.

Nr.	Time/min.	Maximum	Comment
1	28.4	276	
2	30.7	290-306	
3	31.4	255-355	PBS 11
4	31.8	265-300-355	PBS 12
5	33.1	290	
6	34.2	295	
7	35	292	
8	35.2	308	
9	35.6	290	
10	36.2	208	
11	37.1	208	
12	37.9	300-312	

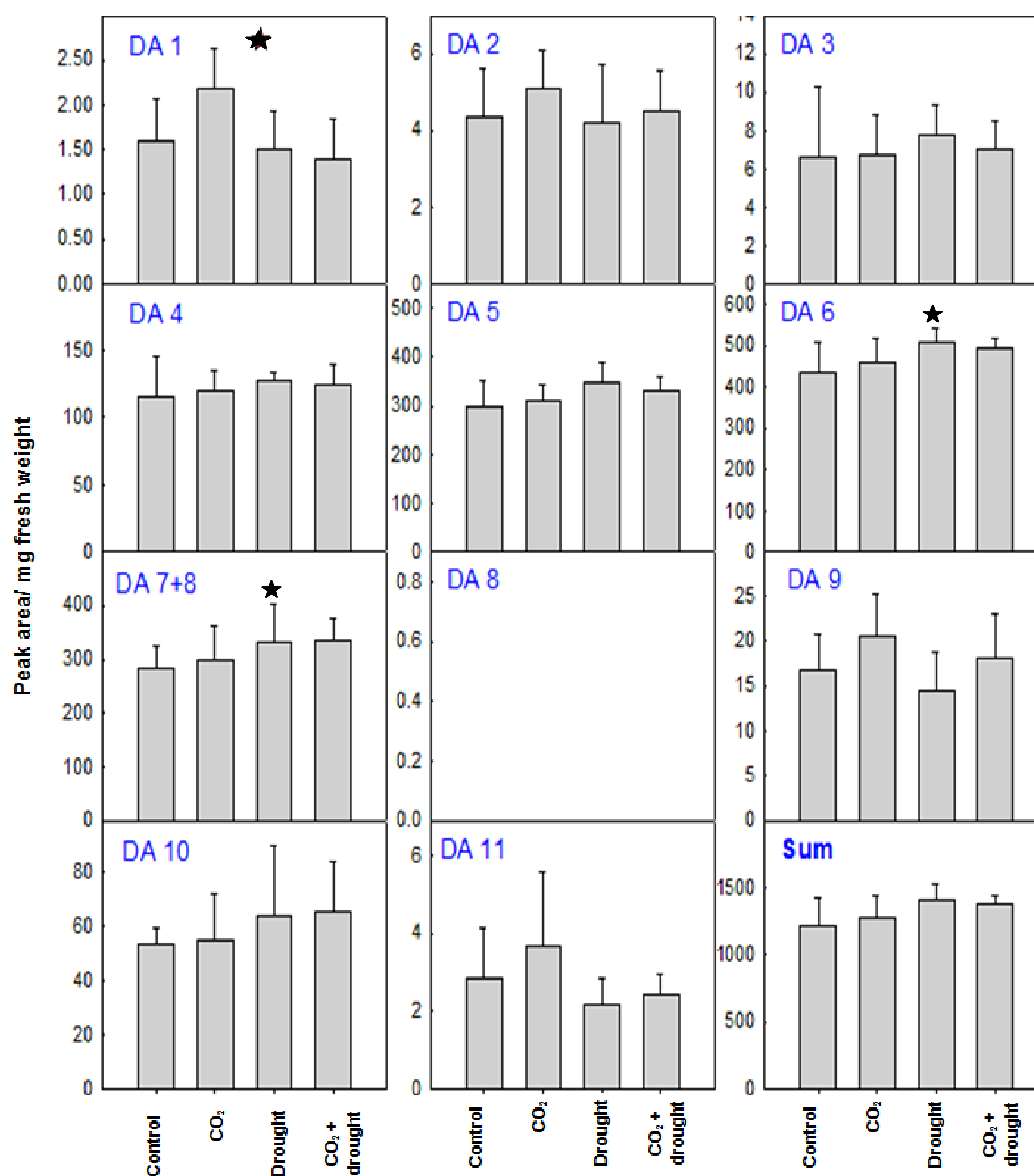

Figure 20: RP-HPLC analysis of methanolic extracts of different ragweed pollen. Bars indicate ± SD; n=5, t-test. Stars refer to significant difference between treatment and control ($P \leq 0.05$).

3.1.6 NAD(P)H oxidase enzymatic activity

The NAD(P)H oxidase enzyme activity in the first generation of ragweed pollen (2011) was determined by the amount of NBT that reduced to formazan (Fig. 21). Initially, the reduction of NBT by the ragweed pollen was just detectable without the NADPH, with no significant difference observed between the control and treated pollen grains (Fig. 21). Upon addition of NADPH (substrate), the activities measured from the elevated CO_2 treated pollen increased by 11% ($P \leq 0.05$), while drought pollen showed non-significant changes. Finally the elevated CO_2 plus drought significantly decreased by 14% (Fig. 21).
This reduction of NBT by the ragweed pollen extract was almost entirely blocked by the addition of SOD , which suggests that NAD(P)H oxidase is the major source of these ROS.

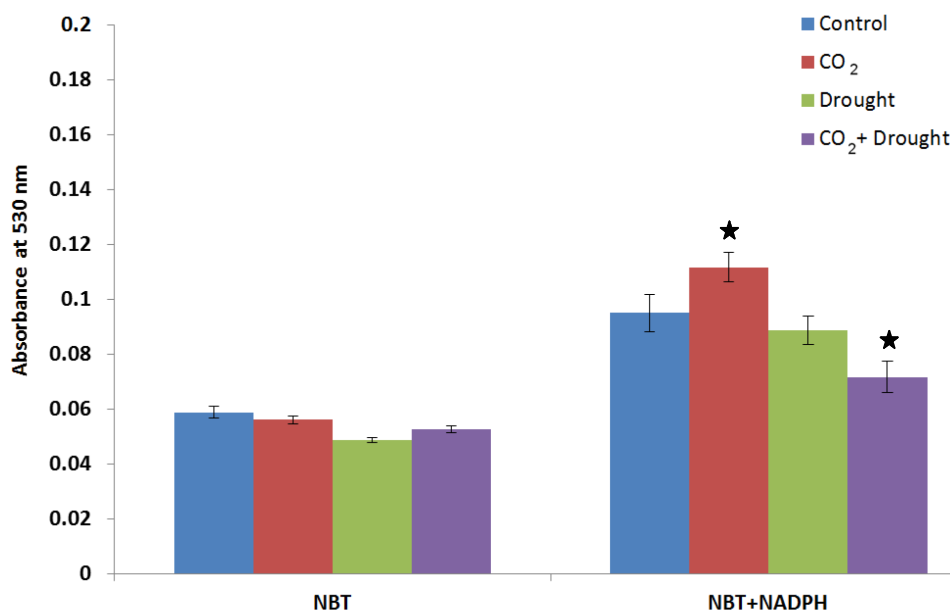

Figure 21: NAD(P)H oxidase activity in pollen from control, elevated CO_2, drought and elevated CO_2 plus drought treatment. The NAD(P)H oxidase activity was assessed in the absence and presence of NADPH. Bars indicate ± SD; n=3, t-test. Stars refer to significant difference between treatments and control ($P \leq 0.05$).

3.1.7 Hydrogen peroxide content

Hydrogen peroxide is the main cause responsible for inflammatory processes, increasing the flow of neutrophils towards the respiratory apparatus (Boldogh et al. 2005). H_2O_2 production in ragweed pollen extract was detected by the conversion of hydroperoxides with ferrous iron (II) to ferric iron (III) at acidic pH. The ferric ion complexes with the xylenol orange dye yielded a purple product with maximum absorbance at 560 nm. For the first generation ragweed pollen (2011), elevated CO_2, drought and elevated CO_2 plus drought ($P \leq 0.000$) significantly increased H_2O_2 production, by 500%, 1200% and 1000% in elevated CO_2, drought and elevated CO_2 plus drought respectively (Fig. 22).

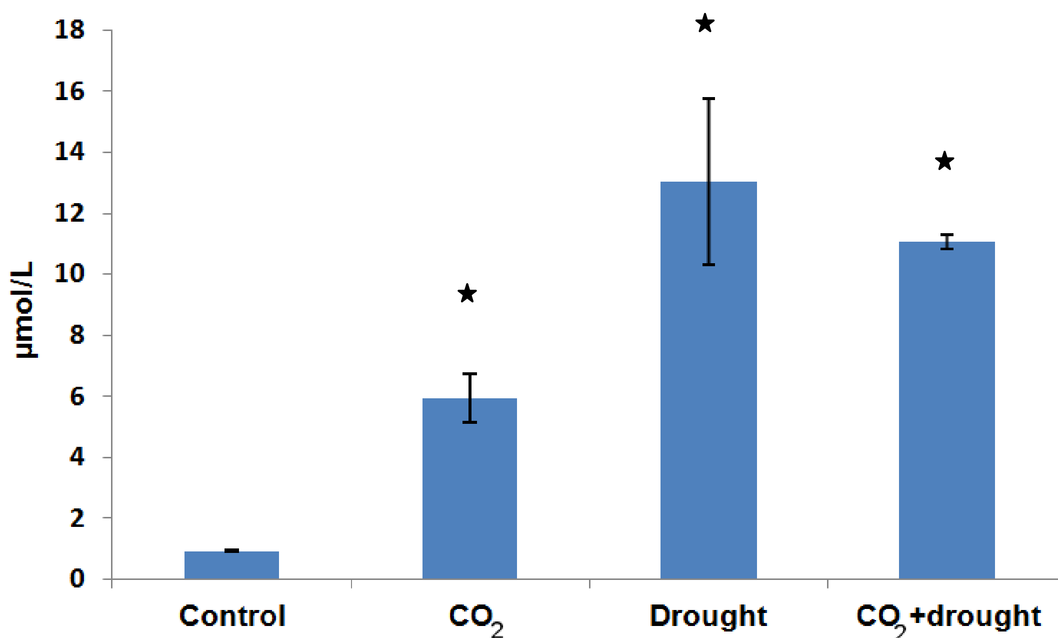

Figure 22: Hydrogen peroxide content in ragweed pollen extract from control, elevated CO_2, drought and elevated CO_2 plus drought. Bars indicate ± SD; n=3, t-test. Stars refer to significant difference between treatments and control ($P \leq 0.05$).

3.1.8 Determination of total oxidant status (TOS)

Oxidant species (OS) or ROS are produced in metabolic and physiological processes, which were subsequently scavengered via enzymatic and non-enzymatic antioxidative mechanisms. At the same time, this oxidative stress was been involved in burst and enhance allergenic potential (Harma and Erel 2005, Yanik M 2004). The total oxidant status of first generation ragweed pollen (2011) showed that no significant changes were observed between the control and elevated CO_2, drought, elevated CO_2 plus drought treated pollen (Fig. 23).

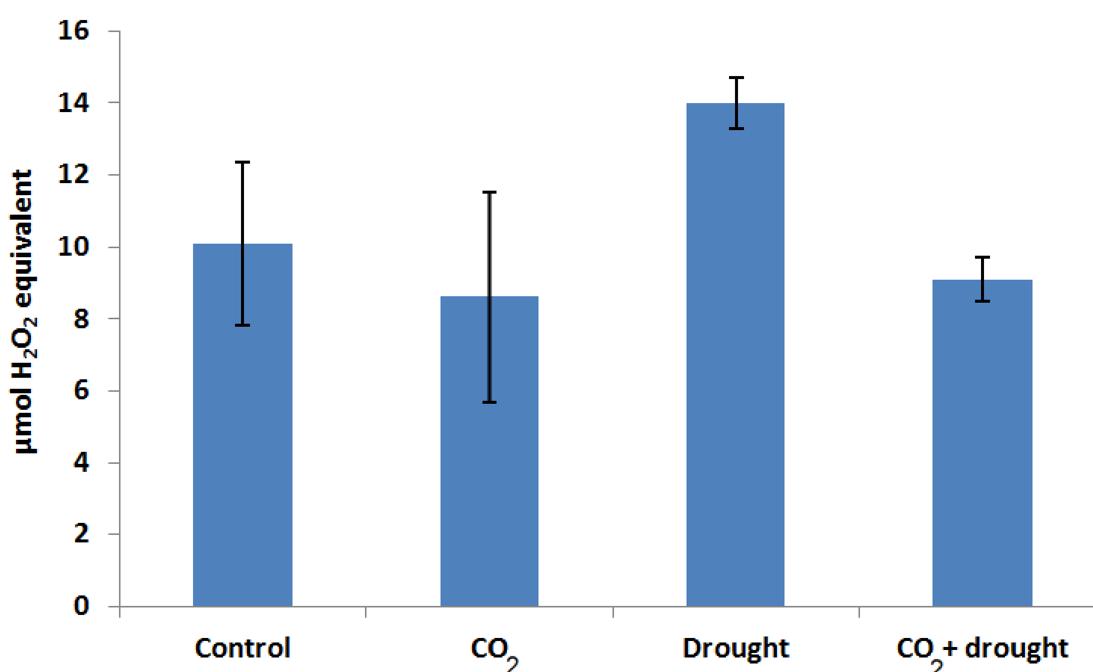

Figure 23: Total oxidant status in pollen of control, elevated CO_2, drought and elevated CO_2 plus drought. Bars indicate ± SD; n=3, t-test.

3.1.9 Determination of total antioxidant status (TAS)

Oxidative stress, reflecting the consequence of an imbalance between ROS generation and antioxidants production in the plant, initiates a series of a harmful biochemical events that are also associated with diverse physiological processes (Sastre et al. 2003).

In pollen grains an efficient antioxidant network is essential in order to effectively scavenge ROS and maintain intracellular ROS pools at low levels (Foyer and Shigeoka 2011). The total amounts of antioxidant in pollen extracts are quantified by the percentage of inhibition of ABTS (Fig. 24). The total antioxidant status of first generation ragweed pollen (2011) of elevated CO_2, drought, elevated CO_2 plus drought compared to the control is displayed in Fig. 24. The elevated CO_2 showed no significant changes compared to the control, however; the amount of antioxidant in drought-treated pollen significantly (p=0.003) reduced from the control, by around 30%. Finally, elevated CO_2 plus drought showed no significant difference from the control pollen in the total antioxidant status (Fig. 24).

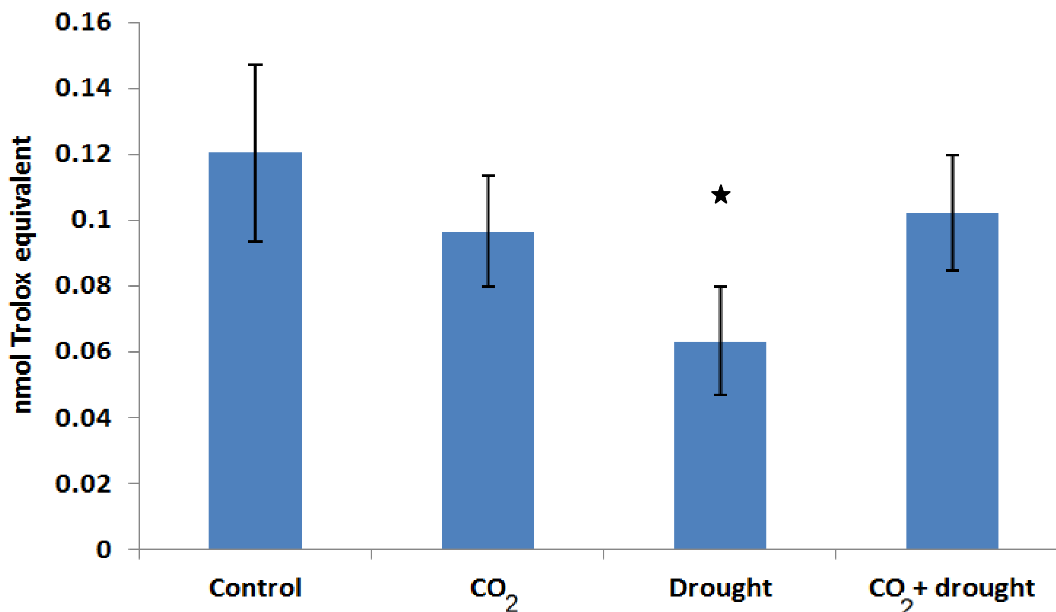

Figure 24: Total antioxidant status in ragweed pollen of control, elevated CO_2, drought and elevated CO_2 plus drought. Bars indicate ± SD; n=3, t-test. Stars refer to significant difference between treatments and control ($P \leq 0.05$).

3.1.10 Determination of oxidative stress Index (OSI)

The percent ratio of the total oxidant status to the total antioxidant status provides the oxidative stress index (OSI), which represents the key indicator of the oxidative stress degree that occurs inside the living cell (Erel 2004, 2005). The total antioxidant status of first generation ragweed pollen (2011) under elevated CO_2, treated with drought and elevated CO_2 plus drought compared to the control is shown in Fig. 25, highlighting that the elevated CO_2 showed no significant increase from the control pollen. Furthermore, it is apparent from Fig. 25 that the OSI of the drought treated pollen significantly (p=0.02) increased by around 28% compared to control pollen, while the OSI of the elevated CO_2 plus drought showed no significant differences compared to the control pollen.

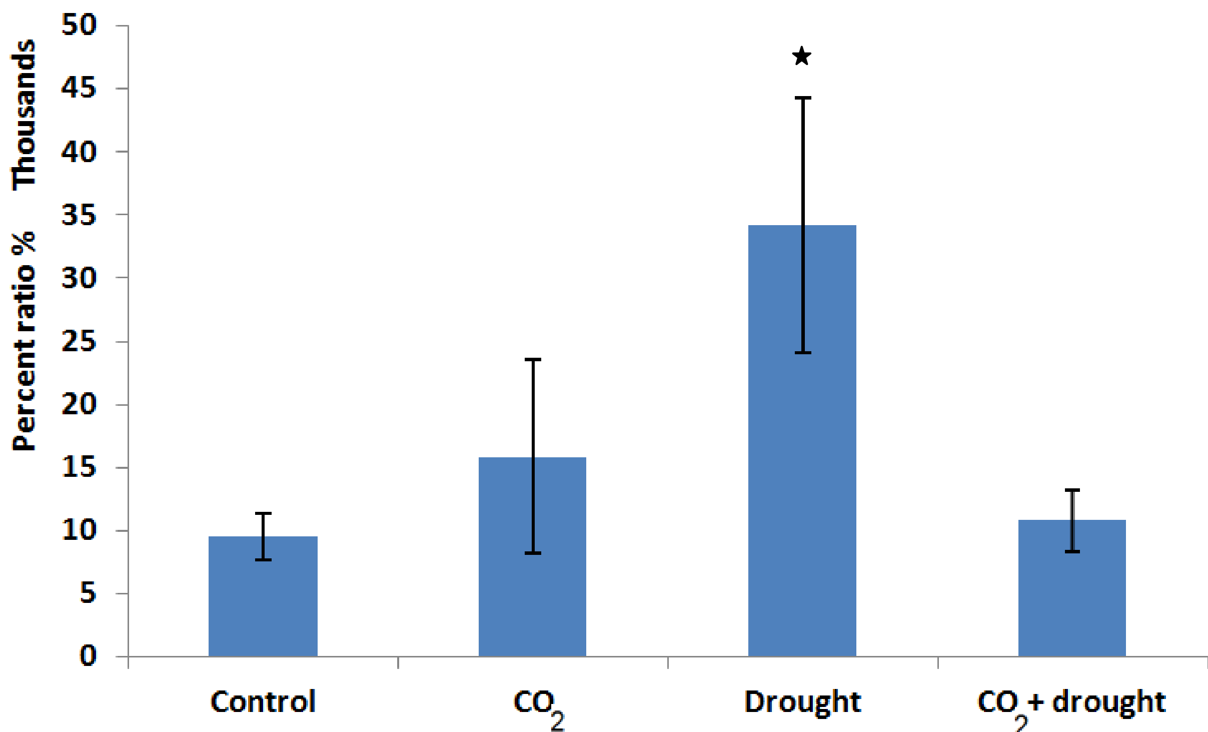

Figure 25: Oxidative stress index (OSI) in ragweed pollen extract of control, elevated CO_2, drought and elevated CO_2 plus drought. Bars indicate ± SD; n=3, t-test. Stars refer to significant difference between treatments and control ($P \leq 0.05$).

3.2 Genomic and Transcriptomic analysis

3.2.1 Quantitative real-time RT–PCR of ragweed pollen grains

The technique of qRT-PCR combined with the use of highly-specific primers designed for each pollen allergen enabled testing relative change in gene expression at the level of messenger RNA in a precise, accurate and sensitive manner. The selection of the optimal reference target was performed by geNorm software (Vandesompele et al. 2002). In this experiment, two housekeeping genes was the optimal number, represented by α-tubulin and 18S rRNA.

The genes were grouped into si classes according to the AllFam database of allergen families (Radauer et al. 2008) (Fig. 26): pectate lyase, which includes Amb a 1.1, 1.2, 1.3, 1.4 and 1.5; group 5 allergen for Amb a 5; non-specific lipid transfers protein (nsLTP) for Amb a 6; profilin, which includes Amb a 8.0101 and Amb a 8.0102; calcium binding protein group which refers to Amb a 9, Amb a 9.0102 and Amb a 10; and finally, the cystatin family for Amb a CPI (cystatin proteinase inhibitor).

The relative expression of different allergens in the first generation (2011), subjected to elevated CO_2, drought and elevated CO_2 plus drought pollen, significantly increased compared to the control pollen (Fig. 26). The pectate lyase allergen gene family is one of the most important allergens, given that 95% of ragweed-sensitive individuals react in skin tests and show high IgE antibody titers towards it (Platts-Mills and Woodfolk 2011). In pectate lyase, profilin, calcium binding protein and cystatin gene families, the drought pollen showed higher relative expression values than the elevated CO_2 (Fig. 26). The CO_2 in the elevated CO_2 plus drought managed and mitigated the drought effect, reducing the relative expression by around 30 to 50% (Fig. 26). By the contrast, the Amb a 10 had a higher relative expression value in the elevated CO_2 plus drought than the drought and elevated CO_2 (Fig. 26).

On the other hand, the group 5 and non-specific lipid transfers protein gene families, showed lower relative expression in the drought and higher expression in the elevated CO_2 and elevated CO_2 plus drought (Fig. 26).

The relative expression of different allergens differed between the first and second generations (2011) (Fig. 27). The ragweed pollen of the drought showed a clear reduction in the relative expression level in most of the allergens transcripts, apart from the Amb a 10, which have a higher expression than the elevated CO_2 and elevated CO_2 plus drought.

Moreover, the elevated CO_2 plus drought showed a higher relative expression level value than that of the elevated CO_2 and drought with all the allergenic gene families, apart from Amb a 1.4 and Amb a 8, where elevated CO_2 was higher than the elevated CO_2 plus drought (Fig. 27).

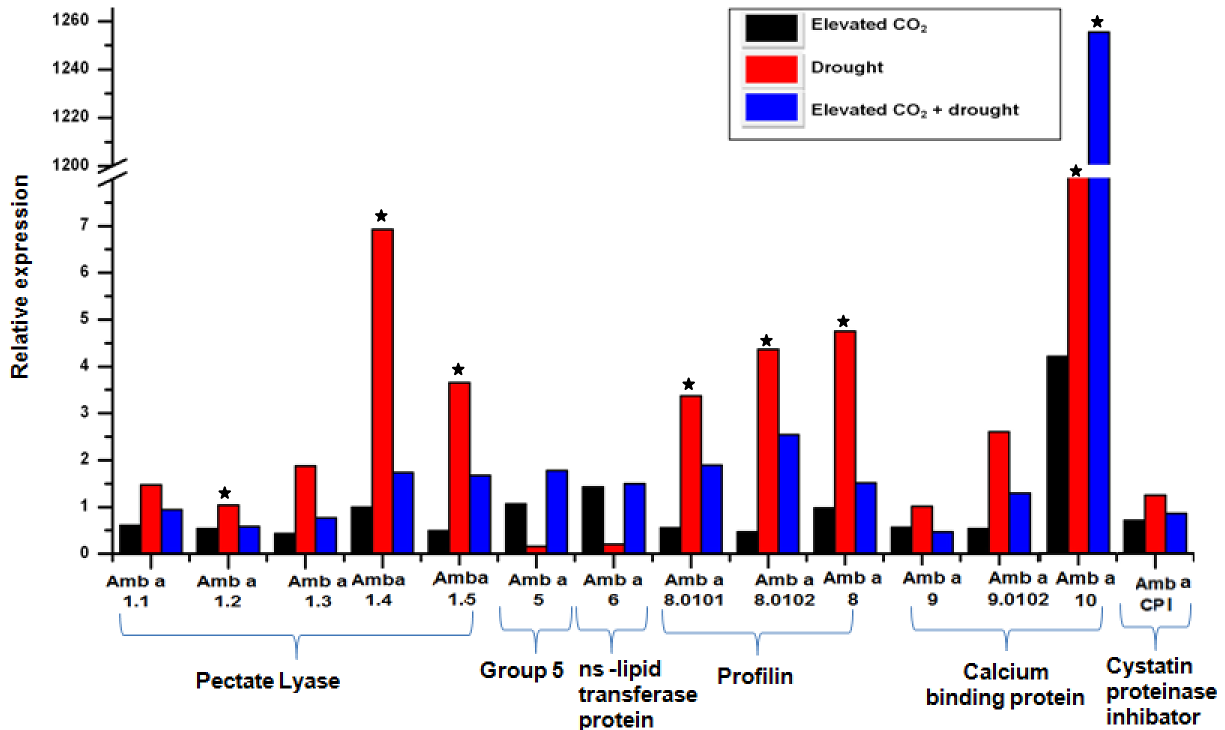

Figure 26: Real-time RT-PCR analysis for pollen allergen genes families: pectate lyase (Amb a 1.12 - 1.2 - 1.3 - 1.4 - 1.5), group 5 (Amb a 5), non-specific lipid transferase protein (nsLTP) (Amb a 6), profilin (Amb a 8.0101-8.0102-8), calcium binding protein (Amb a 9-9.0102-10) and cystatin (Amb a CIP) was represented in first generation ragweed pollen (2011) treatments (elevated CO_2, drought, elevated CO_2 plus drought). Transcript levels were normalised with respect to 16S-rRNA and α-tubulin transcript levels. Mean values were obtained from three independent PCR amplifications. Pair-wise fixed reallocation randomisation test according to Pfaffl et al., (2002). * refers to significant difference between treatments ($P \leq 0.05$) and control.

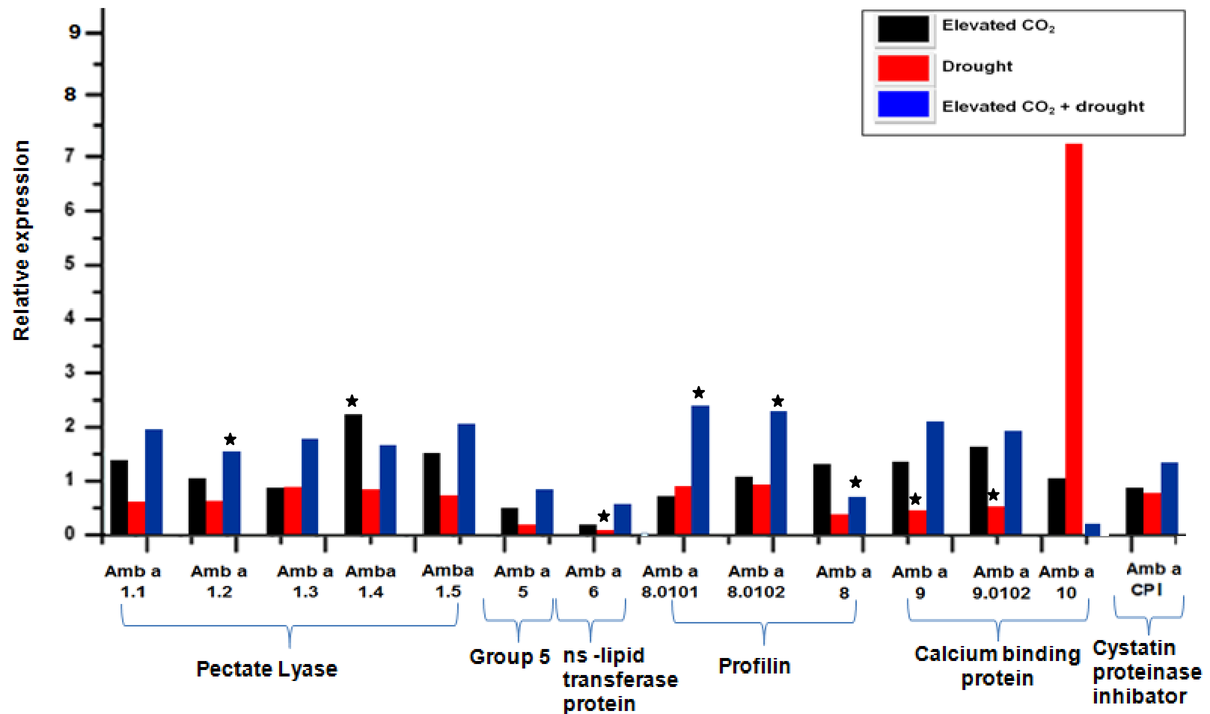

Figure 27: Real-time RT-PCR analysis for pollen allergen genes families, pectate lyase (Amb a 1.12 - 1.2 - 1.3 - 1.4 - 1.5), group 5 (Amb a 5), non specific lipid transferase protein (nsLTP) (Amb a 6), profilin (Amb a 8.0101-8.0102-8), calcium binding protein (Amb a 9-9.0102-10) and cystatin (Amb a CIP) was represented in second generation ragweed pollen (2011) treatments (elevated CO_2, drought, elevated CO_2 plus drought). Transcript levels were normalised with respect to 16S-rRNA and α-tubulin transcript levels. Mean values were obtained from three independent PCR amplifications. Pair-wise fixed reallocation randomisation test according to Pfaffl et al., (2002). * refers to significant difference between treatments (P ≤ 0.05) and control.

3.2.2 SuperSAGE libraries

In the present work, the high resolution power of SuperSAGE coupled with the Illumina - Solexa NGS technology was developed to characterise the complete transcriptome of ragweed pollen subjected to different environmental conditions. The number of sequenced tags ranged from around 4.5 x 10^6 to 17.2 x 10^6 in the four libraries. The SuperSAGE data set included 236,942 unique sequences of 26 base pairs' length in each library. The tag-amounts were provided for each of these sequences, counting how frequently a unique tag was found in each of the four samples (control, elevated CO_2, drought and elevated CO_2 plus drought). One sequence could be found in one, two, three or all samples, as indicated in overlapping regions in Fig. 28, albeit in different quantities (tag-amounts) according to the transcript expression.

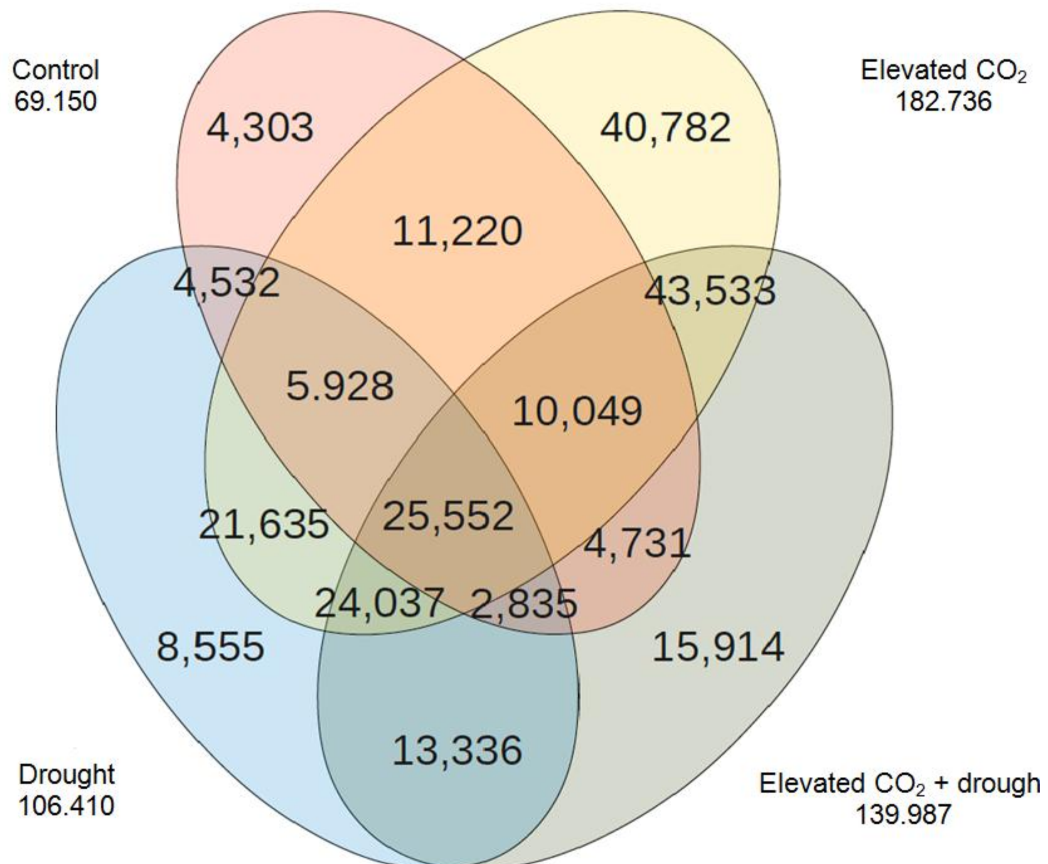

Figure 28: Number of SuperSAGE sequence tags. For each sequence the tag-amount was analysed in the individual samples (control, elevated CO_2, drought, CO_2+ drought). Sequences with ≥ 1 appearances in two, three or all samples are shown by the individual overlapping regions.

It is evident from the data in Tab. 11, that the four libraries vary in terms of the total number of sequenced tags, 69.150 for control, 182.736 for elevated CO_2, 106.410 for drought, and 139.987 for CO_2 plus drought.

Table 11: Summary of the total number of sequenced tags in different libraries and number of unique tags with its percentage.

Treatments	Total no. of Tags	no. of unique-Tags	Percentages of unique tags
Control	69.150	4.303	6.2%
Elevated CO_2	182.736	40.782	22%
Drought	106.410	8.555	8 %
CO_2+Drought	139.987	15.914	11%

The number of unique tags was the highest in the elevated CO_2 (22%), in comparison with 8% and 11% for the drought and elevated CO_2 plus drought libraries respectively (Tab.11).

3.2.2.1 Annotation of SuperSAGE tags

A significant advantage of SuperSAGE compared to conventional SAGE is that annotation becomes more species specific due to the longer tag sequence (Matsumura et al. 2003). On the other hand, investigating the transcriptome of non-model plants reflects a challenge due to the shortage of a complete genome database for accurate annotation, especially in respect of a particular plant part such as pollen grain. After EST annotation by different databases, the chart revealed that Asteraceae Entrez mRNA fast (4.1%) and Asteraceae TIGR (4.9%) possessed the best annotation matching count within these databases (Fig. 29, A). Moreover, the numbers of annotated matched genes were 16,301 for control, 43,552 for elevated CO_2, 25,999 for drought and 34,760 for elevated CO_2 plus drought (Fig. 29, B).

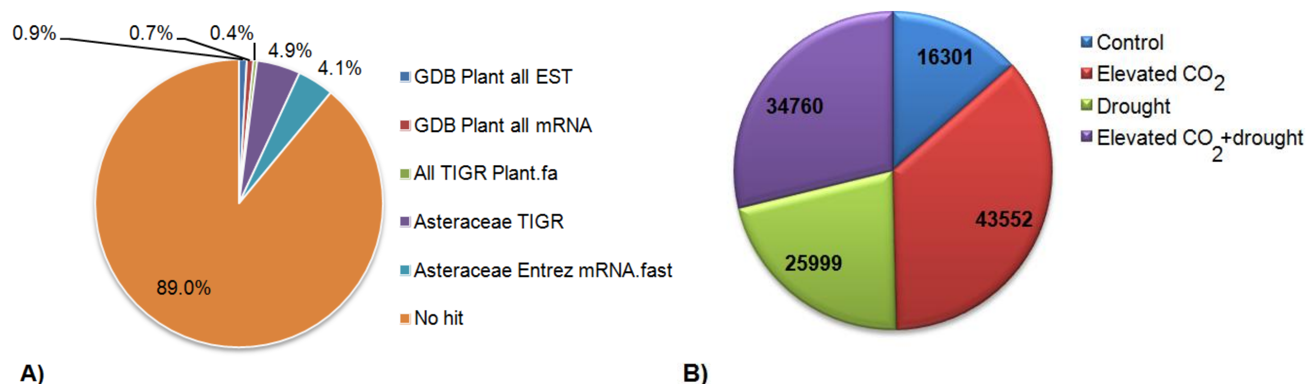

Figure 29: Annotation BLAST searches of the SuperSAGE tags. A) Percentages of genes annotated in different databases. B) Total number of annotated matched genes in each library.

3.2.2.2 Refining criteria and data filtration

The results of each library of the digital gene expression analyses are provided as an Excel sheet, providing the following information:

The first column represent the tag sequence, which gives the nucleotide sequence of each tag; the count, which includes the sum of all tags of the same sequence present in all different samples; followed by the gi (gene ID). The fourth column outlines the short description of the BLAST hit. This precedes the score of the BLAST hit, with a perfect score of 52 (26 of 26 bp), followed by the identity, which describes the identical bp of the tag to the BLAST hit. The seventh column of the database indicates, the database (DB) in which the BLAST hit was found. Next is the tag count, defining how frequently the tag matches a contig, while considering the occurrence of a sequence in the sample. The same applies for the tags per million, which is a normalised value of each tag in relation to one million tags listed (tpm = tags per million). Those tags with a count of 0 were given a false count of 0.05 in order to enable comparisons with other libraries in which the tag was present (White et al. 2006). Subsequently, the p-value of that treatment is compared to the control library, while the final column represents the $\log_2$ fold change, namely the logarithm to the basis 2 of the ratio of normalised X (treatment) divided by normalised Y (control).

Later, the four libraries (control, elevated CO_2, drought and elevated CO_2 + drought) were filtered to refine the data and enhance the libraries quality by removing the singleton tags and biased hits. The criteria used in filtering the libraries were as follows: template per million values ≥ 0.8, fold change ≥ ± 1.5, identity ≥ 23, database of Asteraceae and $P \leq 0.0005$. Any tags that did not follow such standards were filtered from the library.

3.2.2.3 Abundance of 26 bp tags

The 26 bp tag in each library was visualised according to four levels of abundance: very high level (more than 500 copies), high level (499 to 100 copies), moderate level (99 to 10 copies) and low level of abundance (less than 10 copies) (Tab. 12) (Molina et al. 2011).

Table 12: Abundance of 26 bp tags in the ragweed pollen libraries (control, elevated CO_2, drought and elevated CO_2 plus drought). Abundance divided into 4 levels; very high level (≥ 500), high level (499 to 100), moderate level (99 to 10) and low level (≤ 10). Each level is expressed in a percentage of the total number of tags in each library.

Copies number	Control	CO_2	Drought	CO_2 + drought
≥ 500	5%	2%	12%	13%
499 to 100	13%	7%	25%	26%
99 to 10	38%	39%	58%	56%
≤ 10	42%	52%	5%	1%

The abundance of 26 bp in the four libraries is displayed in Tab. 12. In the control library, 5% of the transcripts were at the very high level, 13% at the high level, 38% at the moderate level and 42% were presented in low copies number. The elevated CO_2 library showed similarities with the control library, with 2% for the very high copy number, 7% the high copy number, 39% the moderate copy number and 52% the low copy number of 26 bp abundance tags. On the other hand, the drought and elevated CO_2 plus drought expressed a high percentage at the very high level of 26 bp tags, with 12% and 13%, respectively. In terms of the high level copy number were 25% for the drought and 26% for the elevated CO_2 plus drought. Most of tag abundance was in moderated level by 58% in drought and 56% in elevated CO_2 plus drought. Finally the lowest percentage of the low level copy number was 5% for drought and 1% for elevated CO_2 plus drought (Tab. 12).

3.2.2.4 Up and down regulation of ragweed pollen transcripts

The regulation rate of transcripts expression varied dramatically within libraries in comparison to the control.

The pollen of ragweed subjected to elevated CO_2 and elevated CO_2 plus drought reported the highest number of the up-regulated transcripts by 538 (86%) and 550 transcripts (84%), respectively, followed by the drought with 378 transcripts (70%) (Tab.13).

Nonetheless, the drought treated pollen represented the highest number of down-regulated transcripts, with 164 (30%), followed by elevated CO_2 plus drought with 108 transcripts (16%) and elevated CO_2 with 87 transcripts (14%) (Tab.13).

Table 13: Changes in gene expression profiles among the different treatments of ragweed pollen (compared to control). The number of up-regulated and down-regulated transcripts in different treatments.

Treatment	Tag number	No. of up-regulated transcripts	No. of down-regulated transcripts
Elevated CO_2	625	538 (86%)	87 (14%)
Drought	542	378 (70%)	164 (30%)
CO_2+Drought	658	550 (84 %)	108 (16%)

Molina et al (2011) selected the 8-fold of up-regulation in order to evaluate the most effective treatment between libraries. In terms of ragweed pollen libraries, It were found that elevated CO_2 library showed higher 8-fold of log_2 up-regulated by 279 transcripts (45%), compared to 93 (17%) and 90 transcripts (14%) for the drought and elevated CO_2 plus drought, respectively.

Several common transcripts were found among the top 40 up-regulated transcripts within elevated CO_2, drought and elevated CO_2 plus drought (Tab.14, 15, 16): The DY911281 transcript, which encodes FatB thioestrase fatty acid biosynthesis; the TC45575 transcript, which encodes pectinesterase inhibitor; and the TC4620 transcript, which encodes pollen tube formation. Moreover, the TC4620 transcript, which encodes cysteine proteinase aleuran as well as the 294689814 transcript, which encodes cold acclimated bark, are both involved in signaling pathways and response to biotic and abiotic stresses. Finally the 255779153 (AmbT-237) and 255779301 (AmbT-405), which belongs to *Ambrosia trifida* pollen transcripts.

The top 40 up-regulated transcripts in ragweed pollen under elevated CO_2 compared to the control are detailed in Tab.14. *Ambrosia* genes are represent by 8 transcripts (p- 0.00E+00), Amb T-237 (log_2 fold change= 16.9), Amb T-405 (log_2 fold change= 16.3), Amb a 1.1 (log_2 fold change= 14.3), Amb T-100 (log_2 fold change= 14.1), Amb T-674 (log_2 fold change= 13.9), Amb T-545 (log_2 fold change= 13.6), Amb T-339 (log_2 fold change= 12.5) and Amb T-508 (log_2 fold change= 12.3) (Tab. 14), while stress-related genes are represented by 3 transcripts (p- 0.00E+00), 2 safflower (A-1), drought stressed subtracted transcript (log_2 fold change = 12.4 and 11.9) and cold acclimated bark (log_2 fold change= 14.5) (Tab. 14). The highest log_2 of fold change in the elevated CO_2 ragweed pollen library, was FatB thioestrase with 21.4; followed by; UDP-glucose protein transglucosylase-like with 19.4 and Amb T-237 SF16 protein with 16.9.

In the drought top 40 up-regulated transcripts, *Ambrosia* genes are also represent by 7 transcripts with p-value (0.00E+00 to 0.0034), although with log_2 of fold change lower than the elevated CO_2 library as follows: Amb T-237 (log_2 fold change= 14.5), Amb T-405 (log_2 fold change= 14.4), Amb T-665 (log_2 fold change= 9.3), Amb T-507 (log_2 fold change= 7.5), Amb T-182 (log_2 fold change= 6), Amb T-680 (log_2 fold change= 5.7) and Amb T-575 (log_2 fold change= 5.1) (Tab. 15). Meanwhile stress-related genes represented a higher number by 10 transcripts with p-value (0.00E+00 to 3.77E-36) (Tab. 15), 9 scafflower (A-1) drought stressed subtracted transcript (log_2 fold change = 11.9, 11.7, 9.3, 8.8, 8.1, 7.4, 6.3, 6.0, 5.4) and one cold acclimated bark (log_2 fold change= 9.9). The FatB thioestrase, Amb T-237 SF16 protein and Amb T-405 represented the highest log_2 fold change with 17.0, 14.5 and 14.4 respectively (Tab. 15).

The elevated CO_2 plus drought top 40 up-regulated transcripts are provided in Tab. 16. *Ambrosia* genes are represented by a lower number than elevated CO_2 and drought libraries, by 6 transcripts with p-value (0.00E+00 to 9.70E-31), Amb T-237 (log_2 fold change= 13.8), Amb T-405 (log_2 fold change= 12.8), Amb T-665 (log_2 fold change= 9.2), Amb T-339 (log_2 fold change= 9.1), Amb T-476 (log_2 fold change= 8.5) and Amb T-365 (log_2 fold change= 7.3) (Tab. 16), while stress genes are represented with 6 transcripts with p-value ranging from 0.00E+00 to 9.52E-18 (Tab. 16). 5 scafflower (A-1) drought stressed subtracted transcript (log_2 fold change = 12.1, 10, 9.6, 9.3, 7.7) and one cold acclimated bark (log_2 fold change= 9.6). The highest log_2 fold change showed FatB thioestrase with 17.9, followed by; Amb T-237 SF16 protein with 13.8 and Amb T-405 with 12.8.

Tab. 14, 15 and 16 showed that 8 highly up-regulated *Ambrosia* transcripts were represented within the top up-regulated transcripts in the superSAGE library of ragweed pollen treated by elevated CO_2 or under drought conditions and elevated CO_2 plus drought. The ragweed pollen library under drought library showed a high number of stress-related genes with 10 transcripts, compared with 3 and 5 transcripts in elevated CO_2 and elevated CO_2 plus drought, respectively. FatB thioestrase was one of the most prominent transcripts, with high log_2 fold change in all the libraries.

The highest was in the elevated CO_2 pollen library with 21.4, 17 in the drought pollen library and 17.9 in the elevated CO_2 plus drought pollen library.

Table 14: Top 40 up-regulated annotatable tags in elevated CO_2 ragweed pollen compared to control.

Database-id	Database	Description	Species	Score	p-value	Fold change (log_2)
DY911281	Asteraceae	FatB thioesterase	*Helianthus annuus*	48	0.00E+00	21.4
FS501718	Asteraceae	UDP-glucose:protein transglucosylase-like	*Solanum tuberosum*	52	0.00E+00	19.4
255779153	Asteraceae	AmbT-237 SF16 protein	*Ambrosia trifida*	36	0.00E+00	16.9
255779301	Asteraceae	AmbT-405	*Ambrosia trifida*	36	0.00E+00	16.3
DY930316	Asteraceae	Calcineurin B-like protein	*Populus euphratica*	42	0.00E+00	16.1
TC42145	Asteraceae	ADP ribosylation factor 002	*Daucus carota*	36	0.00E+00	15.1
BQ863099	Asteraceae	Carbohydrate sulfotransferase 2	*Canis lupus familiaris*	44	0.00E+00	14.5
294689814	Asteraceae	Cold acclimated bark	*Parthenium argentatum*	40	0.00E+00	14.5
166433	GDB	PLN ragweed Amb a I.1 (antigen E) mRNA	*Ambrosia artemisiifolia*	44	0.00E+00	14.3
TC4620	Asteraceae	Cysteine proteinase aleuran type	*Nicotiana benthamiana*	52	0.00E+00	14.1
190607080	GDB	AmbT-100 galactan galactosyltransferase	*Ambrosia trifida*	44	0.00E+00	14.1
TC8266	Asteraceae	Probable rRNA-processing protein EBP2 homolog	*Arabidopsis thaliana*	48	0.00E+00	14.1
TC48534	Asteraceae	Calmodulin-like protein	*Musa acuminata*	44	0.00E+00	14.1
TC45575	Asteraceae	Pectinesterase inhibitor	*Medicago truncatula*	52	0.00E+00	13.9
296281913	Asteraceae	AmbT-674 Putative 60S ribosomal protein L36	*Ambrosia trifida*	44	0.00E+00	13.9
BU030160	Asteraceae	Tetratricopeptide repeat protein 21A	*Danio rerio*	48	0.00E+00	13.8
296281816	Asteraceae	AmbT-545	*Ambrosia trifida*	48	0.00E+00	13.6
GE483796	Asteraceae	WD40 protein	*Aegiceras corniculatum*	48	0.00E+00	13.5
TC10769	Asteraceae	Actin	*Striga asiatica*	42	0.00E+00	13.1
TC57407	Asteraceae	Inositol 2-dehydrogenase like protein	*Arabidopsis thaliana*	46	0.00E+00	13.0
FS452053	Asteraceae	Cysteine proteinase inhibitor	*Glycine max*	48	0.00E+00	12.8
TC5518	Asteraceae	Endo-1.4-beta-glucanase	*Pisum sativum*	46	0.00E+00	12.6
GR151050	Asteraceae	BZIP transcription factor	*Nicotiana tabacum*	50	0.00E+00	12.5
255779240	Asteraceae	AmbT-339 Lipid transfer protein	*Ambrosia trifida*	42	0.00E+00	12.5
TC11281	Asteraceae	Ethylene-responsive transcription factor ERF117	*Arabidopsis thaliana*	46	0.00E+00	12.5
TC4990	Asteraceae	Anther-specific protein LAT52 precursor	*Solanum lycopersicum*	44	0.00E+00	12.5
289595400	Asteraceae	Safflower (A-1) drought stressed	*Carthamus tinctorius*	36	0.00E+00	12.4
CV517191	Asteraceae	Pyruvate dehydrogenase E1 alpha subunit	*Arabidopsis thaliana*	48	1.68E-44	12.3
296281782	Asteraceae	AmbT-508 ragweed male flower and pollen	*Ambrosia trifida*	46	3.36E-44	12.3
TC53976	Asteraceae	NADPH-ferrihemoprotein reductase	*Helianthus tuberosus*	48	3.66E-41	12.2
FS447802	Asteraceae	Disheveled-associated activator of morphogenesis 1	*Takifugu rubripes*	46	7.35E-41	12.2
DC241199	Asteraceae	DNA-binding protein Fis / transcriptional regulator	*Anaeromyxobacter*	46	6.13E-39	12.1
DW142617	Asteraceae	Na+-dependent neutral amino acid transporter	*Didelphis virginiana*	44	7.93E-38	12.1
TC53194	Asteraceae	Preprotein translocase secA subunit	*Vitis vinifera*	46	1.05E-35	12.0
261291913	Asteraceae	Safflower (A-1) drought stressed (GEG protein)	*Carthamus tinctorius*	48	5.50E-34	11.9
DY928568	Asteraceae	FAD-linked oxidoreductase BG60	*Cynodon dactylon*	42	5.50E-34	11.9
FS450069	Asteraceae	Serine/threonine-protein kinase SAPK10	*Oryza sativa*	46	2.46E-29	11.7
CJ748618	Asteraceae	Signal recognition particle protein	*Lyngbya sp.*	46	2.52E-28	11.6
TC4778	Asteraceae	ACT domain containing protein	*Oryza sativa*	46	8.70E-25	11.4
TC58643	Asteraceae	ABC transporter of peptides	*Chlamydophila felis*	44	1.79E-23	11.4

Table 15: Top 40 up-regulated annotatable tags in drought stress ragweed pollen compared to control

Database-id	Database	Description	Species	Score	p-value	Fold change (log_2)
DY911281	Asteraceae	FatB thioesterase	*Helianthus annuus*	48	0.00E+00	17.0
255779153	Asteraceae	AmbT-237 SF16 protein	*Ambrosia trifida*	36	0.00E+00	14.5
255779301	Asteraceae	AmbT-405	*Ambrosia trifida*	36	0.00E+00	14.4
FS500194	Asteraceae	Methylthioadenosine/S-adenosyl homocysteine	*Oryza sativa Japonica*	48	0.00E+00	13.1
289595415	Asteraceae	Safflower (A-1) drought stressed subtracted (carbonic anhydrase 3)	*Carthamus tinctorius*	48	0.00E+00	11.9
261291788	Asteraceae	Safflower (A-1) drought stressed subtracted (lipoxygenase)	*Carthamus tinctorius*	36	0.00E+00	11.7
TC47944	Asteraceae	Ubiquitin carrier protein	*Eudicotyledons*	50	0.00E+00	11.0
TC42145	Asteraceae	ADP ribosylation factor 002	*Daucus carota*	36	0.00E+00	10.8
TC11242	Asteraceae	DNA-binding response regulator TorR	*Roseovarius*	50	0.00E+00	10.1
FS451861	Asteraceae	Cell surface flocculin	*Saccharomyces c.*	42	0.00E+00	10.0
TC42756	Asteraceae	NADH-ubiquinone oxidoreductase related-like protein	*Brassica rapa*	44	0.00E+00	9.9
294701547	Asteraceae	Cold acclimated bark cDNA library	*Parthenium argentatum*	44	0.00E+00	9.9
TC1924	Asteraceae	Vesicle transport v-SNARE 11-like	*Solanum tuberosum*	46	0.00E+00	9.9
TC10769	Asteraceae	Actin	*Gossypium hirsutum*	42	0.00E+00	9.7
289595400	Asteraceae	Safflower (A-1) drought stressed subtracted (hypothetical protein)	*Carthamus tinctorius*	36	0.00E+00	9.3
296281905	Asteraceae	AmbT-665 (Putative clathrin assembly protein)	*Ambrosia trifida*	52	0.00E+00	9.3
TC5518	Asteraceae	Endo-1.4-beta-glucanase	*Pisum sativum*	46	9.67E-44	9.2
289595446	Asteraceae	Safflower (A-1) drought stressed subtracted (photosystem I reaction center)	*Carthamus tinctorius*	48	1.32E-33	8.8
TC53211	Asteraceae	Cytochrome b6-f complex iron-sulfur subunit 2	*Nicotiana tabacum*	52	5.43E-32	8.7
TC4620	Asteraceae	Cysteine proteinase aleuran type	*Nicotiana benthamiana*	52	1.57E-31	8.7
TC12549	Asteraceae	3-phosphoshikimate 1-carboxyvinyltransferase	*Dicliptera chinensis*	42	3.78E-30	8.6
NP524518	Asteraceae	gibberellin 2-oxidase No_2	*Lactuca sativa*	44	5.37E-29	8.6
FS450069	Asteraceae	Serine/threonine-protein kinase SAPK10	*Oryza sativa*	46	1.08E-26	8.5
TC45575	Asteraceae	Pectinesterase inhibitor	*Medicago truncatula*	52	5.31E-26	8.4
289595366	Asteraceae	Safflower (A-1) drought stressed subtracted	*Carthamus tinctorius*	42	2.16E-21	8.1
CV517100	Asteraceae	DEAD-box ATP-dependent RNA helicase 27	*Arabidopsis thaliana*	46	2.13E-18	7.9
TC48534	Asteraceae	Calmodulin-like protein	*Musa acuminata*	44	3.03E-17	7.8
DY395377	Asteraceae	Short chain dehydrogenase	*Solanum tuberosum*	48	8.75E-17	7.8
TC40728	Asteraceae	Pollen-specific protein SF3	*Helianthus annuus*	44	8.75E-17	7.8
FS490087	Asteraceae	Auxin-repressed protein like-protein	*Malus x domestica*	48	7.30E-16	7.7
296281781	Asteraceae	AmbT-507 Putative epoxide hydrolase	*Ambrosia trifida*	42	2.99E-14	7.5
289595406	Asteraceae	Safflower (A-1) drought stressed subtracted neurofilament triplet H1-like	*Carthamus tinctorius*	48	4.25E-13	7.4
289595449	Asteraceae	Safflower (A-1) drought stressed subtracted chloroplast protein 12	*Carthamus tinctorius*	50	1.20E-06	6.3
255779129	Asteraceae	AmbT-182 60S ribosomal protein	*Ambrosia trifida*	36	1.71E-05	6.0
261291674	Asteraceae	Safflower (A-1) drought stressed subtracted conserved hypothetical protein	*Carthamus tinctorius*	50	1.71E-05	6.0
296281917	Asteraceae	AmbT-680 DNA-directed RNA polymerase family protein	*Ambrosia trifida*	50	1.42E-04	5.7
261291911	Asteraceae	Safflower (A-1) drought stressed subtracted S-adenosyl-L-methionine synthetase	*Carthamus tinctorius*	36	3.77E-36	5.4
261291882	Asteraceae	AmbT-575 Unnamed protein product, mRNA sequence	*Ambrosia trifida*	46	3.44E-03	5.1
TC10690	Asteraceae	Acyl carrier protein	*Olea europaea*	46	1.04E-14	7.6
TC20143	Asteraceae	Cysteine protease CP14	*Frankliniella occidentalis*	52	1.23E-12	7

Table 16: Top 40 up-regulated annotatable tags in elevated CO_2 plus drought stress ragweed pollen compared to control.

Database-id	Database	Description	Species	Score	p-value	Fold change (log_2)
DY911281	Asteraceae	FatB thioesterase	*Helianthus annuus*	48	0.00E+00	17.9
255779153	Asteraceae	AmbT-237 SF16 protein	*Ambrosia trifida*	36	0.00E+00	13.8
255779301	Asteraceae	AmbT-405 BU-R-405 5'	*Ambrosia trifida*	36	0.00E+00	12.8
TC42145	Asteraceae	ADP ribosylation factor	*eudicotyledons*	36	0.00E+00	12.3
261291788	Asteraceae	Safflower (A-1) drought stressed (lipoxygenase)	*Carthamus tinctorius*	36	0.00E+00	12.1
GE483796	Asteraceae	WD40 protein	*Aegiceras corniculatum*	48	0.00E+00	11.6
TC4620	Asteraceae	Cysteine proteinase aleuran type	*Nicotiana benthamiana*	52	0.00E+00	11.0
DY930799	Asteraceae	ATPase 6	*Chalcites basalis*	46	0.00E+00	10.4
TC45575	Asteraceae	Pectinesterase inhibitor	*Medicago truncatula*	52	0.00E+00	10.3
FS500194	Asteraceae	Methylthioadenosine/S-adenosyl homocysteine nucleosidase	*Oryza sativa*	48	0.00E+00	10.2
289595599	Asteraceae	Safflower (A-1) drought stressed (suppressor of ty)	*Carthamus tinctorius*	44	0.00E+00	10.0
294701547	Asteraceae	Cold acclimated bark cDNA library	*Parthenium argentatum*	52	0.00E+00	9.6
289595446	Asteraceae	Safflower (A-1) drought stressed (photosystem I reaction center subunit IV A)	*Carthamus tinctorius*	48	0.00E+00	9.6
289595601	Asteraceae	Safflower (A-1) drought stressed (ribulose-1, 5-bisphosphate carboxylase/oxygenase)	*Carthamus tinctorius*	42	0.00E+00	9.3
296281905	Asteraceae	AmbT-665 Putative clathrin assembly protein	*Ambrosia trifida*	52	0.00E+00	9.2
TC19597	Asteraceae	Cytochrome c oxidase subunit 2	*Thrips imaginis*	52	0.00E+00	9.2
255779240	Asteraceae	AmbT-339 (Lipid transfer protein)	*Ambrosia trifida*	42	5.61E-45	9.1
TC20143	Asteraceae	Cysteine protease CP14	*Frankliniella occidentalis*	52	1.67E-32	8.6
CV517100	Asteraceae	DEAD-box ATP-dependent RNA helicase 27	*Arabidopsis thaliana*	46	2.41E-32	8.6
GR041038	Asteraceae	Syntaxin-related protein Nt-syr1	*Nicotiana tabacum*	48	1.53E-31	8.6
296281762	Asteraceae	AmbT-476 BU-R-476 5', mRNA sequence	*Ambrosia trifida*	44	9.70E-31	8.5
TC53976	Asteraceae	NADPH-ferrihemoprotein reductase	*Helianthus tuberosus*	48	6.15E-30	8.5
TC7737	Asteraceae	ADP/ATP translocase	*Manduca sexta*	52	5.64E-29	8.5
TC55619	Asteraceae	Cholinephosphate cytidylyltransferase	*Pisum sativum*	44	2.26E-27	8.4
BQ973368	Asteraceae	Cytochrome b6-f complex iron-sulfur subunit 2	*Nicotiana tabacum*	52	1.75E-24	8.2
BQ978369	Asteraceae	Phi-1 protein	*Nicotiana tabacum*	36	1.95E-21	8.0
NP524518	Asteraceae	gibberellin 2-oxidase No_2	*Lactuca sativa*	44	7.82E-20	7.9
TC55302	Asteraceae	F6 protein	*Gossypium hirsutum*	50	1.50E-18	7.8
TC23176	Asteraceae	Acyl carrier protein	*Coriandrum sativum*	48	3.14E-18	7.8
289595400	Asteraceae	Safflower (A-1) drought stressed (hypothetical protein)	*Carthamus tinctorius*	36	9.52E-18	7.7
TC22805	Asteraceae	Cellulose synthase CesA1	*Boehmeria nivea*	46	6.03E-17	7.7
GR048420	Asteraceae	Ubiquitin carboxyl-terminal hydrolase	*Vitis vinifera*	50	2.42E-15	7.5
TC46307	Asteraceae	NADPH cytochrome P450 reductase	*Stevia rebaudiana*	44	3.21E-14	7.4
TC7402	Asteraceae	Hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyltransferase	*Coffea canephora*	44	9.73E-14	7.3
255779264	Asteraceae	AmbT-365 Hypothetical protein	*Ambrosia trifida*	44	0.00E+00	7.3
DW123906	Asteraceae	Histone H3	*Oryza sativa*	36	4.26E-13	7.3
CD856477	Asteraceae	Skp1-like protein 3	*Petunia integrifolia*	48	6.17E-13	7.2
TC46600	Asteraceae	Ubiquitin carrier protein	*Vitis vinifera*	36	1.29E-12	7.2
TC17825	Asteraceae	Actin	*Striga asiatica*	36	2.70E-12	7.2
DY907343	Asteraceae	Fiber annexin	*Gossypium hirsutum*	44	1.18E-11	7.1

3.2.2.5 Terms related to ragweed pollen

The STDGE2GO-toolkit was used to unveil special interested terms, which are closely related to ragweed pollen under different environmental conditions. For each term, only those genes that are highly significant (p-value ≤ 0.00005) will be considered. For each term, the number and top up- and down-regulated transcripts are summarised for each treatment.

3.2.2.5.1 Pollen grain

The result of the search on pollen grain in libraries indicated that over 99% are annotated according to the available EST of *Ambrosia trifida*, which is very close to ragweed (*Ambrosia artemisiifolia*). The dataset is summarised in Tab.18, reflecting that the elevated CO_2 have the highest number of transcripts representing the pollen grain, with 53, as well as the highest number of up-regulated transcripts, with 37. On the other hand, the highest up-regulated log_2-fold change was reported for the drought ragweed pollen of log_2 fold change 19 (AmbT-660 Ragweed pollen clone BU-R-660 5' similar to Putative CBS domain-containing protein), while the lowest was found for elevated CO_2 plus drought of 9.1 log_2-fold change (Amb T-339 gene similar to lipid transfer protein) (Tab.17). Furthermore the drought pollen recorded a higher down regulation log_2-fold change than elevated CO_2 and elevated CO_2 plus drought by -14.7, for a 60S ribosomal protein L35a gene.

Table 17: Top up- and down-regulated transcripts in different libraries related to pollen grains and performed by STDGE2GO-Toolkit.

Library	No. of genes	Up regulated	Down regulated	Max. up-regulation	Max. down-regulation
CO_2	53	37	16	10.3 AmbT-545 Ragweed pollen clone BU-R-545 5'	-13.7 AmbT-668 Ragweed pollen clone BU-R-668 5' similar to Putative 60S ribosomal protein L34
Drought	41	19	22	19 AmbT-660 Ragweed pollen clone BU-R-660 5' similar to Putative CBS domain-containing protein	-14.7 AmbT-396 Ragweed pollen clone BU-R-396 5' similar to 60S ribosomal protein L35a
CO_2 + drought	47	32	15	9.1 AmbT-339 Ragweed pollen clone BU-R-339 5' similar to Lipid transfer protein	-9.5 AmbT-470 Ragweed pollen clone BU-R-470 5' similar to Putative ribokinase

3.2.2.5.2 Stress

For the stress genes, the CO_2 plus drought and drought ragweed pollen proved to face the stress with a high number of transcripts, 100 and 90 transcripts, respectively, amongst which 59 in the CO_2 plus drought and 66 in drought were highly up-regulated. The highest log_2-fold change of up-regulated transcripts was found in drought by 14.7 for safflower (A-1) drought stressed subtracted cDNA library *Carthamus tinctorius* cDNA, mRNA sequence, while the elevated CO_2 possessed a lower number and lower log_2 fold changes of up- regulated transcripts than the drought (Tab. 18).

Table 18: Top up- and down-regulated transcripts in different libraries related to stress and performed by STDGE2GO-Toolkit.

Library	No. of genes	Up regulated	Down regulated	Max. up-regulation	Max. down-regulation
CO_2	88	43	45	9.2 Safflower (A-1) drought stressed subtracted cDNA library *Carthamus tinctorius* cDNA similar to hydrolase, acting on ester bonds, putative	-11.9 Safflower (A-1) drought stressed subtracted cDNA library *Carthamus tinctorius* cDNA similar to hypothetical protein ANACAC_00331
Drought	90	66	24	14.7 Safflower (A-1) drought stressed subtracted cDNA library *Carthamus tinctorius* cDNA, mRNA sequence	-9.4 Safflower (A-1) drought stressed subtracted cDNA library PTC-225 *Carthamus tinctorius* cDNA similar to chloroplast ribulose-1.5-bisphosphate carboxylase/oxygenase small subunit.
CO_2 + drought	100	59	41	14 Safflower (A-1) droughtstressed subtracted cDNA library PTC-225 Carthamus tinctorius cDNA, mRNA sequence	-9.4 Safflower (A-1) drought stressed subtracted cDNA library PTC-225 *Carthamus tinctorius* cDNA similar to chloroplast ribulose-1.5-bisphosphate carboxylase/oxygenase small subunit. mRNA sequence

3.2.2.5.3 Allergen

The usage of the STDGE2GO-toolkit in the superSAGE libraries data effectively enhanced the capacity to list all the allergen genes that might be involved in the allergenic potential of ragweed pollen. The allergenicity was preliminary visualised as a total number of allergenic genes hits, assigned homologous to plant genes (Fig. 30). It was found that the elevated CO_2 plus drought possessed the highest number of normalized tags per million for allergen transcripts with 4199 hits, followed by drought, elevated CO_2, and control, respectively.

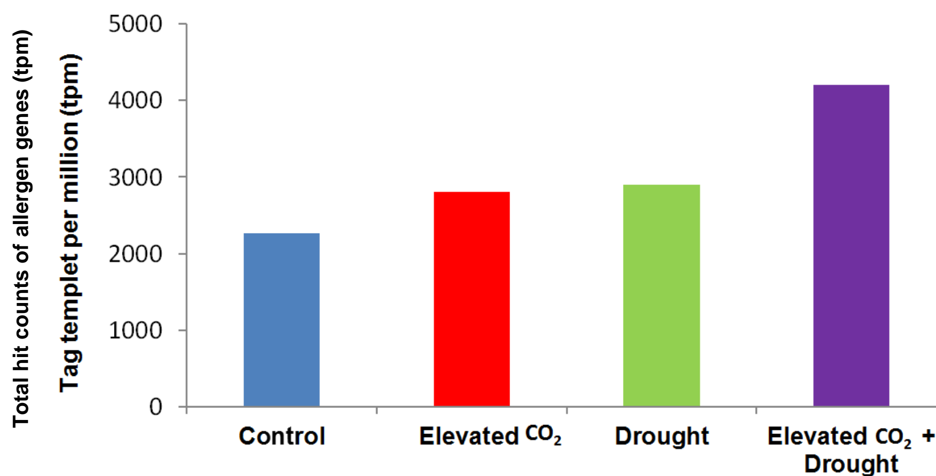

Figure 30: Total hit counts of the tags per million values of the allergen genes assigned homologous to plant genes within ragweed pollen libraries.

The allergen transcripts that appeared in superSAGE pollen libraries (elevated CO_2, drought and elevated CO_2 plus drought) are provided in Tab. 19, 20 and 21. According to the AllFam allergen database, these transcripts are represented by 9 major allergenic families (calcium binding protein, cystatin proteinase inhibitor, defensin-like protein, expansins, group 5/6, lipid transfer protein, pectate lyase, ploygalacturonase, profilin), in addition to other minor allergens transcripts that did not match any of the known families. Within the 9 major allergenic families, 5 were assigned to *Ambrosia sp.*, namely calcium binding protein, cystatin proteinase inhibitor, lipid transfer protein, pectate lyase and profilin. Each family can be represented with more than one transcript or isoform, and thus only the significant values ($P \leq 0.00005$) will be considered.

4 families in the elevated CO_2 ragweed pollen library showed up-regulation compared to the control (calcium binding protein, cystatin proteinase inhibitor, lipid transfer protein and pectate lyase), while only the profilin gene family showed down-regulation (Tab. 19). The allergen transcripts of the drought library are provided in Tab. 21. Within the 5 allegens families, the calcium binding protein, cystatin proteinase inhibitor and pectate lyase are highly up-regulated, while the profilin familie are down-regulated (Tab. 20). Finally, the cystatin proteinase inhibitor, lipid transfer protein, pectate lyase and profilin are up-regulated in the elevated CO_2 plus drought (Tab. 21).

The aforementioned allergen results in ragweed pollen libraries revealed that pectate lyase family (Amb a 1 isoallergen) proved the most prominent family within the libraries. The transcripts in tags per million for the *Ambrosia artemisiifolia* Amb a 1 isoallergen detailed in Tab. 22 showed that elevated CO_2, and elevated CO_2 plus drought recorded a higher number of tpm than the drought (Amb a 1.1, 1.2, 1.3 and 1.5).

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Table 19 : Summary of the superSAGE library allergen of ragweed pollen under elevated CO_2 condition. Database id, Datbase, allergen family, description, organism, p-value and log_2 of fold change compared to the control library. Only the significant values ($P \leq 0.00005$) will be considered.

Database-id	Database	Allergenic family	Common name	Description	Organism	p-value	Fold change (log_2)	Regulation
TC52169	Asteraceae_TIGR	Calcium binding protein	Amb a 9 isoform	Calcium-binding protein isoallergen 1	*Ambrosia artemisiifolia*	0.0000000	2.4	Up
DY929617	Asteraceae_TIGR		Amb a 9 isoform	Calcium-binding protein isoallergen 2	*Ambrosia artemisiifolia*	0.0862419	-2.1	
TC42736	Asteraceae_TIGR		Amb a 9 isoform	Calcium-binding pollen allergen	*Ambrosia artemisiifolia*	0.9975530	0.1	
437311	GDB_Plant_all_mRNA	Cystatin	Amb a CPI	PLN cystatin proteinase inhibitor	*Ambrosia artemisiifolia*	0.0000000	0.2	Up
TC51843	Asteraceae_TIGR	Defensin-like protein	Art v 2	Art v 2 allergen precursor	*Artemisia vulgaris*	0.2229080	-1.9	Down
3901093	GDB_Plant_all_mRNA	Expansins	PhlpI	PLN P.pratense mRNA for pollen allergen PhlpI.	*Phleum pratense*	0.2461810	3.2	Up
3004466	GDB_Plant_all_mRNA	Group 5/6	Phl p6	PLN Phl p6 allergen, isolate c142.	*Phleum pratense*	0.2229080	-1.9	Down
255779240	Asteraceae_Entrez	Lipid transfer protein	Amb a 6	AmbT-339 ,Lipid transfer protein	*Ambrosia tifida*	0.0000000	9.2	Up
226766874	GDB_Plant_all_EST		Mal d 3	Mdfrb3146A08 Nonspecific lipid-transfer protein	*Ambrosia artemisiifolia*	0.9948820	1.2	
166433	GDB_Plant_all_mRNA	Pectate lyase	Amb a 1.1	PLN Ragweed Amb a I.1 (antigen E) mRNA.	*Ambrosia artemisiifolia*	0.0000000	0.8	Up
166442	GDB_Plant_all_mRNA		Amb a 1.1	PLN Amb a I.1 (antigen E) mRNA	*Ambrosia artemisiifolia*	0.9948820	1.2	
302127811	GDB_Plant_all_mRNA		Amb a 1.2	PLN pollen allergen Amb a 1.2, variant 2.	*Ambrosia artemisiifolia*	0.0000000	1.3	
302127825	GDB_Plant_all_mRNA		Amb a 1.3	PLN E (al.3) mRNA, complete cds	*Ambrosia artemisiifolia*	0.0000000	1.0	
166440	GDB_Plant_all_mRNA		Amb a 1.3	PLN Amb a I.3 (antigen E) mRNA, complete cds	*Ambrosia artemisiifolia*	0.1224610	3.7	
166444	GDB_Plant_all_mRNA		Amb a 1.4	PLN allergen(Amb a I.4) mRNA, complete cds.	*Ambrosia artemisiifolia*	0.1545550	3.5	
302127823	GDB_Plant_all_mRNA		Amb a 1.4	PLN pollen allergen Amb a 1.4, variant 2.	*Ambrosia artemisiifolia*	0.6210290	-0.1	
166433	GDB_Plant_all_mRNA		Amb a 1.5	PLN pollen allergen Amb a 2.01, variant 2.	*Ambrosia artemisiifolia*	0.0980341	2.3	
TC5912	Asteraceae_TIGR	Polygalacturonase	PEST459 isoform	Oil palm polygalacturonase allergen	*Elaeis guineensis*	0.1950600	3.4	Up
TC6003	Asteraceae_TIGR		PEST459 isoform	Oil palm polygalacturonase allergen	*Elaeis guineensis*	0.7443810	0.2	
34851181	GDB_Plant_all_mRNA	Profilin	Amb a 8 isoform	PLN profilin-like protein (D03) mRNA, complete cds.	*Ambrosia artemisiifolia*	0.0000000	0.9	Down
TC43769	Asteraceae_TIGR		Amb a 8 isoform	Profilin isoallergen 1	*Ambrosia artemisiifolia*	0.0000000	-2.9	
TC48587	Asteraceae_TIGR		Amb a 8 isoform	Profilin isoallergen 1	*Ambrosia artemisiifolia*	0.0513399	-1.1	
CD851748	Asteraceae_TIGR		Amb a 8 isoform	Profilin-2	*Ambrosia artemisiifolia*	0.1791080	-0.8	
TC48587	Asteraceae_TIGR		Amb a 8 isoform	Profilin isoallergen 1	*Ambrosia artemisiifolia*	0.7443810	0.2	
TC49246	Asteraceae_TIGR		Amb a 8 isoform	Profilin	*Helianthus annuus*	0.7882890	1.8	
TC68483	all_TIGR_Plant.fa		Amb a 8 isoform	Pollen profilin variant 7	*Corylus avellana*	0.9948820	1.2	
28513232	GDB_Plant_all_EST	Others	-	cDNA clone PP043C12 similar to MINOR ALLERGEN,	*Pinus pinaster*	0.3690750	-0.6	Up
FS446595	Asteraceae_TIGR		BRSn20	Allergen-like protein BRSn20	*Sambucus nigra*	0.7882890	1.8	
TC430677	all_TIGR_Plant.fa		Def2/Der2	E1 protein and Def2/Der2allergen family protein	*Oryza sativa*	0.9948820	1.2	

Table 20 : Summary of the superSAGE library allergen of ragweed pollen under drought condition. Database id, Datbase, allergen family, description, organism, p-value and log_2 of fold change compared to the control library. Only the significant values ($P \leq 0.00005$) will be considered

Database-id	Database	Allergenic family	Common name	Description	Organism	p-value	Fold change (log_2)	Regulation
TC42736	Asteraceae_TIGR	Calcium binding protein	Amb a 9 isoform	Calcium-binding pollen allergen	*Arachis hypogaea*	0.009741	-4.5	Up
DY929617	Asteraceae_TIGR		Amb a 9 isoform	Calcium-binding protein isoallergen 1	*Ambrosia artemisiifolia*	0.000000	2.7	
TC52169	Asteraceae_TIGR		Amb a 9 isoform	Calcium-binding protein isoallergen 2	*Ambrosia artemisiifolia*	0.737897	-0.5	
437311	GDB_Plant_all_mRNA	Cystatin	Amb a CPI	PLN cystatin proteinase inhibitor	*Ambrosia artemisiifolia*	0.000000	0.2	Up
TC51843	Asteraceae_TIGR	Defensin-like protein	Art v 2	Art v 2 allergen precursor	*Artemisia vulgaris*	0.339018	-2.1	Down
3901093	GDB_Plant_all_mRNA	Expansins	Phlpl	PLN P.pratense mRNA for pollen allergen Phlpl.	*Phleum pratense*	0.082901	4.0	Up
3004466	GDB_Plant_all_mRNA	Group 5/6	Phl p6	PLN Phl p6 allergen, isolate c142.	*Phleum pratense*	0.339018	-2.1	Down
255779240	Asteraceae_Entrez	Lipid transfer protein	Amb a 6	AmbT-339 ,Lipid transfer protein	*Ambrosia trifida*	0.670000	-0.5	Down
302127823	GDB_Plant_all_mRNA	Pectate Lyase	Amb a 1.1	PLN Ragweed Amb a I.1 (antigen E) mRNA.	*Ambrosia artemisiifolia*	0.000000	0.5	Up
166433	GDB_Plant_all_mRNA		Amb a 1.2	PLN Amb a 1.2, variant 2.	*Ambrosia artemisiifolia*	0.000007	0.7	
302127813	GDB_Plant_all_mRNA		Amb a 1.3	PLN Amb a 1.3, variant 2	*Ambrosia artemisiifolia*	0.000000	0.6	
302127811	GDB_Plant_all_mRNA		Amb a 1.3	PLN Amb a I.3 (antigen E) mRNA, complete cds	*Ambrosia artemisiifolia*	0.692159	1.6	
302127825	GDB_Plant_all_mRNA		Amb a 1.4	PLN pollen allergen Amb a 1.4, variant 2.	*Ambrosia artemisiifolia*	0.000621	-1.2	
166440	GDB_Plant_all_mRNA		Amb a 1.5	PLN Amb a 2.01	*Ambrosia artemisiifolia*	0.431011	1.5	
TC6003	Asteraceae_TIGR	polygalacturonase	PEST459 isoform	Oil palm polygalacturonase allergen	*Elaeis guineensis*	0.140919	3.6	Up
TC43769	Asteraceae_TIGR	Profilin	Amb a 8 isoform	PLN profilin-like protein (D03) mRNA, complete cds.	*Ambrosia artemisiifolia*	0.000019	0.5	Down
CD851748	Asteraceae_TIGR		Amb a 8 isoform	Pollen profilin variant 7	*Corylus avellana*	0.407187	2.6	
TC43769	Asteraceae_TIGR		Amb a 8 isoform	Profilin isoallergen 1	*Ambrosia artemisiifolia*	0.000126	-2.3	
TC48587	Asteraceae_TIGR		Amb a 8 isoform	Profilin isoallergen 1	*Ambrosia artemisiifolia*	0.019232	-0.8	
34851181	GDB_Plant_all_mRNA		Amb a 8 isoform	Profilin isoallergen 1	*Ambrosia artemisiifolia*	0.923553	0.0	
TC68483	all_TIGR_Plant.fa		Amb a 8 isoform	Profilin-2	*Ambrosia artemisiifolia*	0.139656	-1.2	
28513232	GDB_Plant_all_EST	Others	-	cDNA clone PP043C12 similar to MINOR ALLERGEN	*Pinus pinaster*	0.810577	0.3	Up
TC430677	all_TIGR_Plant.fa		Def2/Der2	E1 protein and Def2/Der2allergen family protein	*Oryza sativa*	0.407187	2.6	

Table 21 : Summary of the superSAGE library allergen of ragweed pollen under elevated CO_2 plus drought condition. Database id, Datbase, allergen family, description, organism, p-value and log_2 of fold change compared to the control library. Only the significant values ($P \leq 0.00005$) will be

Database-id	Database	Allergenic family	Common name	Description	Organism	p-value	Fold change (log_2)	Regulation
TC42736	Asteraceae_TIGR	Calcium binding protein	Amb a 9 isoform	Calcium-binding pollen allergen	*Arachis hypogaea*	0.1020370	1.2	Down
DY929617	Asteraceae_TIGR		Amb a 9 isoform	Calcium-binding protein isoallergen 2	*Ambrosia artemisiifolia*	0.1907100	-2.1	
437311	GDB_Plant_all_mRNA	Cystatin	Amb a CPI	PLN cystatin proteinase inhibitor	*Ambrosia artemisiifolia*	0.0000000	0.2	Up
TC51843	Asteraceae_TIGR	Defensin-like protein	Art v 2	Art v 2 allergen precursor	*Artemisia vulgaris*	0.1907100	-2.1	Down
3901093	GDB_Plant_all_mRNA	Expansins	Phlpl	PLN P.pratense mRNA for pollen allergen Phlpl.	*Phleum pratense*	0.1042030	3.8	Up
3004466	GDB_Plant_all_mRNA	Group 5/6	Phl p6	PLN Phl p6 allergen, isolate c142.	*Phleum pratense*	0.1907100	-2.1	Down
3309042	GDB_Plant_all_mRNA		Phl pl 5	PLN Phleum pratense group V allergenPhl p 5.0204 mRNA, partial cds	*Phleum pratense*	0.6604620	2.0	
255779240	Asteraceae_Entrez	Lipid transfer protein	Amb a 6	AmbT-339 ,Lipid transfer protein	*Ambrosia trifida*	0.0000000	9.1	UP
166442	GDB_Plant_all_mRNA	Pectate Lyase	Amb a 1.1	PLN Ragweed Amb a I.1 (antigen E) mRNA.	*Ambrosia artemisiifolia*	0.0000000	1.6	Up
302127811	GDB_Plant_all_mRNA		Amb a 1.2	PLN pollen allergen Amb a 1.2, variant 2.	*Ambrosia artemisiifolia*	0.0000000	1.7	
302127823	GDB_Plant_all_mRNA		Amb a 1.3	PLN Amb a I.3 (antigen E) mRNA, complete cds	*Ambrosia artemisiifolia*	0.0000000	1.2	
302127825	GDB_Plant_all_mRNA		Amb a 1.4	PLN pollen allergen Amb a 1.4, variant 2.	*Ambrosia artemisiifolia*	0.0693065	-0.5	
166433	GDB_Plant_all_mRNA		Amb a 1.5	PLN pollen allergen Amb a 2.01, variant 2.	*Ambrosia artemisiifolia*	0.0604899	2.5	
TC6003	Asteraceae_TIGR	polygalacturonase	PEST459 isoform	Oil palm polygalacturonase allergen	*Elaeis guineensis*	0.2181050	3.3	Up
34851181	GDB_Plant_all_mRNA	Profilin	Amb a 8 isoform	PLN profilin-like protein (D03) mRNA, complete cds.	*Ambrosia artemisiifolia*	0.0000000	1.4	Up
TC52169	Asteraceae_TIGR		Amb a 8 isoform	Profilin isoallergen 1	*Ambrosia artemisiifolia*	0.0000000	3.7	
TC48587	Asteraceae_TIGR		Amb a 8 isoform	Profilin isoallergen 1	*Ambrosia artemisiifolia*	0.4419600	-0.4	
CD851748	Asteraceae_TIGR		Amb a 8 isoform	Profilin-2	*Ambrosia artemisiifolia*	0.0056155	-4.1	
EE554793	all_TIGR_Plant.fa	other	-	Allergen; n=1; Periplaneta americana	*Periplaneta americana*	0.0000000	7.6	Up
FS446595	Asteraceae_TIGR		BRSn20	Allergen-like protein BRSn20	*Sambucus nigra*	0.6604620	2.0	
28513232	GDB_Plant_all_EST		-	cDNA clone PP043C12 similar to MINOR ALLERGEN,	*Pinus pinaster*	0.4601300	-0.6	
TC430677	all_TIGR_Plant.fa		Def2/Der2	E1 protein and Def2/Der2allergen family protein	*Oryza sativa*	0.6604620	2.0	

Table 22: Transcript amounts in tags per million (tpm) for Ambrosia artemisiifolia Amb a 1 isoallergen

Allergen	Control	Drought	Elevated CO_2	Elevated CO_2 + Drought
Amb a 1.1	615.2	852.4	1102.8	1817.5
Amb a 1.2	30.1	47.4	71.7	98.3
Amb a 1.3	113.9	168.2	233.7	262.9
Amb a 1.4	9.1	3.9	9	6.3
Amb a 1.5	0.2	0.6	1.1	1.3

3.2.2.6 Functional annotation

Gene ontology (GO) assignments were used to classify the functions of the predicted ragweed transcripts. Based on sequence homology, the 236,942 sequences can be categorised into three main levels: biological process, cellular components and molecular function (Fig. 31). The number of transcripts in each level showed the elevated CO_2 and drought libraries having a similar number of transcripts in the biological (298,293 transcripts), cellular (331,327 transcripts) and molecular level (316,302 transcripts), respectively, while the elevated CO_2 plus drought reported a higher number of transcripts in the biological (335 transcripts), cellular (380) and molecular level (345 transcripts) (Fig. 31). As shown in Fig. 32, the GO levels can be categorised according to 54 functional groups. It was found that the metabolic process and cellular process are dominant in the biological level, whereas cell, cell part and organelle are dominant in the cellular level, and binding and metallochaperone activity are dominant in the molecular level. Moreover, a high amount of transcripts were found in categories of response to stimulus, macromolecular complex and structural molecular activity, while a few transcripts were found in antioxidant activity, symplast and carbon utilisation (Fig. 32).

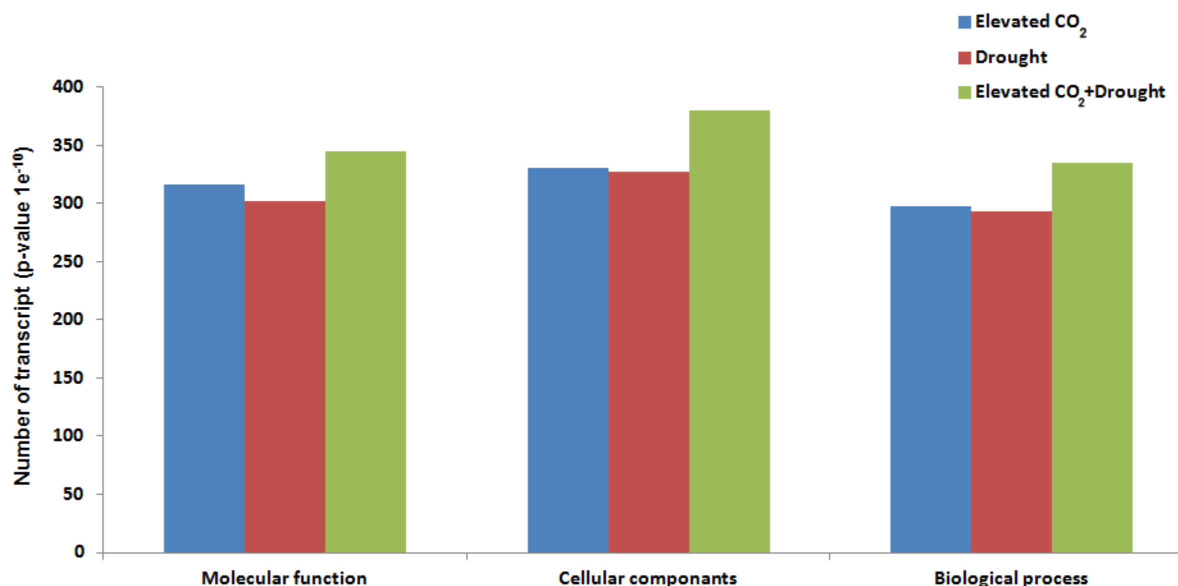


Figure 31: Functional categories of the genes annotated from the ragweed pollen tags. Number of transcripts under molecular function, cellular components and biological process corresponding to ragweed pollen libraries (elevated CO_2, drought and elevated CO_2 plus drought)

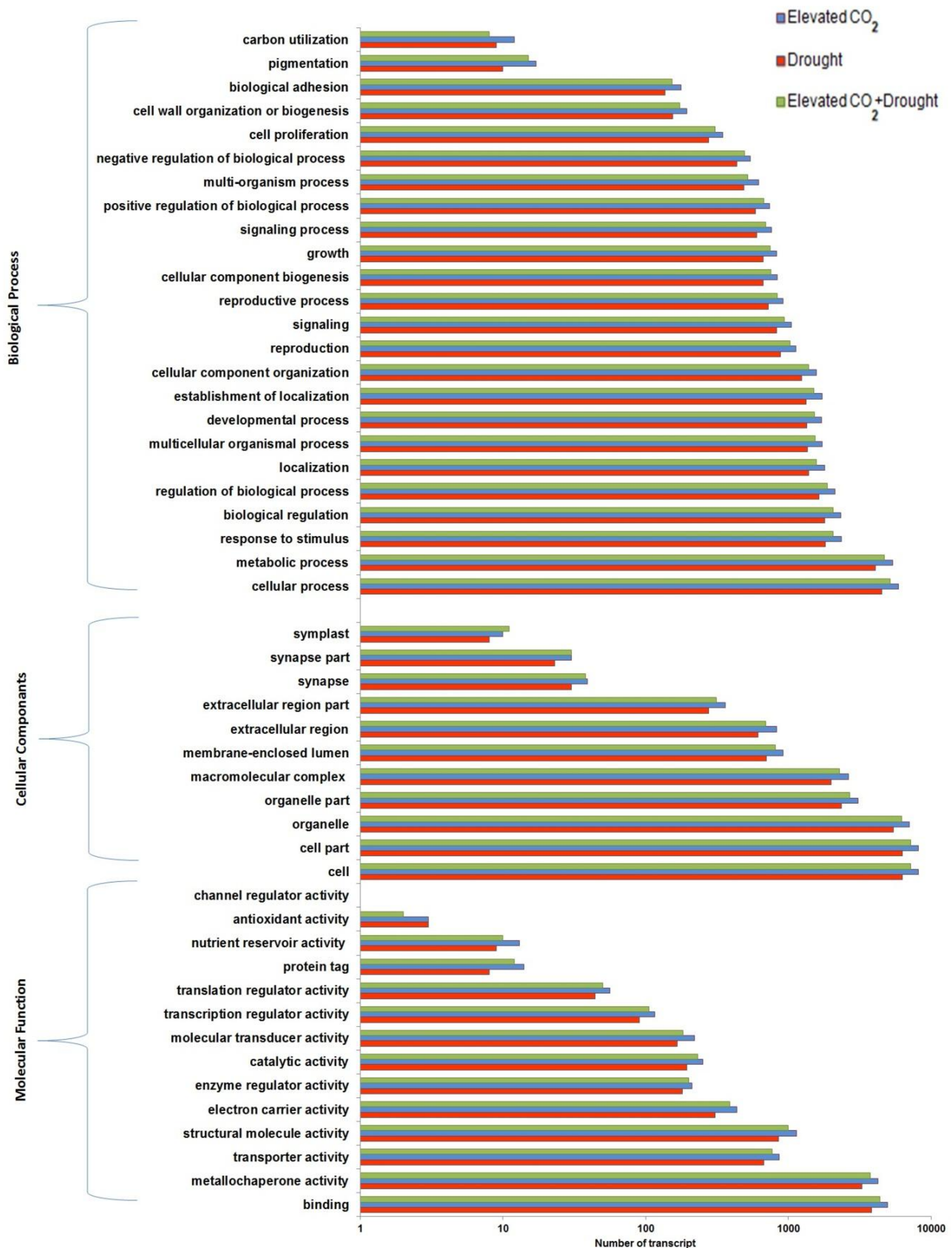


Figure 32: Functional categories of the genes annotated from the ragweed pollen tags. Genes corresponding to ragweed pollen tags were categorised according to their function, based on their annotation as described in the methods section. The tags with different annotations were grouped into the corresponding categories.

In the most enriched GO terms in the ragweed pollen libraries (Tab. 23), elevated CO_2 are represented with 92 GO terms that comprised 14,397 transcripts, including 872 significant transcripts ($P \leq 1E^{-10}$), 563 of which are up-regulated while 309 are down-regulated. In drought library, it is represented with more terms by 195 GO terms, comprising 14,206 transcripts; albeit only 1,254 of which are highly significant ($P \leq 1E^{-10}$), 544 are up-regulated and 710 are down-regulated (Tab. 23). Elevated CO_2 plus drought showed 165 GO terms, with the highest number of transcripts by 25,300, within which 1,801 are highly significant ($P \leq 1E^{-10}$). The elevated CO_2 plus drought have the highest number of up-regulated and down-regulated transcripts, by 586 and 1,215 respectively (Tab. 23).

Table 23: Summary of the most enriched GO terms tables of elevated CO_2, drought and elevated CO_2 plus drought ragweed pollen libraries

	GO-Term	Transcripts	Differential expressed p-Value <1e-10	Up-regulated	Down-regulated
Elevated CO_2	92	14397	872	563	309
Drought	195	14206	1254	544	710
Elevated CO_2 + drought	165	25300	1801	586	1215

The most enriched GO terms in the ragweed pollen libraries with $P \leq 0.05$ enrichment values are provided in Tab. 24, 25 and 26. Several GO terms are related to stress response in the elevated CO_2 library (GO: 0010288 responses to lead ion, GO: 0006974 responses to DNA damage stimulus, GO: 0006950 responses to stress). Furthermore, other GO terms are related to pollen and seed development (GO: 0019953 sexual reproduction, GO: 0035239 tube morphogenesis and GO: 0009555: pollen development) (Tab. 24).

However, the drought library showed another distribution in its enriched GO terms (Tab. 25). The GO terms involved in stress response are more than that in elevated CO_2 and elevated CO_2 plus drought libraries (GO: 0051788 response to misfolded protein, GO: 0050896 response to stimulus, GO: 0009650 UV protection, GO: 0009411 response to UV, GO: 0009631 cold acclimation, GO: 0009611 response to wounding, GO: 0010288 response to lead ion, GO: 0009737 response to abscisic acid stimulus) (Tab. 25). Moreover, the pollen and seed development are represented with more GO terms (GO:0048455 stamen formation, GO:0048448 stamen morphogenesis, GO:0048449 floral organ formation, GO:0048444 floral organ morphogenesis, GO:0035239 tube morphogenesis, GO:0035295 tube development, , GO:0009845 seed germination, GO:0090351 seedling development, , GO:0009555 pollen development) (Tab. 25).

Finally, the elevated CO_2 plus drought shared some GO terms found in the elevated CO_2 and drought libraries (Tab. 26). The stress response is represented by new GO terms that is not found in the other libraries (GO:0009626 plant-type hypersensitive response, GO:0009408 response to heat, GO:0030682 evasion or tolerance of host defence response, GO: 0006974 responses to DNA damage stimulus) (Tab. 26). By contrast, the pollen and seed development GO terms are similar to the drought library (GO:0048448 stamen morphogenesis, stamen formation, GO:0009555 pollen development, GO:0009845 seed germination, GO:0090351 seedling development, GO:0048449 floral organ formation, GO:0048444 floral organ morphogenesis) (Tab. 26).

Table 24: Most enriched GO terms related to stress response, pollen and seed development in elevated CO_2 library compared to control

GO-Term	Description	Transcripts	Differential expressed p-Value <1e-10	Up-regulated	Down-regulated	Enrichment p-value
Stress response						
GO:0010288	Response to lead ion	1	1	1	0	0.0343
GO:0006974	Response to DNA damage stimulus	218	13	8	5	0.0373
GO:0006950	Response to stress	1627	68	50	18	0.0434
Pollen and seed development						
GO:0019953	Sexual reproduction	435	24	16	8	0.0146
GO:0035239	Tube morphogenesis	34	4	1	3	0.0281
GO:0009555	Pollen development	35	4	2	2	0.0308

Table 25: Most enriched GO terms related to stress response, pollen and seed development in drought treated library compared to control

GO-Term	Description	Transcripts	Differential expressed p-Value <1e-10	Up-regulated	Down-regulated	Enrichment p-value
Stress response						
GO:0051788	Response to misfolded protein	5	2	1	1	0.0173
GO:0050896	Response to stimulus	1820	95	35	60	0.0235
GO:0009650	UV protection	6	2	1	1	0.0252
GO:0009411	Response to UV	28	4	1	3	0.0316
GO:0009631	cold acclimation	7	2	0	2	0.0343
GO:0009611	Response to wounding	153	12	6	6	0.0343
GO:0010288	Response to lead ion	1	1	0	1	0.0435
GO:0009737	Response to abscisic acid stimulus	161	12	5	7	0.0477
Pollen and seed development						
GO:0048455	Stamen formation	2	2	1	1	0.0019
GO:0048448	Stamen morphogenesis	2	2	1	1	0.0019
GO:0048449	Floral organ formation	3	2	1	1	0.0055
GO:0048444	Floral organ morphogenesis	3	2	1	1	0.0055
GO:0035239	Tube morphogenesis	28	5	3	2	0.0065
GO:0035295	Tube development	75	8	5	3	0.0157
GO:0009845	Seed germination	23	4	1	3	0.0162
GO:0090351	Seedling development	23	4	1	3	0.0162
GO:0009555	Pollen development	31	4	2	2	0.0439

Table 26: Most enriched GO terms related to stress response, pollen and seed development in elevated CO_2 plus drought library compared to control

GO-Term	Description	Transcripts	Differential expressed p-Value <1e-10	Up-regulated	Down-regulated	Enrichment p-value
Stress response						
GO:0009626	Plant-type hypersensitive response	26	4	0	4	0.0267
GO:0009408	Response to heat	168	13	7	6	0.0371
GO:0030682	Evasion or tolerance of host	1	1	0	1	0.0446
GO:0006974	Response to DNA damage stimulus	227	16	6	10	0.0468
Pollen and seed development						
GO:0048448	Stamen morphogenesis	4	2	1	1	0.0112
GO:0048455	Stamen formation	4	2	1	1	0.0112
GO:0009555	Pollen development	32	5	3	2	0.0128
GO:0009845	Seed germination	24	4	2	2	0.0204
GO:0090351	Seedling development	24	4	2	2	0.0204
GO:0048449	Floral organ formation	6	2	1	1	0.0264
GO:0048444	Floral organ morphogenesis	6	2	1	1	0.0264

3.2.2.7 Functional Categorization of SuperSAGE Tags

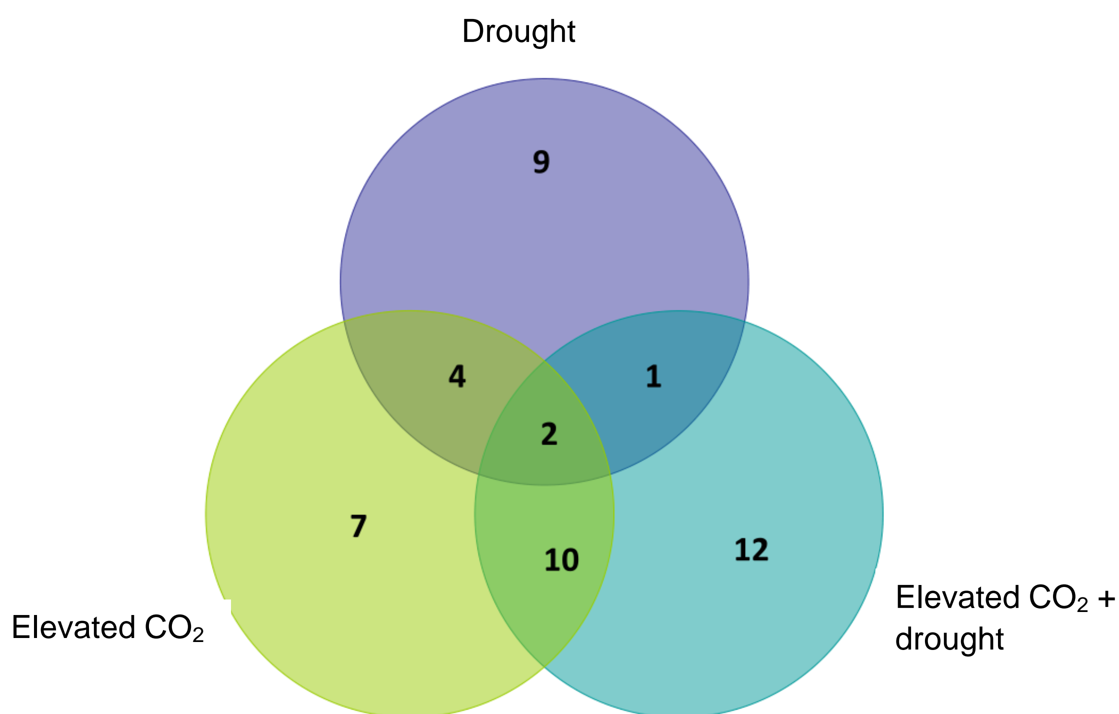
The wide range of the STDGE2GO toolkit enabled searching for terms of interest based on the transcript description (section 3.2.2.5) and/or it's GO-terms that shared the same process or function.

According to the GO-term categorisation at the biological process level, subcategories in response to stress were considered more informative to evaluate the ragweed pollen response to elevated CO_2 and/or drought conditions.

In response to stress in the GO-terms level, responding to water deprivation and oxidative stress will be considered. Through analysis of the up-regulated tags observed in the aforementioned subcategories, it was possible to generate the Venn diagrams presented in Fig. 33.

Fig. 33(A) represents the up-regulated transcripts related to water deprivation in the ragweed pollen of elevated CO_2, drought and elevated CO_2 plus drought. For the common up-regulated transcripts represented in all libraries, 2 structural transcripts (Chromosome chr19 scaffold_4 and Expressed protein - *Oryza sativa*) were recorded (Fig. 33(A)). Meanwhile 4 up-regulated transcripts were found within the transcripts between the drought and elevated CO_2 pollen, (PLN *Picea sitchensis* clone WS02723_P04, PLN *Hordeum vulgare* subsp. vulgare cDNA clone: FLbaf19k05) (Fig. 33(A)). Interestingly, 10 transcripts were common between the elevated CO_2 treated pollen and the elevated CO_2 plus drought, within these transcripts (histone H4, ribosomal protein L25-like protein, Cluster: Actin, cytochrome c) (Fig. 33(A)). Each library showed unique transcripts that up-regulated under each condition. The elevated CO_2 showed 7 distinctive transcripts, the most prominent of which were (PLN *Lactuca sativa* Ls2ox2 mRNA for gibberellin 2-oxidase NO_2, histone H4.2, actin and major intrinsic protein PIPB) (Fig. 33(A)). Moreover, there were 9 exclusive up-regulated drought transcripts, of which the most prominent were PLN *populus* EST from severe drought-stressed leaves, soybean seeds containing globular-stage embryos, UDP-glucose: protein transglucosylase-like and peroxidase 1 (Fig. 33(A)). Finally, the elevated CO_2 plus drought, showed the highest number of exclusive up-regulation, by 12 tags, prominently including cytochrome c biogenesis, protein kinase 3, mitochondrial import inner membrane translocase and low temperature and salt responsive protein (Fig. 33(A)).

In Fig. 33(B), which represents the oxidative stress on the GO validation, 5 common transcripts represented the oxidative stress in the different libraries, with the most prominent being thioredoxin h2, UDP-glucuronate 4-epimerase, ATP synthase subunit beta, mitochondrial precursor. Meanwhile, 7 up-regulated transcripts were found between the drought and elevated CO_2 pollen (Fig. 33(B)), most prominent including cold acclimation responsive protein BudCAR5, actin-depolymerizing factor 12, fructose-bisphosphate aldolase (Fig. 33(B)). 5 transcript were found between the drought treated pollen and elevated CO_2 plus drought, with the most prominent being extensin protein-like (Fig. 33(B)). Interestingly, no shared transcripts were found between elevated CO_2 and elevated CO_2 plus drought (Fig. 33(B)). Each library showed unique transcripts that up-regulated and were based on the oxidative stress GO-terms. Elevated CO_2 showed 14 distinctive transcripts, the most prominent being peptide methionine sulfoxide reductase, nucleoside diphosphate kinase, ATPase, Thioredoxin-dependent peroxidase, aquaporin, actin and cytochrome P450 (Fig. 33(B)). By contrast, the drought library displayed 11 transcripts, with the most prominent ATP synthase subunit 9, DNA-binding response regulator TorR, ATP-dependent Clp protease proteolytic subunit and cysteine proteinase inhibitor 6 precursors (Fig. 33(B)). Finally, elevated CO_2 plus drought possessed a higher number of up-regulation than elevated CO_2 and the drought library, by 22 transcripts. These transcripts mostly represent thioredoxin peroxidase, NADP isocitrate dehydrogenase, ribonucleoside diphosphate reductase small chain, Ser/Thr specific protein phosphatase 2A B regulatory subunit beta isoform and copper chaperone (Fig. 33(B)).



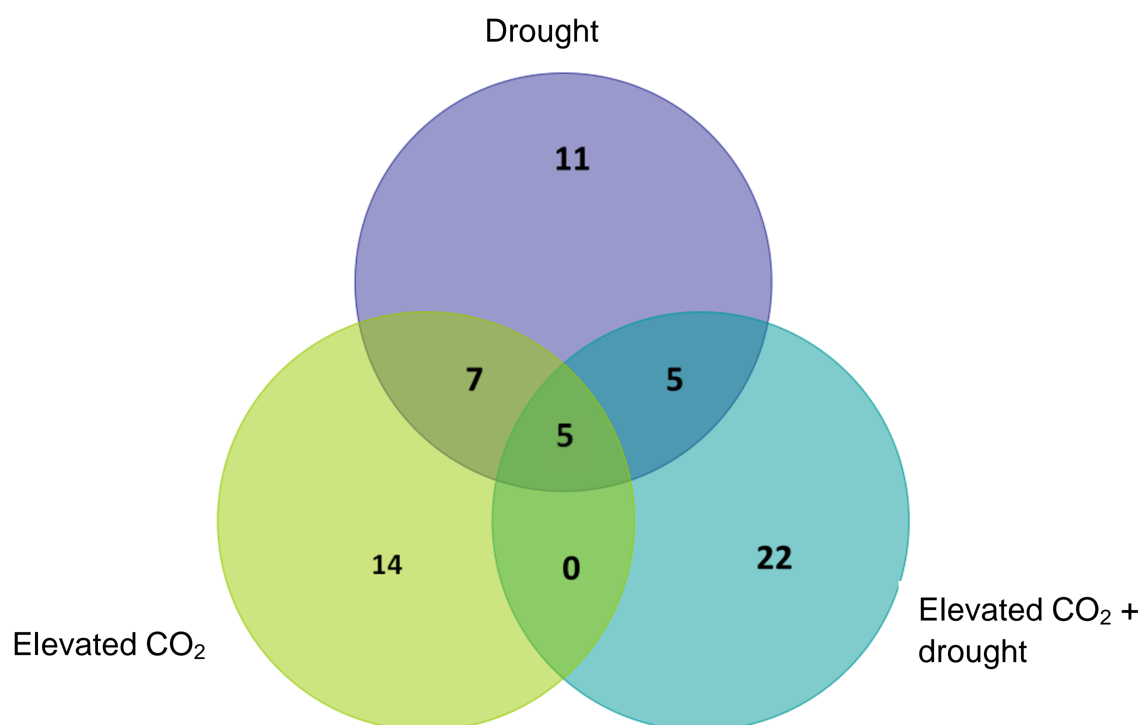


Figure 33: Venn diagrams of differentially up-regulated tags from ragweed pollen ($P \leq 0.05$) under elevated CO_2, drought and elevated CO_2 plus drought associated with gene ontology (GO) response to (A) water deprivation; (B) oxidative stress.

3.2.3 Global DNA methylation

Global DNA methylation levels were measured in ragweed pollen of control, elevated CO_2, drought, elevated CO_2 plus drought in both first generation pollen (2011) and second generation pollen (2011), using an enzyme-linked immunosorbent assay method technology (Fig. 34).

Fig. 34 shows the global DNA methylation patterns of first generation ragweed pollen (2011). There was no significant difference in the global DNA methylation patterns of pollen exposed to elevated CO_2, drought, or elevated CO_2 plus drought compared to control pollen (Fig. 34). Furthermore, no significant differences were observed between the global DNA methylation patterns of second generation pollen (2011) exposed to elevated CO_2, drought, elevated CO_2 plus drought, and control pollen (Fig. 34).

The comparison of global DNA methylation between the first and second generation showed that the control and elevated CO_2 of the first and second generation reported no significant difference. By contrast, the global DNA methylation of drought of the first generation had a significantly higher methylation rate than that of the second generation ($p= 0.02$) (Fig. 34). Finally, no significant difference was found between elevated CO_2 plus drought in the first and second generation pollen.

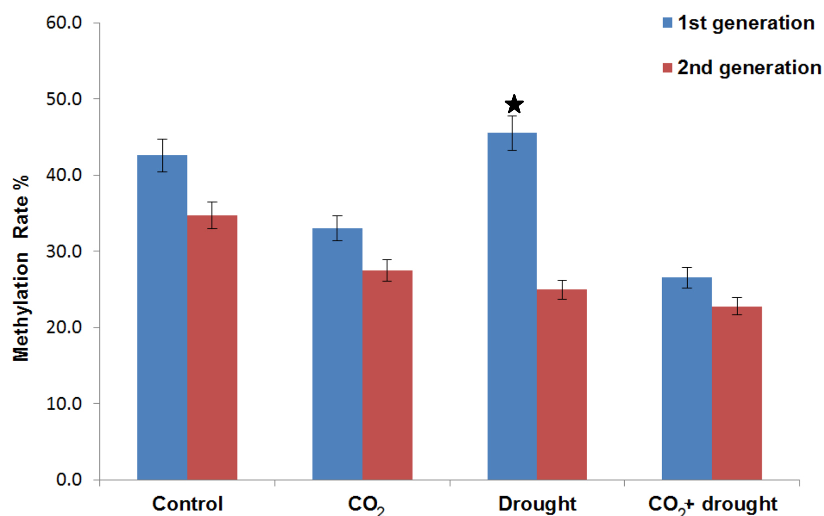


Figure 34: Global DNA methylation of genomic ragweed pollen DNA of control, elevated CO_2, drought and CO_2 plus drought in first generation and second generation pollen. Bars indicate ± SD; n=5, t-test. Stars refer to significant difference between treatments in the first and second generation.

3.3 Proteomic analysis

3.3.1 One-dimensional polyacrylamide gel electrophoresis

SDS-PAGE was regarded as a primary investigation, to demonstrate the protein profile of plants grown under different environmental conditions. Fig. 35 shows the ragweed pollen protein bands in different lanes, whereby each sample lane initially contained homogenous (10µg) pollen proteins (lanes 1-4). During the course of investigation, the analysis of SDS-PAGE data showed different proteins fractions in the studied treatments (elevated CO_2, drought, elevated CO_2 plus drought) compared to control.

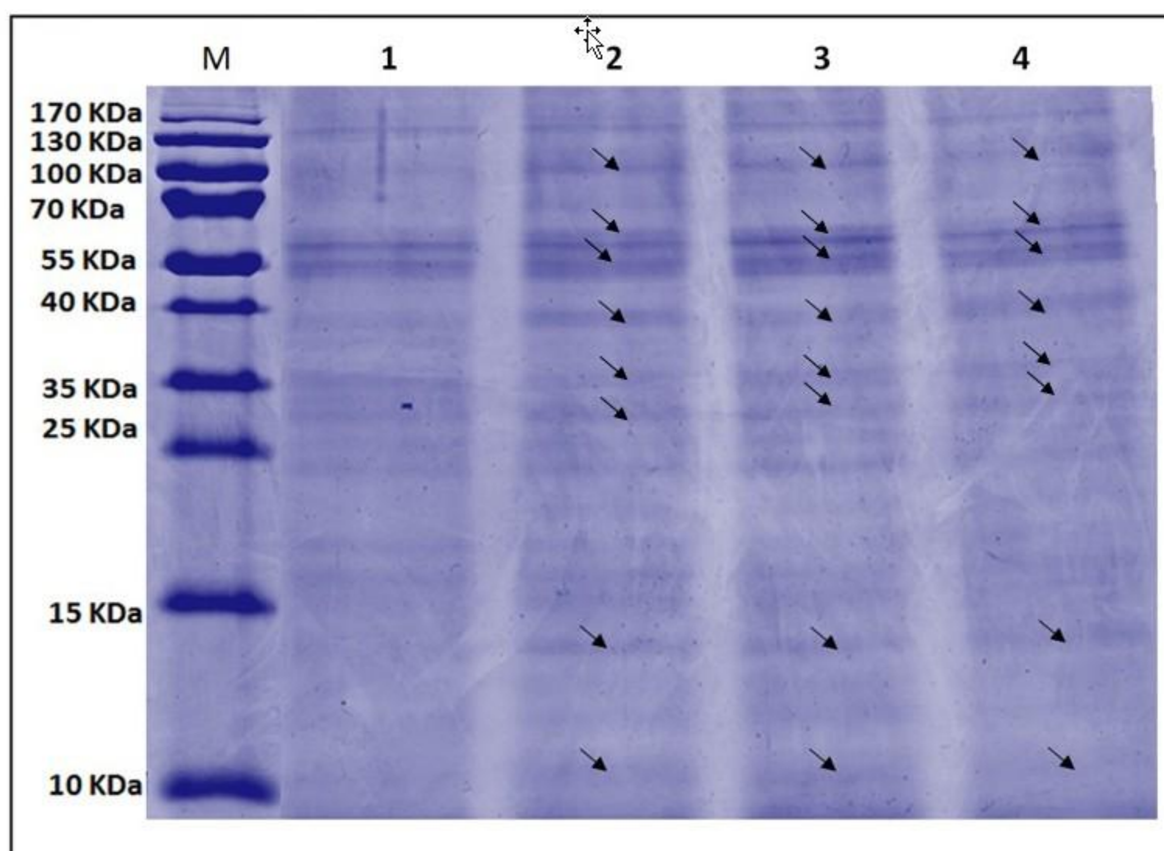


Figure 35: Image of the stained SDS-PAGE gel showed several protein bands in all samples of the ragweed pollen. M: markers, lane 1: control pollen extract, lane 2: elevated CO_2 pollen extract, lane 3: drought pollen extract and lane 4: elevated CO_2 + drought pollen extract. Black arrows indicate different bands.

3.3.2 2D-Difference-in-gel-electrophoresis (2D-DIGE)

The high resolution 2D-DIGE was used to separate soluble proteins from ragweed pollen under elevated CO_2, drought and elevated CO_2 plus drought. The DeCyder 2D software quantified the spot areas in each image, expressing these values as a ratio (fold change), comparing spot volumes of the sample to those corresponding in the internal standard (Fig. 36).

Around 3,164 and 2,975 spots for respective first and second generation were highly resolved and detected by silver staining, over a pH range of 4–7 and a size range of 10–100 kDa. All spots were matched by gel-to-gel comparisons, with differences in the relative abundance (vol %) of each spot analysed. Attention was focused on spots whose abundance varied at least ±1-fold between the treated and non-treated pollen. The differentially expressed proteins are marked on the representative 2D-DIGE gel shown in Fig. 37 and Fig. 39.

The spots of interest were digested and analysed by LC-MS/MS, followed by a homology-driven search identification (Tab. 27, 28). 34 spots were been analysed in the first generation pollen, within which 86 proteins were identified. By contrast, 35 spots were analysed in the second generation pollen, albeit including only 36 identified proteins (Fig. 37 and Fig. 39). The predicted molecular masses and pIs were generally consistent with the experimental data for the majority of the identified proteins, as judged from the location of spots on 2-D gels. The identified proteins were categorised according to several functional groups based on their predicted protein function. Several of the identified proteins were present in more than one spot, which might have arisen from post-translational protein modifications (PTMs) and proteolytic degradation of the protein, or alternatively due to these proteins being products of different genes (Jorge et al., 2005).

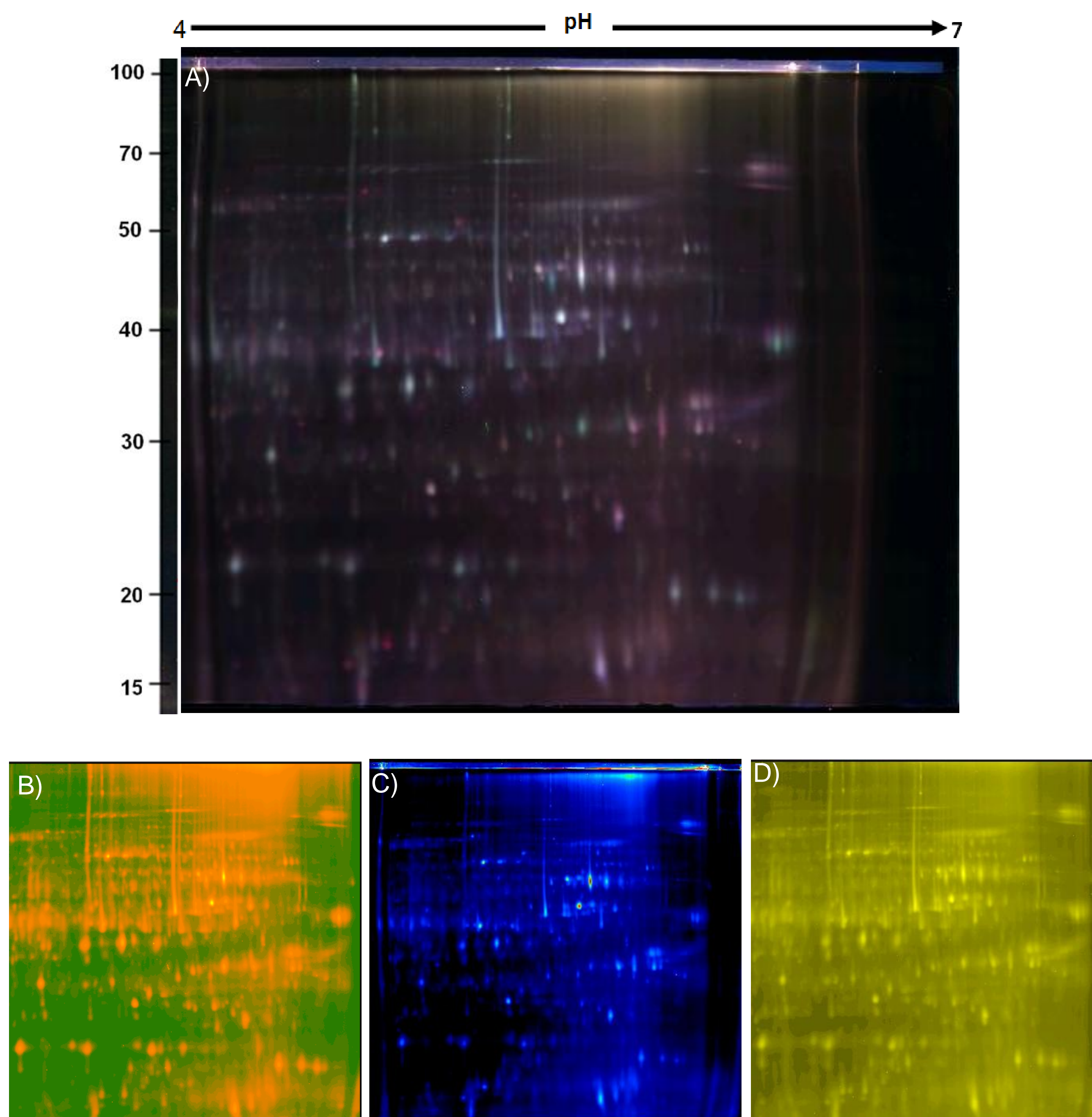


Figure 36: Images from a pH 4–7 2D-DIGE gel of two protein samples isolated from ragweed pollen labelled with minimal CyDye DIGE fluors. A) The three overlaid photos. B) The Cy3 image corresponding to sample 1 (control, drought). C) The Cy5 image corresponding to sample 2 (elevated CO_2, elevated CO_2 plus drought) D) The Cy2 image corresponding to internal standard (mixture of all treatments.

The differentially expressed spots present in mature first generation pollen grains (Fig. 38) could be divided into the following functional categories: (1) allergenic, (2) metabolism, (3) cytoskeleton, (4) protein fate, (5) signaling, (6) stress and defence and (7) cell wall remodeling.

Proteomic analysis of the differentially expressed 34 spots indicated that the largest pool comprised 14 spots responsible for metabolism (41%) and 3 spots (9%) belonging to the protein fate category. Moreover, allergenic protein spots were well represented (seven spots; 20%).

As shown in the heat map of first generation ragweed pollen (Fig. 38), expression of 65% of the spots in the pollen exposed to elevated CO_2 increased. Expression of four allergenic protein spots accumulated, though only Amb a 1.3 that was significant upregulated (p=0.01), and expression three other allergenic protein spots reduced, though only cysteine proteinase inhibitor that was significantly reduced (p=0.006) (Tab. 27). Of the protein spots related to metabolism, the expression of nine spots increased, though only enolase and glutamate dehydrogenase were significantly increased (Tab. 27). Moreover, expression of five metabolism spots reduced; the most prominent significantly reduced metabolism spots were 2,3 bisphosphoglycerat and triosephosphate isomerase (Tab. 27). Finally, the signaling spot Ras-related protein and stress spot unknown protein 1 were significantly reduced (Tab. 27).

In the pollen exposed to drought, expression of 83% of the spots increased (Fig. 38). All allergenic protein spots except for one accumulated, though only Amb a 1.3 accumulated significantly; cysteine proteinase inhibitor was the only significantly reduced allergenic protein spot (Tab. 27). Of the spots related to metabolism, the expression of twelve spots accumulated; the most prominent significantly accumulated metabolism protein spots were 2,3 bisphosphoglycerat and glutamate dehydrogenase (Tab. 27). Moreover, expression of two spots related to metabolism reduced, though only enolase was significantly reduced (Tab. 27). Finally, the signaling spot Ras-related protein significantly reduced, while expression of the stress spot unknown protein 1 accumulated (Tab. 27).

In the pollen exposed to elevated CO_2 plus drought, expression of 70% of the spots increased (Fig. 38). Expression of five allergenic protein spots accumulated, though only Amb a 1.3 was significantly increased, and expression of two allergenic protein spots reduced, though only cysteine proteinase inhibitor was significantly reduced (Tab. 27).

Of the metabolism spots, expression of eleven protein spots accumulated, with the most significant and prominent being 2,3 bisphosphoglycerat and glutamate dehydrogenase; expression of three other metabolism spots decreased, though only enolase was significantly decreased (Tab. 27). Finally, expression of the signaling spots Ras-related protein and stress spot unknown protein 1 decreased (Tab. 27).

In the second generation pollen (Fig. 39), the proteins present in mature pollen could be divided into the same functional categories as the proteins in the first generation pollen, with exception of the absence of a cell wall related protein and the presence of a protein related to reproduction.

As showen in the heat map of second generation ragweed pollen (Fig. 40), expression of 80% of the spots in the pollen exposed to elevated CO_2 increased. Of these, three allergenic protein spots accumulated, though only Amb a 1.1 accumulated significantly ($p = 0.02$), while expression of one allergnic spot protein (Amb a 1.2) reduced non-significantly (Tab. 28). Of the spots related to metabolism, the expression of all ten protein spots accumulated, with the most significant and prominent being beta amylase 1 and malate dehydrogenase 1 (Tab. 28). The expression of two metabolism spots was non-significantly reduced (triosephosphate isomerase and IAA amino acid hydrolase ILR1-like 2). Finally, expression of 14-3-3 like protein (signaling protein spot), and regulatory associated protein Tor 1 and unknown protein 1 (stress protein spots) accumulated significantly (Tab. 28).

In total, the expression of 57% of the spots in the pollen exposed to drought increased (Fig. 40). All of the allergenic protein spots accumulated; Amb a 1.1 and 1.5 accumulated significantly (Tab. 28). Of the metabolism spots, seven protein spots accumulated; glyceraldehyde 3-phosphate dehydrogenase and beta amylase 1 accumulated significantly (Tab. 28). Additionally, the expression of five other metabolism spots decreased, though only malate dehydrogenase 1 decreased significantly.

Finally, of the signaling and stress spots, expression of 14-3-3 like protein and bZIP transcription factor 60 decreased (Tab. 28).

In total, the expression of 65% of the spots in the pollen exposed to elevated CO_2 plus drought increased (Fig. 40). Four allergenic proteins accumulated; Amb a 1.1 and 1.5 accumulated significantly (Tab. 28). The expression of seven metabolism spots accumulated; the most significantly accumulated were beta-amylase 1 and malate dehydrogenase 1 (Tab. 28). In contrast, a further five metabolism spots were non-significantly reduced. Finally of the signaling spot, expression of 14-3-3 like protein was accumulated, while expression of the stress spots regulatory associated protein Tor 1 and unknown protein 12 accumulated, and expression of bZIP transcription factor 60 and unknown protein 1 decreased (Tab. 28).

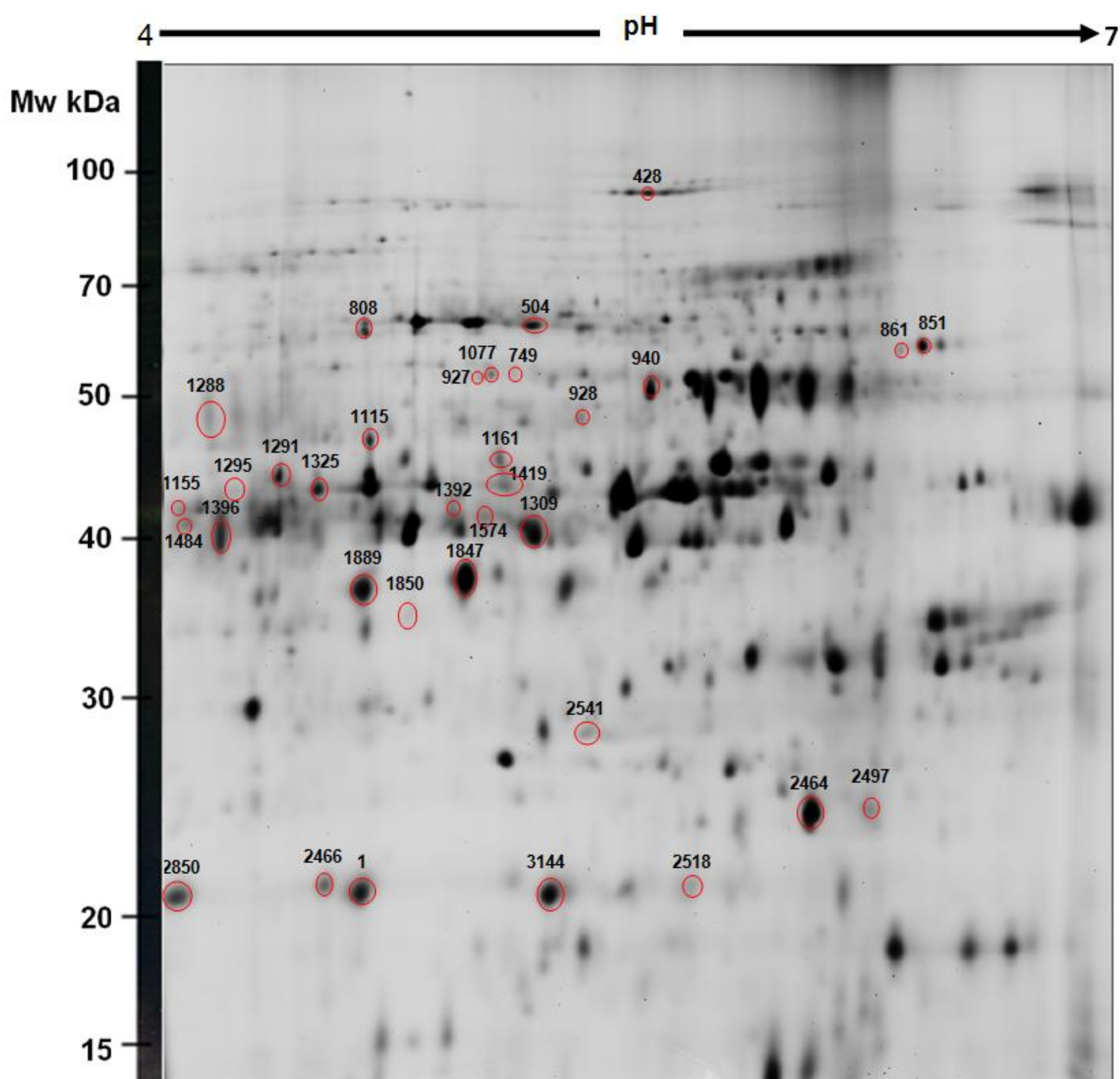


Figure 37: Master 2D-DIEG gel of first generation ragweed pollen extract. Protein were separated on 24 cm, pH 4-7 IPG strips (first dimension), and 13% SDS-PAGE (second dimension). Red circles represent identified protein spots which are numbered according to table 27.

Table 27: Summary of differentially regulated proteins of first generation ragweed pollen in elevated CO_2, drought and elevated CO_2+drought that appeared in Fig. 37, and separated by 2D-DIEG and identified by LC-MS/MS.

Spot number	Identified proteins	Best matching gene products	Accession number	Observed		Spot ratio			p-value
				Molecular weight	PI	CO_2	Drought	CO_2 + drought	
1- Pollen allergen									
1850	10	Cysteine proteinase inhibitor	CYT6_ARATH (+2)	35	4.8	-1.7	-1.5	-1.0	0.006
1309	15	Pollen allergen Amb a 1.1	MPA11_AMBAR	40	5.2	1.3	1.1	1.3	0.800
1484	10	Pollen allergen Amb a 1.2	MPA12_AMBAR	40	4.1	-1.1	1.1	-2.3	0.150
1392	15	Pollen allergen Amb a 1.3	MPA13_AMBAR	44	4.9	1.7	2.5	2.1	0.010
1574	6	Pollen allergen Amb a 1.4	MPA14_AMBAR	41	5.1	1.1	1.4	1.2	0.600
1115	22	Pollen allergen Amb a 1.5	MPAA2_AMBAR	42	4.6	1.5	1.8	1.4	0.300
3144	9	Profilin	PROF_HELAN	21	5.2	-1.8	2.0	2.2	0.700
2- Metabolism									
749	17	2,3-bisphosphoglycerate	PMG2_ARATH	58	5.1	-1.7	1.3	1.4	0.001
1419	15	Malate dehydrogenase 1	MDHM1_ARATH	43	5.1	1.6	2.0	2.0	0.190
928	17	UDP-glucose 6-dehydrogenase	UGDH_SOYBN	59	5.3	-1.1	2.1	1.1	0.050
1288	14	Aspartate aminotransferase	AATC_DAUCA	49	4.1	1.1	1.2	-1.2	0.070
927	13	ATP synthase subunit alpha	ATPAM_HELAN	51	5.0	1.1	1.9	-1.2	0.100
1889	9	Enolase	ENO1_HEVBR	38	4.6	2.4	-1.3	-1.1	0.004
861	5	Glutamate dehydrogenase	MDHC1_ARATH	55	6.3	1.2	3.4	2.3	0.009
1295	11	Glyceraldehyde-3-phosphate	G3PC_MAIZE (+1)	44	4.2	1.0	1.2	1.0	0.490
1396	12	Glyceraldehyde-3-phosphate	G3P_ATRNU	40	4.2	-1.4	-1.2	0.2	0.760
1291	11	Pyruvate dehydrogenase E1	ODPA_SOLTU	44	4.4	1.1	1.4	1.4	0.800
1325	15	Pyruvate dehydrogenase E1	ODPA1_ARATH	43	4.5	1.5	1.8	1.1	0.400
1155	14	Alcohol dehydrogenase	ADH_FRAAN	42	4.1	1.1	1.8	1.6	0.610
2518	4	Triosephosphate isomerase	TPIS_LACSA	21	5.7	-1.8	2.0	1.5	0.020
940	9	UTP--glucose-1-phosphate	UGPA_SOLTU	50	5.5	-1.0	2.1	1.3	0.036
3- Cytoskeleton									
1161	24	Actin-1	ACT1_ORYSI (+3)	55	5.1	1.5	1.8	-1.1	0.070
2541	2	Actin-97	ACT11_SOLTU	28	5.3	1.6	1.4	1.4	0.660
2466	10	Actin-depolymerizing factor 7	ADF7_ARATH	22	4.5	2.2	-1.1	-1.2	0.063
I- Protein fate									
1	5	Ubiquitin protein ligase	Q65XV2	21	4.6	-1.1	1.4	-1.2	0.130
808	10	26s Proteasome regulatory	PRS4A_ARATH (+1)	63	4.6	1.1	1.1	1.1	0.800
428	15	Luminal-binding protein 5	BIP5_TOBAC	92	5.5	1.2	1.6	1.2	0.110
5- Signaling									
2497	10	14-3-3-like protein	1433A_VICFA	24	6.2	1.5	1.1	1.8	0.400
1847	6	Ras-related protein RABD2a	RAD2A_ARATH (+4)	37	5.0	-2.2	-1.9	-1.1	0.000
6- Stress									
2850	3	Unknown protein 1 (Fragment)	UP01_VITRO (+1)	21	4.1	-1.2	-2.1	1.1	0.017
2464	10	Superoxide dismutase [Cu-Zn]	SODC1_BRAJU	24	6.0	1.6	1.3	1.0	0.200
7- Cell Fate									
851	7	Calreticulin	CALR_PRUAR	59	6.4	1.3	2.9	2.0	0.082
504	13	Tubulin beta-1 chain	TBB1_ARATH	62	5.1	2.1	1.3	1.4	0.040
8- Cell wall									
1077	13	Alpha-1,4-glucan-protein synthase	UPTG2_SOLTU	51	5.1	-1.5	1.5	1.3	0.500

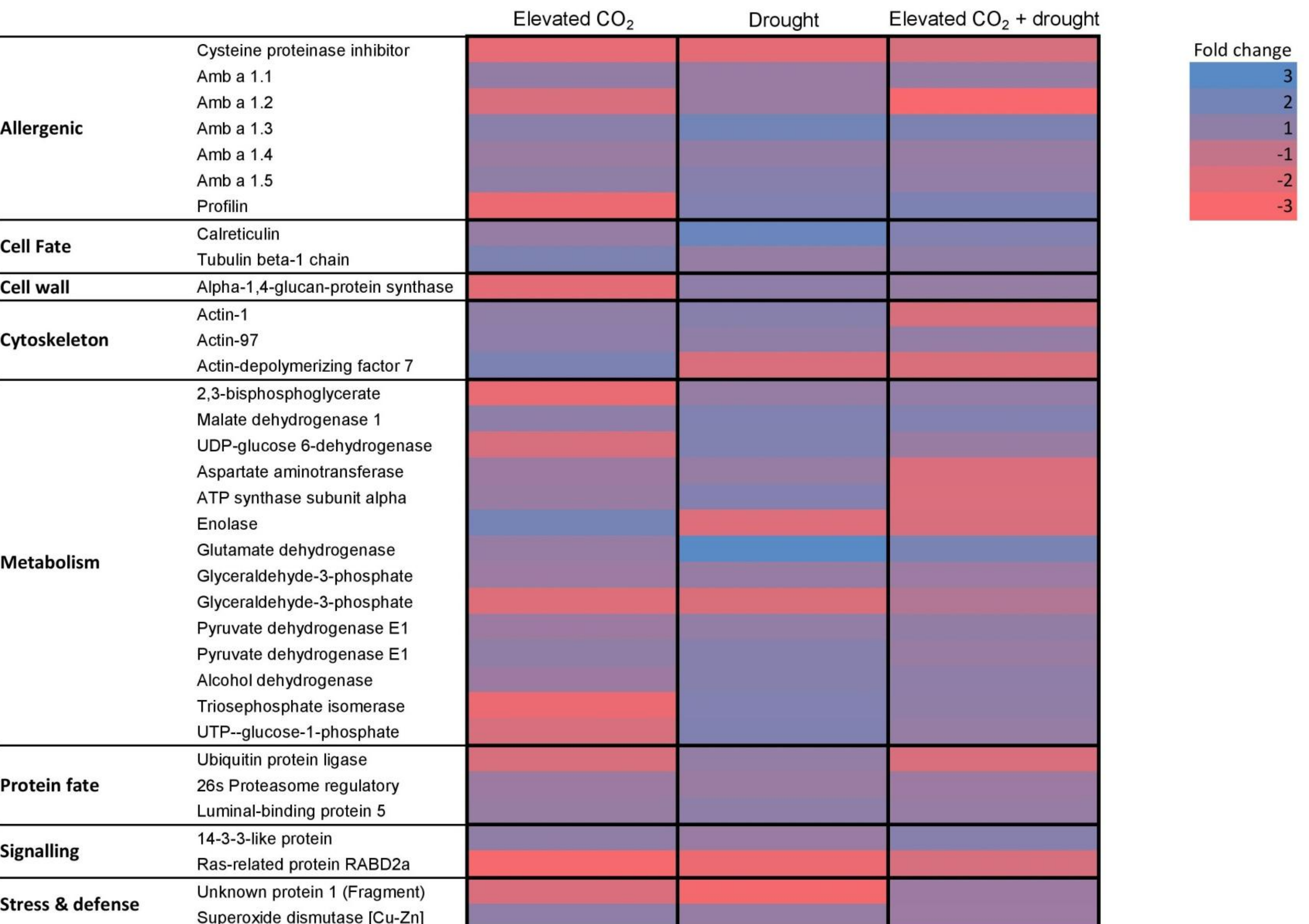


Figure 38: Heat map for the identified protein spots of first generation of ragweed pollen subjected to different environmental conditions. (elevated CO_2, drought treated and elevated CO_2 plus drought) This map showed the up-regulated and down-regulated spots in each treatment and the functional group to which it belongs.

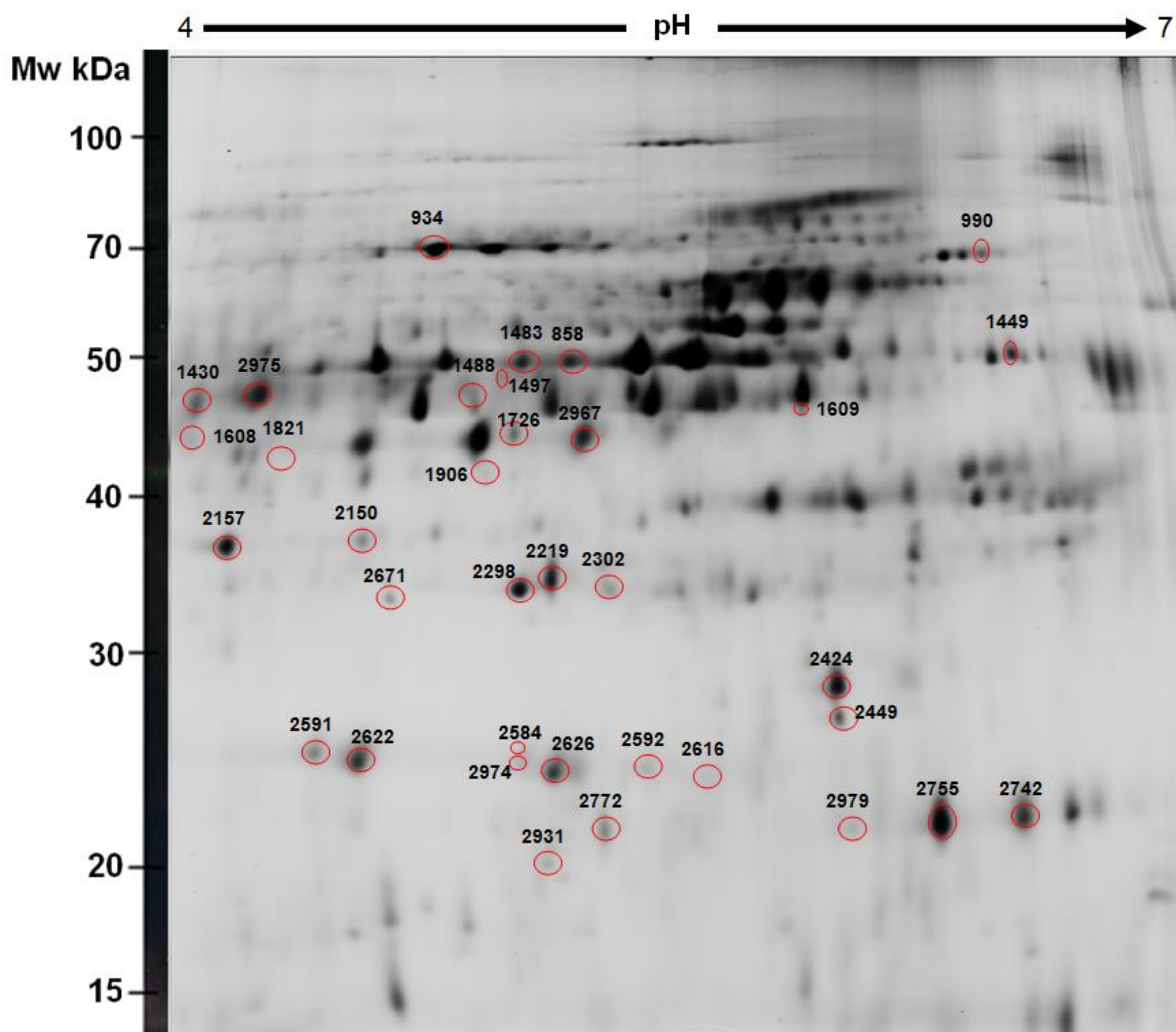


Figure 39: Master 2D-DIEG gel of second generation ragweed pollen extract. Protein were separated on 24 cm, pH 4-7 IPG strips (first dimension), and 13% SDS-PAGE (second dimension). Red circles represent identified protein spots which are numbered according to table 28.

Table 28: Summary of differentially regulated proteins of second generation ragweed pollen in elevated CO_2, drought and elevated CO_2+drought that appeared in Fig. 39, and separated by 2D-DIEG and identified by LC-MS/MS.

Spot number	Identified protein	Best matching gene products	Accession number	Observed		Spot ratio			p-value
				Molecular weight	PI	CO_2	Drought	CO_2 + drought	
1- Pollen allergen									
1726	6	Pollen allergen Amb a 1.1	MPA11_AMBAR	43	4.9	1.5	1.8	2.1	0.022
1430	10	Pollen allergen Amb a 1.2	MPA12_AMBAR	47	4.1	-1.0	1.0	1.4	0.140
1483	11	Pollen allergen Amb a 1.3	MPA13_AMBAR	49	5.1	1.4	1.2	1.4	0.280
858	14	Pollen allergen Amb a 1.5	MPAA2_AMBAR	48	5.2	1.4	1.1	1.2	0.050
2- Metabolism									
1488	9	Fructose-bisphosphate aldolase	ALFC_SPIOL	46	4.8	1.5	1.4	1.8	0.150
1609	12	Glyceraldehyde-3-phosphate dehydrogenase	G3PC2_HORVU	45	5.8	1.6	1.4	1.7	0.022
2302	7	Triosephosphate isomerase	TPIS_LACSA	33	5.4	-1.2	-1.2	-2.2	0.062
1821	7	Beta-amylase 1	BAM1_ARATH	42	4.3	1.6	1.8	2.6	0.002
2219	7	Cysteine-rich repeat secretory protein	CRR30_ARATH	34	5.1	1.3	1.6	1.5	0.120
2622	5	IAA-amino acid hydrolase ILR1-like 2	ILL2_ARATH	24	4.6	-1.3	-1.6	-1.5	0.190
2975	14	Malate dehydrogenase 1	MDHC_MAIZE	48	4.3	1.1	-1.1	1.7	0.001
990	6	NAD-dependent malic enzyme 65 kDa isoform	MAOM_AMAHP	68	6.4	1.2	1.1	1.3	0.470
2755	4	Phosphoenolpyruvate carboxylase 2	MDHC1_ARATH	22	6.4	1.2	2.8	-1.0	0.540
2584	6	Phytochrome B	PHYB_SOLTU	25	5.0	1.3	-1.0	1.2	0.620
2967	10	Protein SPA1-RELATED 4	SPA4_ARATH	42	5.3	1.1	1.0	-1.1	0.300
2298	7	Soluble inorganic pyrophosphatase	IPYR_SOLTU	33	5.0	1.1	-1.2	-1.1	0.250
3- Cytoskeleton									
2424	6	Actin	ACT_CHLRE	28	5.9	1.1	-1.1	-1.2	0.440
2979	5	Actin-depolymerizing factor	ADF_BRANA	20	6.0	-	-	-	-
2591	5	Pumilio homolog 5	PUM5_ARATH	25	4.5	1.3	1.1	1.1	0.007
4- Protein fate									
2671	5	Calcium-dependent protein kinase 1	CDPK1_ARATH	33	4.7	1.1	-1.0	-1.4	0.059
2931	5	Eukaryotic translation initiation factor 5A-1	IF5A1_SOLLC	21	5.1	-1.2	1.3	1.2	0.230
2974	5	Mitochondrial import inner membrane translocase	TIM9_MESCR	24	5.0	-1.0	1.1	1.8	0.025
934	14	T-complex protein 1 subunit alpha	TCPA_ARATH	70	4.7	1.0	1.2	1.5	0.006
2157	6	Ubiquitin protein ligase	Q65XV2	38	4.2	1.3	-1.1	1.1	0.220
5- Reproduction									
1608	8	Protein RIK	RIK_ARATH	45	4.1	2.0	1.0	1.5	0.014
2150	5	Lysine--tRNA ligase	SYK_SOLLC	38	4.6	1.0	1.5	1.4	0.290
2742	5	Histone H4	H41_WHEAT	22	6.5	1.0	-1.2	-1.1	0.830
2626	7	Probable histone H2A.2	H2A2_MEDTR	24	5.1	1.0	1.1	-1.1	0.830
2616	4	Retinoblastoma-related protein	RBR_CAMSI	23	5.6	1.3	1.1	1.1	0.026
6- Signaling									
1449	7	14-3-3-like protein	1433A_VICFA	49	6.5	1.4	-1.6	1.0	0.034
7- Stress									
2772	4	bZIP transcription factor 60	BZI60_ARATH	22	5.3	-1.2	-2.1	-2.4	0.016
2592	4	Regulatory-associated protein of TOR 1	RTOR1_ARATH	24	5.4	1.4	-1.1	1.4	0.025
1906	6	Unknown protein 1 (Fragment)	UP01_VITRO	41	4.8	1.4	-1.4	-3.2	0.001
1497	6	Unknown protein 12 (Fragment)	UP12_PSEMZ	48	4.9	1.1	-1.0	1.3	0.019
8- Cell fate									
2449	4	Tubulin alpha-3/alpha-5 chain-like protein	H9B8F5_MISSI	26	5.9	-1.1	-1.7	-1.3	0.570

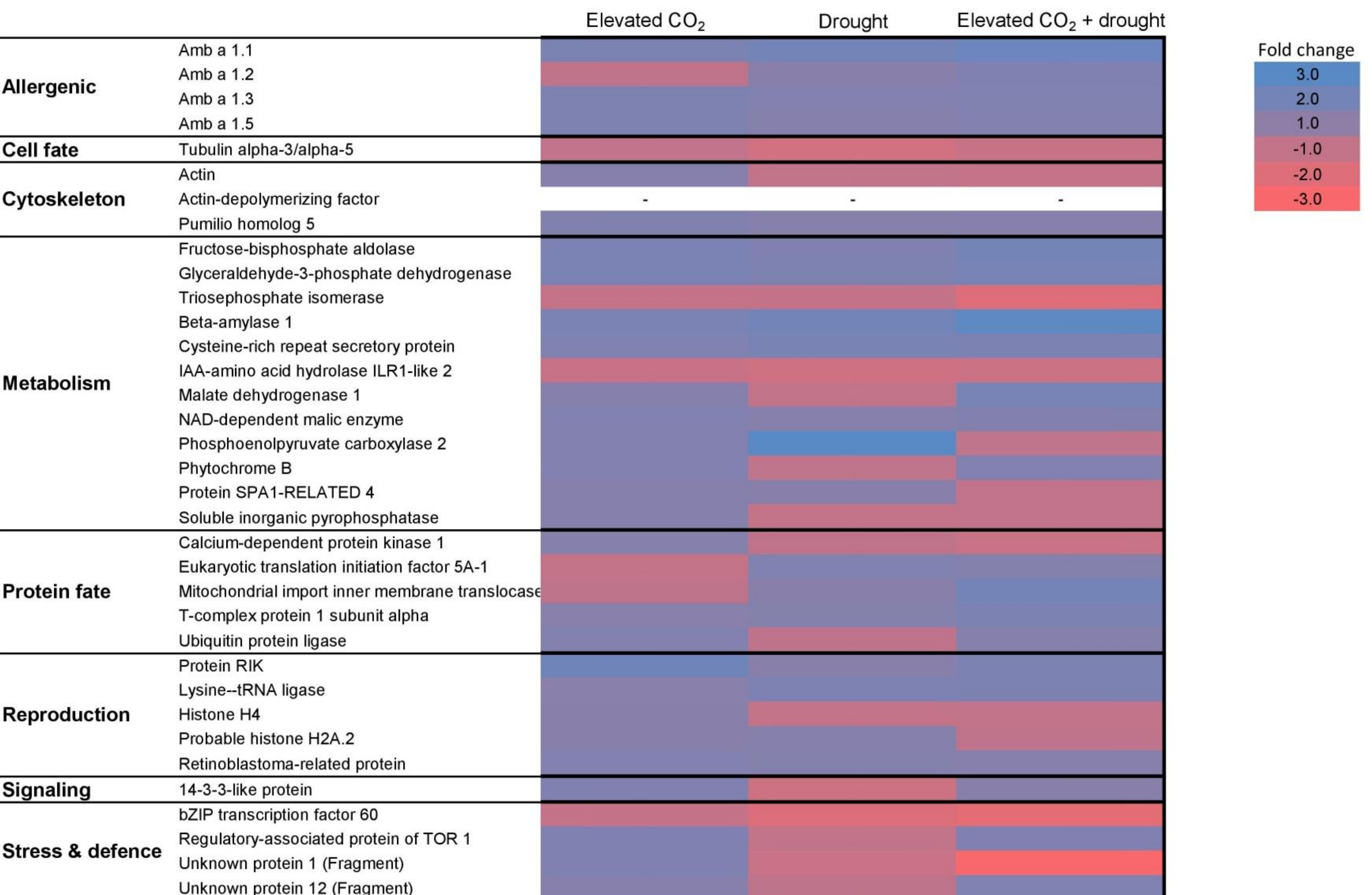


Figure 40: Heat map for the identified protein spots of second generation of ragweed pollen subjected to different environmental conditions. (elevated CO_2, drought treated and elevated CO_2 plus drought) This map showed the up-regulated and down-regulated spots in each treatment and the functional group to which it belongs.

4. Chapter – DISCUSSION

4.1 Morphological and enzymatic analysis

4.1.1 Effect of climate change on the morphological parameters of ragweed

Generally elevated CO_2 levels obviously promoted the growth of *Raphanus sativus* (Marshall et al, 2010); similarly, exposure to 700 ppm CO_2 increased the growth of ragweed roots and shoots. Increased plant growth under elevated CO_2 levels is well recognized, though plant growth is also influenced by other factors such as nitrate availability or water supply (Bloom et al, 2010; Long et al, 2004; Woodward, 2002). In contrast, and as predicted, drought reduced ragweed stem growth; however, elevated CO_2 could partially ameliorated the effect of drought by slightly increasing plant growth. This might be due to the fact that ragweed plants exposed to elevated CO_2 level may have improved water usage, as previously reported in crop species (Burkart et al, 2011). Moreover inflorescence length was reduced under drought conditions, in agreement with a previous report of reduced flower numbers and pod production in *Cicer arietinum* under drought stress conditions (Fang et al, 2010). The negative effect of drought on inflorescence length in ragweed was alleviated by elevated CO_2 and resulted in approximately the same inflorescence length as that of plants grown under normal CO_2 concentrations. Pollen production was enhanced by elevated CO_2, and reduced under drought conditions; similarly Wayne and his coworkers (2002) previously showed that elevated CO_2 stimulated pollen production in ragweed (Ziska & Caulfield, 2000). Moreover Ladeau and Clark (2006) performed a FACE experiment which demonstrated that the number of pollen grains was not significantly different in *Pinus taeda* plants grown in ambient and CO_2 fumigated plots in the year 2004; however, in 2005, the number of pollen grains produced by plants in fumigated plots increased. Generally, exposure to drought stress during the developmental phases altered the initiation and duration flowering. In most cases, the time difference in time between floral initiation (panicle initiation) to anthesis (panicle exsertion) was reduced by mild drought stress, but is increased by acute drought stress (Craufurd, 1993). Panicle initiation is normally retarded by drought and heat stress; however, these factors can postpone panicle development at any stages between panicle initiation and flowering.

Acute drought or heat stress block panicle exsertion and also can lead to defective fertilization by diminishing the pollen content or altering ovule function (O'Toole, 1984; Prasad et al, 2006). Koti and colleagues (2005) described the interactive effects of different parameters under realistic conditions, and interactive effects of CO_2, temperature and UV-B radiation on pollen morphology and pollen production in soybean. They found that the damaging effects of temperature and UV-B were not ameliorated by CO_2 conditions.

In second generation ragweed plants, elevated CO_2 and elevated CO_2 plus drought led to similar trends in increasing stem and inflorescence length as in the first generation plants; however, drought lead to an increase in stem and inflorescence growth.

Stress memory storage is well recognized as an acclimation response in plants (Chinnusamy & Zhu, 2009); (Iba, 2002; Thomashow, 1999). Stress memory storage can be extended over a long period of time if it induces reprogramming in the morphology and phenology of the plants. Chinnusamy and Zhu (2009) proved that reprogramming of cell differentiation in response to drought stress leads to phenological and developmental plasticity, which are notable mechanisms of stress resistance .

Furthermore, the improved in their performance and enhanced of their relative fitness of plants in response to drought may occur due to selection and may eventually result in populations or species with suites of traits that can withstand drought as an unfavourable condition (Chaves et al, 2003; Juenger et al, 2005). Such traits, including acclimation responses, can provide tolerance to tissue desiccation allowing the leaves to function for longer during drought periods or enhance the ability of the plants to avoid water loss, allowing the leaves to preserve a high water potential during drought (Kramer & Boyer, 1995).

4.1.2 100 seed weight

In the future, elevated atmospheric CO_2 level and drought may affect seed production and have important consequences on plants species (Ladeau & Clark, 2006). Compared to control plants, the 100 seed mass of ragweed seeds significantly increased under elevated CO_2, drought and elevated CO_2 with drought conditions.

Although many species produce more seeds under high CO_2 conditions (Jablonski et al, 2002), increased seed numbers under elevated CO_2 levels often correlated with a lower seed quality, as indicated by the seed nitrogen concentration, seed weight and/or seed viability (Jablonski et al, 2002; Thürig et al, 2003). Normally, developing seeds act as a large carbon sink in plants; therefore, elevated CO_2 level can lead to heavier seeds (Darbah et al, 2008; Hussain et al, 2001; Thürig et al, 2003). In some plants including legumes, increased carbon allocation to seeds can be matched by complementary nutrient allocation, resulting in no net change in the seed nutrient content (Miyagi et al, 2007). However, a reduction in seed quality will occur if nutrient allocation cannot keep pace with the higher carbon allocation (Thürig et al, 2003).

The results of several studies have revealed that drought can influence sexual reproduction and/or affect seed filling in a positive manner, negative manner, or have no effect. Drought significantly increased the 100 seed weight in ragweed. Yang and Zhang (2006) provided evidence that the reduction in the seed-filling duration during drought conditions can often be compensated for by an increased seed-filling rate, especially when there is access to carbohydrates are available, either directly from leaf photosynthesis or from carbohydrates pre-stored in the stems or leaves. Several processes are probably involved in this process, including storage, phloem loading, carbohydrate hydrolysis, and phloem unloading into the seed (Prasad et al, 2008). Nevertheless, altering the hormonal balance in the seeds during seed filling, especially reduced gibberellic acid levels and increased abscisic acid (ABA) level, enhances the remobilization of pre-stored carbohydrates to seed (Yang et al, 2001).

4.1.3 Test of pollen viability

The viability of pollen produced by ragweed plants exposed to the drought ,elevated CO_2 and elevated CO_2 with drought conditions were significantly lower than that of control plants. Control ragweed pollen, had a rather a low viability compared to fresh pollen (90-98% viability); reflecting the fact that the pollen was stored at -80°C for more than 4 months before the viability assays were carried out. Many studies have shown that a significant loss of viability and loss of membrane integrity occurs during the storage of pollen (Jain, 1989; Pasqualini et al, 2011). Reduced pollen viability is a common symptom in angiosperms under the stress environments (Porch & Jahn, 2001).

For example, drought stress during reproductive development resulted in reduced pollen viability and pollen germination in the common bean (*Phaseolus vulgaris*) (Porch & Jahn, 2001). Numerous hypotheses have been suggested to explain the mechanisms responsible for reduced pollen viability under drought stress, including developmental abnormalities in the anthers leading to premature dislocation of the microspores (Saini, 1984), tapetal cells dysfunction due to abnormal vacuolization (Lalonde, 1997); premature degeneration of tapetal cells and a lack of endothecial development (Ahmed et al, 1992); and altered carbohydrate accumulation and metabolism (Jain, 2007; Saini, 1997).

4.1.4 Scanning electron microscopy (SEM)

Pollen development inside the anther is a highly complex and depends on several processes which occur in parallel to enable pollen development and prepare the anther for pollen release. The wall of pollen grains is totally different from the walls of other plant cells (Heslop-Harrison, 1968; Heslop-Harrison & Heslop-Harrison, 1985). The pollen wall is multilayered and is highly resistant to degradation, making the pollen cell wall an important determinant of pollen viability (Heslop-Harrison & Heslop-Harrison, 1985). In several species, on the pollen wall surface is coated with a layer termed the pollen coat (Taylor & Hepler, 1997). The functions of the pollen coat include providing a sticky surface to facilitate pollen transfer by insects and protect against stressful conditions (UV-radiation,drought, heat) (Heslop-Harrison & Heslop-Harrison, 1985). Singh et al. (2010) reported that elevated CO_2 level did not reduce the damaging effects of abiotic stresses on reproductive traits, particularly viable pollen production and seed yield. While Shen and Webster (1986) demostrated that *Phaseolus vulgaris* produced abnormal exines with deeply pitted and smooth regions under drought stress. The exines arise from the tapetum and the development of normal pollen depends on the relative interaction with the tapetal tissue that composes the innermost layer of the anther (Dickinson & Potter, 1976). Therefore, premature degeneration of the tapetal layer could lead to the observed pollen abnormalities (Ahmed et al, 1992). Scanning electctron microscopy did not reveal any significant difference between ragweed pollen produced by plants under elevated CO_2, drought and elevated CO_2 plus drought conditions compared to control pollen.

4.1.5 Analysis of phenolic metabolites by reverse-phase HPLC (RP-HPLC)

Secondary metabolites within the chemical compounds present in ragweed pollen solutes, particularly phenolic compounds (e.g. phenols and flavonoids), are of a great importance in plant-environment relationships (Rhodes, 1994). Flavonoids play key roles in plant growth and development, including male fertility, pollen germination, tube growth, pollen development, protection against ultraviolet radiation and the cell cycle regulation (Woo et al, 2005).

Analysis of ragweed pollen by reverse-phase HPLC identified that 17 buffer-soluble compounds could be separated, with quercetin and kaempferol derivatives present at the highest levels. Significant differences were observed between the control, elevated CO_2, drought and elevated CO_2 + drought samples. Mo Y *et al.* (1992) confirmed a role for flavonols in functional pollen and stated that the ability to undergo pollen germination and restore full tube growth in vitro were flavonol aglycones-dependant. Furthermore, Mo Y *et al.* (1992) screened several phenolic compounds and demostrated that the amount of kaempferol in wild-type pollen was adequate to support germination and tube growth in the first few hours after pollination. Changes in the level of secondary plant products, such as allergens, were expected in pollen under elevated CO_2 level (Levetin & Van de Water, 2008). Poison ivy (*Toxicodendron radicans*) grown under elevated atmospheric CO_2 contains altered levels of secondary plant metabolites (Levetin & Van de Water, 2008); plants grown at twice the ambient CO_2 levels exhibited higher production of the allergenic form of urushiol, the cause of contact dermatitis (Mohan et al, 2006).

The production of various secondary metabolites is triggered by abiotic stresses (Wahid & Ghazanfar, 2006). For example, drought stress increased the concentration of plant phenolics in cotton (Hura et al, 2006; Yildiz-Aktas et al, 2009). Several lines of evidence indicate that the production of secondary metabolites increases in water-deprived plants, as carbohydrates are redirected to the synthesis of these products (Kujawski, 2011).

4.1.6 Reactive oxygen species and their allergenic potential

Reactive oxygen species (ROS) is a collective term used to describe oxygen radicals (e.g., superoxide, hydroxyle, hydroperoxyle) and specific non-radicals (e.g., H_2O_2, singlet oxygen) which act as oxidising agents and/or are readily converted into radicals (Speranza & Scoccianti, 2012). ROS are constantly produced in plants as a result of the partial O_2 reduction during normal metabolic processes, such as respiration, photosynthesis and photorespiration (Foyer & Noctor, 2009; Noctor et al, 2007).

Plants contain a sophisticated battery of ROS scavenging enzymes (e.g.,superoxide dismutase, ascorbate peroxidase, glutathione peroxidase and catalase), and low molecular weight non-enzymatic antioxidants (e.g., ascorbate and glutathione, and also tocopherol, flavonoids, alkaloids and carotenoids), that play a major role in the control of cellular levels (Alscher et al, 1997; Apel & Hirt, 2004). Accumulation of excess ROS is harmful to many plant cell components, including lipids, proteins and nucleic acids, and may induce plant cell death (Levine et al, 1994; Mittler & Rizhsky, 2000). Until recently, ROS were considered to be undesirable by-products of metabolism. However, further information is emerging on the range of ROS, and has indicated that ROS play an important role in pollen development and as growth regulators via processes closely associated with hormone signaling pathways (Kwak et al, 2006). When the pollen make contact with the airway epithelial mucosa, a number of allergenic proteins, including lipid immunomodulators (Traidl-Hoffmann et al, 2002), and nicotinamide adenine dinucleotide phosphate (NAD[P]H) oxidases are released in the mucosa (Boldogh et al, 2005). In turn, NAD[P]H oxidases are a major source of ROS and lead to an increase in the levels of ROS in the epithelium of the respiratory apparatus, which promotes the recruitment of neutrophils into the respiratory apparatus (Boldogh et al, 2005).

In order to investigate the effect of climate change on the regulation of ROS levels in ragweed pollen, NADPH oxidase activity, H_2O_2, total oxidant status (TOS), total antioxidant status (TAS) and oxidative stress index (OSI) were essayed to evaluate the allergenicity of the pollen in the lung rather from the allergenic protein,

The pollen produced by plants subjected to drought conditions demonstrated a higher capability for ROS production compared to the control (high H_2O_2 ,low TAS), as did the pollen produced by plants subjected to elevated CO_2 and elevated CO_2 with drought (higher NADPH and H_2O_2).

Oxidative stress is induced by drought stress in plant cells as a result of enhanced generation of ROS such as O_2^- and hydrogen peroxide (H_2O_2) within the cell (Navari-Izzo et al, 1994; Tambussi et al, 2000). Antioxidant scavengers such as glutathione and ascorbate; antioxidant enzymes including superoxide dismutase; H_2O_2-scavenging enzymes like ascorbate peroxidase, catalase and non-specific peroxidases can neutralize such toxic reactive species (Tambussi et al, 2000).

In the plant reproductive system, abiotic stresses such as drought and high or low temperatures can lead to reduced grain yield, especially when the stress occurs during the reproductive stage of plant development (Saini et al, 1984). For example drought induces spikelet sterility in both rice and wheat (O'Toole & Moya, 1981; Saini et al, 1984). Nguyen and his co-workers (2009) demonstrated that drought stress induced oxidative stress in rice anthers, which in turn increased the levels of hydrogen peroxide in the stressed anthers at all stages of plant development. Accumulation of ROS in mucosa cells exerts two actions, firstly causing damage to a variety of biological molecules (cell membranes, lipoproteins) which may lead to programmed cell death (Moller et al, 2007; Ryter et al, 2007), and secondly, enhancing the recruitment of inflammatory cells towards the respiratory apparatus.

According to Dharajiya et al., (2008) suggested that the production of ragweed pollen is likely to increase under predicted future climatic conditions (Rogers et al, 2006). These findings also suggest that global warming (high CO_2 levels and high temperatures) may increase the total environmental pollen NAD(P)H oxidase which in turn may contribute to an increased prevalence of allergic disorders. Polymorphonuclear leukocytes (white blood cells) contribute to the inflammatory response, through the production of ROS by nicotinamide adenine dinucleotide phosphate (reduced) (NAD(P)H) oxidase (Boldogh et al, 2005). Bacsi (2005) suggested that grass pollen already contain sufficient endogenous NAD(P)H oxidase activity to trigger the induction of granulocytes, and initiate of oxidative stress, even in the absence of the adaptive immune response. It was also confirmed that the ROS generated by pollen NAD(P)H oxidases provide a signal that enhances allergic inflammation of the airway (Bacsi et al, 2005).

4.2 Genomic and transcriptomic analysis

4.2.1 SuperSAGE libraries and quantitative real-time RT–PCR of ragweed pollen grains

4.2.1.1 SuperSAGE advantages and disadvantages

SAGE is a highly advanced method of gene expression analysis, which can be used to compare different samples; for example, various developmental time points, or treated and untreated samples (White et al, 2006). An entire SAGE library must be created for each sample of interest. Developments in the SAGE protocol, including the analysis of longer tag concatemers (Crawford et al, 2005), and the increased throughput and efficiency of modern sequencing platforms, have reduced both the cost of sequencing and the time taken to generate large amounts of tag sequence data. However, the ability of SAGE to detect low abundance expressed transcripts remains limited. The sage method also has the advantage of reducing the amplification bias caused by high copy number transcripts which occurs in occurs in other methods such as microarray (White et al, 2006).

SAGE technology may result in the under- or overestimation of gene expression levels due to the generation of antisense tags, or the existence of numerous members in a gene family or alternative splice variants (White et al, 2006). SuperSAGE tags are 26bp long; which, theoretically, is long enough to differentiate in between different transcripts. However very similar or exact sequences are likely to arise particularly from members of the same gene family. Therefore, if more than one gene family member shares the same tag sequence, their counts would be combined resulting in an overestimate of the frequency of that gene transcript (Wahl et al, 2005).

SAGE results can also be underestimated when a transcript is represented by multiple tags. Alternative splicing, incomplete digestion by the anchoring enzyme, alternative polyadenylation and antisense tags have been reported as sources of multiple tags; these events are a common problem in SAGE studies(Gibbings et al, 2003; Ibrahim et al, 2005; Pauws et al, 2001).

Despite its downfalls, SAGE is still a useful tool for gene expression analysis as it has the ability to capture expression data for all transcripts in a particular tissue, at a give time point or under specific conditions.

4.2.1.2 Comparative analysis of the elevated CO_2, drought and elevated CO_2 plus drought samples provides insight into the genetic network of ragweed pollen.

The total number of sequenced tags varied in the different libraries varied: 182,736 for elevated CO_2 pollen, followed by 139,987 for elevated CO_2 plus drought pollen and finally 106,410 and 69,150 for drought and control pollen, respectively. The pollen of ragweed subjected to elevated CO_2 showed the highest number of up-regulated transcripts (86%), followed by the elevated CO_2 plus drought pollen (84%) and the drought pollen (70%). Elevated CO_2 has been proven to increase the reproductive potential (flowering and fruit and seed production) of plants (Bunce, 2005; Edwards et al, 2001; Johnson & Lincoln, 2000). Furthermore, early maturation of the reproductive parts has also been reported under elevated CO_2 conditions, which is likely to be due to disproportionate allocation of carbon to reproduction (Darbah et al, 2008; Ward & Strain, 1999). Taylor et al., (2005) found strong evidence to indicate that gene expression is highly influenced by the carbon status in plant, as plants display some plasticity in gene expression in response to altered CO_2.

Any shift in the plant carbohydrate status can differentially regulate photosynthetic pathways (Moore et al, 1999), biosynthetic activity (Yu, 1999), growth and organogenesis (Pien et al, 2001), and contribute as a co-regulator of the responses to biotic and abiotic stresses (Roitsch, 1999). Changes in gene expression are one response to higher atmospheric CO_2 level. Due of the large number of genes involved in the response to CO_2, several techniques used to monitor global gene expression have revealed a significant increase in gene expression in Arabidopsis (Li et al, 2006), maize (Kim et al, 2006), poplar (Druart et al, 2006) and soybean in response to altered CO_2 level (Ainsworth et al, 2006).

Plant growth is generally affected by different environmental abiotic stresses, such as water stress, high salinity, and low temperature (Seki et al, 2001). Among these stresses, drought or water deficit is the most sever limiting factor to plant growth and reproduction (Ingram & Bartels, 1996). Drought stress triggers a number of different physiological and biochemical responses in plants. Recently, a number of genes that respond to drought stress at the transcriptional level have been described (Bray, 1997; Shinozaki & Yamaguchi-Shinozaki, 2000; Shinozaki & Yamaguchi-Shinozaki, 2007).

The finding of enhanced gene expression levels in the pollen subjected to drought in this study are consistent with Wang et al., (2008), who reported that the number of expressed genes, either as the total number of expressed genes or specifically expressed genes, increased significantly from desiccated mature pollen to hydrated pollen in *Arabidopsis*. Moreover, increased gene expression was observed in both the pollen and ovary of maize under drought stress (Kakumanu et al, 2012).

4.2.1.3 Numerous unassigned SuperSAGE tags

The finding of a large proportion (more than 80%) of experimental tags which could not be assigned to any gene needs an extensive and detailed explanation. Unassigned genes are routinely observed in SAGE studies, even when using complete genome sequence data for gene identification (Pleasance et al, 2003). There are three hypotheses to explain this unusual observation: first, the unassigned tags may be artifactual; second, they may be true SAGE tags, but emerged from transcripts with an wrong/absent virtual tag in the reference database; third, the unassigned tags may a result of dealing with non-model plant such as ragweed.

Artifactual tags can be generated during several steps of the SAGE procedure. One of the main sources of artifactual tags is the production of internal tags upstream from the correct SAGE tag. This can be due to incomplete digestion (the anchoring enzyme) or mispriming by the oligo(dT) during internal extension of the mRNA poly(A) during the synthesis of cDNA (Jones et al, 2001; Welle et al, 1999). Nevertheless, as in other studies (Piquemal et al, 2002), artifactual tags comprised a very low percentages (2%) of the correct tags. Therefore, generation of artifactual tags by the aforementioned process is not sufficient to explain the high percentage of unassigned tags in this study. Artifactual tags can also be generated by PCR errors and during the sequencing steps of the SAGE process. However, sequencing errors do not occur frequently enough to explain such a large proportion of unmatched tags (Wang, 2003), and elimination of the tags which were only found once from our analysis allowed us to dismiss the large majority of such these erroneous tags (Lash et al, 2000; Piquemal et al, 2002).

The fact that a number of unassigned tags were found more than 20 times clearly indicates that PCR or sequencing errors were not responsible for the unassigned tags rather that the genes corresponding to the unassigned tags could not be identified (Fig. 41). Taken together, these considerations support the hypothesis that most of the unassigned SAGE tags are not artifacts (Chen et al, 2002b).

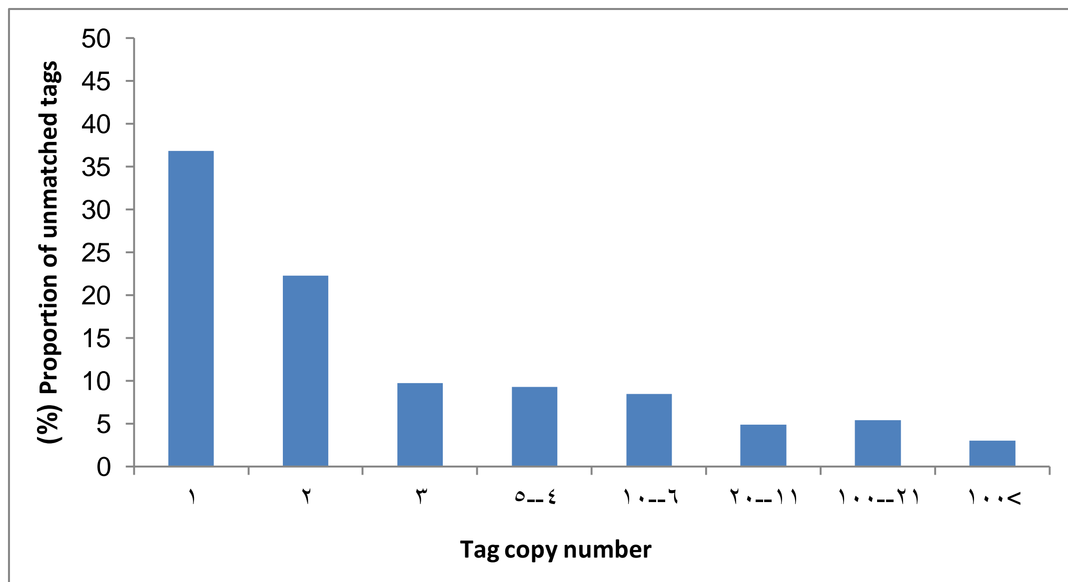


Figure 41: Proportion of unmatched tags as a function of the tag copy number in the four SAGE libraries.

The second explanation that the unassigned tags are correct tags generated from transcripts with an incorrect/absent virtual tag in the reference database (Fizames et al, 2004). This is much more likely to be true for several reasons. Firstly, the virtual tag database may not be complete, in particular because it relies on the assumption that the 5′- or 3′-UTRs of the mRNAs are not longer than 400 bp (Fizames et al, 2004). Additionally, polymorphisms could also partly explain the lack of tag assignment to genes, especially if the SNPs are located in the areas corresponding to the unassigned SAGE tags (Haas et al, 2002). Moreover, a number of the transcripts may undergo alternative splicing (Zhu et al, 2003), gene disruption, or be transcribed in the antisense orientation, preventing them from being assigned (Jones et al, 2001).

Finally, when investigating the transcriptome in a species lacking complete genomic data such as ragweed, we face a series of unanswered questions (Hornett & Wheat, 2012). Additionally, concerns exist over the quality of *de novo* transcriptomes and their usefulness when used as scaffolds for mapping RNA-Seq reads for a genome with incomplete sequence data, compared to a genome for which high quality sequence data is available

An additional problem faced by the analysis of non-model species is the reduced ability to extract functional information from the *de novo* assembly and expression data (Hornett & Wheat, 2012).

Our data suggests that, if they correspond to actual transcripts, the unmatched tags mainly arose from genes expressed at low levels (Fig. 41). This may explain why the genes corresponding to the unassigned tags could not been correctly identified, as the structure of genes with a low expression level is often not supported by any cDNA or EST data, and is mainly derived from computer predictions which are far from fully reliable (Haas et al, 2002; Zhu et al, 2003).

These suggestions strongly support the idea that SAGE, particularly in the absence of previous knowledge concerning the tested genes, is a valuable tool for characterizing all of the actual transcripts present in a sample, and therefore enhances genome annotation when these transcripts do not match any predicted gene (Boheler & Stern, 2003; Chen et al, 2002b; Saha et al, 2002).

4.2.1.4 Top up regulated tags

Several transcripts among the top 40 up regulated annotatable tags within the elevated CO_2, drought and elevated CO_2 plus drought libraries share some common traits (Tab.14, 15, 16). These transcripts include FatB thioesterase, pectinesterases inhibitor, and cysteine proteinase aleuran, and their function and role in allergenicity will briefly discussed.

4.2.1.4.1 FatB thioestrase gene

Generally, thioesterases play an essential role in the partitioning of de novo–synthesized fatty acids between the prokaryotic and eukaryotic pathways; unsaturated oleate (18:1) and saturated palmitate (16:0) are the major products of plastid fatty acid synthesis (Bonaventure et al, 2003). Furthermore, it specificity determines saturation of fatty acids and the chain length imported from the plastid (Pollard et al, 1991). There are two classes of acyl-acyl carrier protein (acyl-ACP) thioesterases have been described in plants (Voelker et al, 1997). First FATA class has highest in vitro activity for unsaturated acyl-ACP and lower activity for saturated acyl-ACP substrates.While second class of thioesterases, FATB, prefer saturated acyl groups but also have activity for unsaturated acyl-ACPs (Salas & Ohlrogge, 2002; Voelker et al, 1997).

Early studies of FATB over-expression in *Arabidopsis* showed that this enzyme is involved in the in vivo production of saturates in flowers and seeds (Dormann et al, 2000). Similarly, down-regulation of *FATB* expression in soybean also resulted in partial reduction of seed palmitic acid (Buhr et al, 2002; Wilson et al, 2001). Bonaventure et al., (2003) stated that one of the vital roles for the thioesterases is to provide saturated/unsaturated mixture balance of membrane fatty acids. Such a mixture is found to be essential in stabilizing the membrane physical properties (e.g., fluidity) and to adapt the membrane in facing the environmental changes (e.g., drought or temperature). Beside its role as main components of membranes, some play more specialized roles as precursors for signal molecules (e.g., linolenic acid for jasmonate), or precursors for sphingolipids, surface waxes, and cutin and in protein acylation (Wallis & Browse, 2002).

Graham (1988) found that in *Cuphea*, the trend from longer, unsaturated fatty acid (FA) to short-chain saturated FA is generally correlated with increasing size and zygomorphy of flowers, increased seed size, and other characters associated with phylogenetic advancement

Earlier it was known that the pollen coat contains several lipids that are required for pollen hydration, germination, and penetration of the stigma by pollen tubes (Dickinson, 1993; Piffanelli et al, 1997; Wolters-Arts et al, 1998). Very-long chain lipids contribute a fundamental component of the extracellular pollen coat in the Brassicaceae (Fiebig et al, 2000; Preuss et al, 1993) and loss of any of such pollen-coat lipids can disrupt cell

signalling with the stigma during fertilization, inhibiting pollen hydration and causing sterility (Hulskamp et al, 1995).

Bashir et al., (2013) largely overlooked on the extracellular pollen matrix, of a lipidic nature and are potentially immunogenic. They found that when lipid being in close vicinity to allergenic proteins on the pollen coat, when interacting with host cells, lipids might modify the antigenic properties of proteins. Furthermore it also raised the possibility of a “two-hit” signal composed of pollen proteins (allergens) and pollen lipophilic components to trigger an allergic response and enhance the effect of each other. So, the pollen lipid became a rich source of immune-stimulatory molecules that may play an essential role in the immune-regulation and controlling the allergic response (Bashir et al, 2013).

The effect of the elevated CO_2 on lipid production is confirmed, in a biodiesel production experiments that were using different techniques to stimulate lipid biosynthesis in microalgae. It is clear that different microalgae species react to different stresses by producing different fatty acids or by altering their composition of fatty acids (Sharma et al, 2012). Lv et al., (2010) found that when adjusting the culture conditions with low nitrogen (1 mM KNO_3), and higher CO_2 (1.0%), the lipid production of *Chlorella vulgaris* was increased by 2.5-fold.

Drought is one of the major challenges that facing the plant in the twenty-first century. In response to this drought, some genes are up-regulated, which can mitigate the detrimental effect of stress, lead to the adjustment of the cellular medium and thus contribute to plant tolerance (Mahajan & Tuteja, 2005). Adaption to stress, achieved by displaying specific traits such as improved water use efficiency and/or osmotic adjustment (Bargmann et al, 2009; Yang et al, 2010). Furthermore, plants drought tolerance depends on complex signalling network pathways (Mahajan & Tuteja, 2005). The lipid signalling (phospholipid signalling) is one of the main pathways with its double functions, as structural role and a signal-transducing property. The metabolism of membrane lipids is highly affected by drought stress through changing in the membrane lipid composition (sphingolipids, galactolipids and phosphoglycerolipids) (Gigon et al, 2004; Repellin et al, 1997; Torres-Franklin et al, 2007). These lipid signalling allows the plant to overcome the stress conditions (Wang, 2004; Welti et al, 2002).

Drought-tolerant plants tend to increasing levels of unsaturated polar lipids implying higher membrane fluidity and physiological efficiency (Guerfel et al; Torres-Franklin et al, 2007). In many plant species, drought has been reported to stimulate digalactosyldiacylglycerol (DGDG) biosynthesis and accumulation in extrachloroplastic membranes that contribute to drought tolerance in plants (De Paula et al, 1990; Gigon et al, 2004; Larsson et al, 2006; Torres-Franklin et al, 2007). This DGDG is initially biosynthesised from FAT thioestrase genes (Arunga & Morrison; Browse et al, 1986).

4.2.1.4.2 Pectinesterase inhibitor

Pectinesterases (PEs), also known as pectin methyl esterases (PMEs), are ubiquitous cell wall-associated enzymes that involved in pectin de-methylesterification and subsequent breakdown (Liu & Bennett, 2011). They are regulated by post-translational control through association with proteinaceous inhibitors, PEIs or PMEIs (Wolf et al, 2003)

Pectinesterase inhibitor been found in higher plants and are also produced by certain plant pathogenic fungi and bacteria (Jiang, 2001). Pectinesterase inhibitor participates in the conversion of protopectin to soluble pectin and pectate, and they are important in the fruit maturation process(Jiang, 2001). Pectinesterase inhibitor mainly involved in cell wall metabolism (Day et al, 2013).

Investigation the molecular mechanisms in Aspen trees growing under elevated CO_2, showed that the genes involved in hemicellulose and pectin biosynthesis were enhanced, but some genes that catalyze important steps in lignin biosynthesis pathway were inhibited (Wei et al, 2013). The expected explanation that, these increases can contributes to cell division and expansion by supporting the formation of new wall material (Wei et al, 2013).

The Pectinesterase inhibitor transcripts are up-regulated in the drought pollen, this results follow Verelst et al (2012) when they found that the expression of various genes related to cell wall formation and expansion was elevated by drought, such as a pectinesterase inhibitor (Bradi3g45080), a 1,3-β-glucan synthase (Bradi1g76620), and a xyloglucan galactosyltransferase (Bradi1g75450) gene.

The Arabidopsis cDNA microarray of the expression profiles of genes under drought, cold and high-salinity stress conditions found that, pectinesterase inhibitor transcripts increased more than fivefold after drought treatments, and was also identified as drought stress-inducible gene (Seki et al, 2002).

Liu and Bennett (2011) found that pectinesterase inhibitor domain containing protein whose expression was up-regulated only in drought-stressed IR64 anthers after re-watering. The high PEI activity in anthers/pollens indicated that the de-methylestrification process in IR64 was inhibited by drought and/or re-watering.

Higher pectin methylesterification activity and lower de-methylesterification activity in anthers may be one of the reasons for the reduction in pollen releasing, germination, and pollen tube growth in drought-stressed IR64 (Liu et al, 2006).

4.2.1.4.3 Cysteine proteinase aleuran

Cysteine proteinases playing an essential role in the proteolytic pathways in various cellular compartments and it is responsible for up to 30% of total proteolytic activity in mature non-senescing organs (Grudkowska & Zagdanska, 2004). In addition, they are involved in signalling pathways and in the biotic and abiotic response (Grudkowska & Zagdanska, 2004).

Grobe and his co-workers (2002) proved that expansins are proteinases that arose from the wall-digesting cysteine proteinase family of *Giardia*. Expansins are extracellular proteins that promote plant cell wall enlargement, evidently by disrupting non-covalent bonding between cellulose microfibrils and matrix polymers (McQueen-Mason & Cosgrove, 1994; McQueen-Mason et al, 1992). Group I pollen allergens are actually structurally and functionally linked to expansins and that they and their vegetative homologs includes a second family of expansins (Grobe, 2002). Furthermore, group I allergens facilitate penetration of the pollen tube into the maternal tissues by breakthrough the cell walls of the stigma and style (Grobe, 2002).

Their expression is usually limited to specific organs or to particular phases during plant growth: germination (Botella et al, 1996), drought (Waldron et al, 1993) or cold and salt stresses (Grudkowska & Zagdanska, 2004; Pernas et al, 2000; Van der Vyver et al, 2003).

Zavala et al (2009) found in soybeans that the cysteine proteinase accumulated under elevated CO_2. This may be resulted from the down-regulation in the expression of genes related to defense signalling (jasmonic acid and ethylene) (Casteel, 2008; Zavala et al, 2008).

Nevertheless the expression of cysteine proteinase inhibitors is regulated directly by jasmonic acid (Bolter & Jongsma, 1995). In turn, reduced the expression of the two inducible cysteine proteinase inhibitors genes (N2 and R1) and reduces the corresponding enzyme activity (Zavala et al, 2008; Zhao et al, 1996).

This are being confirmed when gut cysteine proteinase activity was higher in beetles consuming foliage of soybeans grown under elevated CO_2 than in beetles consuming soybeans grown in ambient CO_2.

The clones rd19 and rd21 which encoding different cysteine proteinases in *Arabidopsis* were induced by water deficit (Koizumi et al, 1993; Yamaguchi-Shinozaki et al, 1992). Moreover cysteine proteinases were induced in wheat leaves both non-acclimated and acclimated to drought and their contribution in the total proteolytic activity significantly increased (Zagdanska & Wisniewski, 1996). Subsequence's experiments to cultivars of different drought resistance showed that, once the cysteine proteinases induction takes place, their level of induction is negatively related to the drought resistance and positively correlated with extravacuolar ATP-dependent proteolysis (Grudkowska & Zagdanska, 2004; Wiśniewski & Zagdańska, 2001).

4.2.1.5 Pollen grain and stress responsive genes

Plants are frequently exposed to a various external conditions that can affect their growth, development and productivity. Their ability to adapt and survive in a changing environment depends on their tolerance or resistance to such adverse growing conditions. Plants have evolved specific acclimation and adaptation mechanisms to face and survive these stresses (Thippeswamy et al, 2013).

The functional and biochemical features of ragweed pollen may be determined by their particular gene expression profiles (Lee & Lee, 2003). The pollen transcriptome can be modified by both internal and external factors.

Such patterns of global gene expression can be represented by transcriptome analysis, which reveals the identity and the level of expression of each expressed gene (Velculescu et al, 1997), and provides useful information on basic cell biology and a global vision of the biological responses to environmental stimuli (Lee & Lee, 2003).

The pollen grain represents the male gametophyte of flowering plants, and includes all of the genetic information required to combine with the female gamete at fertilization and form a new sporophyte (Lee & Lee, 2003). Recently, investigation of the processes underlying pollen development and function has been expanded by the studying of pollen-specific gene expression.

In flowering plants, male gametogenesis includes a series of complicated events beginning with development of archesporal cells and leading to the formation of mature pollen (Goldberg et al, 1993). The differentiation and development of pollen in angiosperms depends on expression of the haploid genome after meiosis (Lee & Lee, 2003). The function of the protein products encoded by mature pollen mRNAs appear to extend to the late maturation stages of pollen tube growth and germination (Mascarenhas, 1975).

During investigation of the ragweed pollen libraries for up- and down-regulated genes that appeared under library search of term pollen grain, it was found that most of the transcripts in the different libraries were putative *Ambrosia trifida* genes linked to variable aspects of ribokinase and/or transferase proteins. No distinctive traits could be recognized between treatments and control.

At a molecular level, the activation of numerous stress responsive genes is a critical event during the response to stress (Cushman & Bohnert, 2000). In ragweed superSAGE pollen libraries, It was found that Safflower (A-1) drought-stressed subtracted cDNA library always appeared under library search of term stress.

Drought tolerance is a multigenic process which involves the participation of a complex set of genes (Thippeswamy et al, 2013). A large number of pollen drought-induced genes have been studied in a wide range of plant species (Seki et al, 2002; Shinozaki & Yamaguchi-Shinozaki, 2007).

These genes co-operate to protect the cells against a deficit of water by producing important metabolic proteins and by regulating the genes which control signal transduction in response to drought stress response (Thippeswamy et al, 2013).

4.2.1.6 Allergenic potential of ragweed pollen

Pollen allergens represent only a tiny fraction of the proteins which the human population is exposed to via ingestion, inhalation, or direct contact. Therefore, allergens possess specific structural properties that are responsible for sensitizing an individual (Aas, 1978).

The close interaction between allergens and the immune system may explain the allergenicity of an allergen. As a whole, allergens are a very diverse group of proteins from a variety of environmental sources including plants, fungi, insects, and mammals (Aas, 1978). The function of allergenic proteins in their respective hosts varies, and ranges from structural proteins to proteases (Bysice, 2012). Several studies aimed to identify the common properties which define the allergic potential of allergen's. Eventually, it was demonstrated that specific epitopes recognized by T and B cells are the reason for an allergen's allergic potential (Karp, 2010). Recently, other evidence has supported the hypothesis that allergens possess intrinsic properties which activate the innate immune system and promote a Th2-type cytokine immune response (Karp, 2010; Wills-Karp, 2010).

In this study, analysis of the superSAGE expression profiles and quantitative real-time RT-PCR of the pollen allergens in the four libraries showed that the elevated CO_2, drought and elevated CO_2 plus drought condition significantly increased the allergenic potential of ragweed pollen. The differentially expressed allergenic genes could be grouped into six classes according to the AllFam database of allergen families (Radauer et al, 2008). The six classes were pectate lyases, which including Amb a 1.1, 1.2, 1.3, 1.4 and 1.5; group 5 allergens including Amb a 5; non-specific lipid transfers proteins (nsLTP) including Amb a 6; profilin including Amb a 8.0101 and Amb a 8.0102; calcium binding proteins including Amb a 9, Amb a 9.0102 and Amb a 10; and finally members of the cystatin family including Amb a CPI (cystatin proteinase inhibitor).

The superSAGE analysis and RT-PCR results confirmed that Amb a 1 isoallergen from the pectate lyase family was the most prominently upregulated major allergen in the elevated CO_2, drought and elevated CO_2 plus drought conditions.

The relative expression of different in the allergens in elevated CO_2, drought and elevated CO_2 plus drought was higher in first generation than second generations (2011). Moreover, the second generation drought-exposed pollen showed a clear reduction in the relative expression level of most allergen families compared to the elevated CO_2 and elevated CO_2 plus drought samples from the same generation. Bhalla and co-worker (1999) successfully generated ryegrass specifically devoid of Lolp5 in pollen using antisense RNA approach. The transgenic ryegrass plants displayed normal fertile pollen development, thus indicating the feasibility of generating genetically-engineered hypoallergenic ryegrass. The reduction in the relative expression of allergens observed in drought-exposed pollen may be due to the fact that the seeds of the second generation plants were generated from self-pollinated plants, although most weeds are known to be self-incompatible (Bhalla et al, 1999). Furthermore, stress memory may extend over longer period by inducing phenological reprogramming in the plants (Chinnusamy & Zhu, 2009).

Chinnusamy and Zhu (2009) showed that the reprogramming of cell differentiation in response to drought stress leads to phenological and developmental plasticity, including changes in cell wall composition, which had a remarkable ability to ameliorate the effect of stress.

In the same study, differences can be observed between superSAGE and RT-PCR results (Kavsan et al, 2007); some of the differences between the results obtained by SAGE analysis and RT-PCR in different investigations can be explained by the use of different statistical methods. Hamada et al (2008) showed that the result of RT-PCR were consistent for approximately 90% of the genes annotated from SuperSAGE tags. In this study of ragweed pollen, the RT-PCR primers for the tested allergens were designed using EST sequences available in the NBCI database. Furthermore, as annotation of the tags in the SuperSAGE libraries largely depends on existing cDNA libraries or EST collections (Coemans et al, 2005), the use of superSAGE in plants has mainly been limited to model organisms such as *Arabidopsis* and rice (Fizames et al, 2004; Lee & Lee, 2003). In non-model organisms, i.e., plants with limited or no genomic DNA and cDNA/EST sequences available, analysis of SAGE short tags may not be efficient due to the very low chance and accuracy of annotating the sequenced tags (Coemans et al, 2005). However, non-model plant species possess numerous important traits which cannot be studied in model plants.

These traits may include the organs that possess different properties in different plants (e.g., pollen grains), special developmental processes (e.g., apomixis or parthenocarpy), and distinct quality traits such as flavour or the presence of nutrients or medicinal substances.

Single-molecule sequencing will enhance whole-genome analysis, even de novo sequencing of genomes, owing to efficient sequence assembly and the presence of fewer errors due to PCR amplification (Matsumura et al, 2008). Silva et al (2013) stated that RT-PCR must be carried out to achieve the best confirmation of SuperSAGE results. For this reason, the 26-bp tag sequences obtained by SuperSAGE were used directly as 3-RACE PCR primers to amplify the regions between the tag and poly-A tail in this study.

During the last century, a number of allergens from diverse weeds have been studied, the most extensively studied weed is short ragweed (*Ambrosia artemisiifolia*) (Mohapatra et al, 2004). A total of 22 distinct proteins which could bind to specific human IgE antibodies were detected by crossed-radioimmunoelectrophoresis of aqueous short ragweed pollen extracts (King et al, 1964; King et al, 1967). These allergens families include pectate lyase, group 5 allergen, non-specific lipid transfer protein, profilin, cystatin protein inhibitors and calcium binding proteins and their function and role in allergenicity will briefly discussed.

4.2.1.6.1 Pectate lyase family

The Amb a 1 formerly known as antigen E was the most predominant allergen from short ragweed pollen; it was purified and identified as α- and β-chain (King et al, 1967; Rogers et al, 1996). Amb a 1 constitutes about 6% of the protein content of short ragweed pollen, and the antibodies directed to Amb a 1.were found in 90% of ragweed-sensitive patient. Number of major and minor antigenic determinants were found on the native Amb a 1 molecule (Smith et al, 1988).

The cloning of Amb a 1 polypeptide subunits revealed that short ragweed pollen contained four isoforms of Amb a 1, designated as 1.1, 1.2, 1.3, 1.4 and recently 1.5 (Amb a 2 previously)(Rogers et al, 1996). Since the examination of the Amb a 1 isoform on the IgE patien, it was shown that all three forms of the recombinant Amb a 1 (1.1, 1.2, 1.3) were capable of stimulating T-cell proliferation assay (Bond et al, 1991). While the Amb a 1.4 appeared to be only a minor component of the Amb a 1 family (Griffith et al, 1991).

Amb a 1.5 or Amb a 2 (formerly antigen K) is another major ragweed-pollen allergen and immunoprecipitation analysis showed that it comprises 1.2% of the soluble protein in an aqueous extract of ragweed pollen (Gadermaier et al, 2004).

Amb a 1 belong to the pectate lyases (PL) family, which are enzymes that catalyze the cleavage of pectin, the major constituent of the primary cell walls of many higher plants (Gadermaier et al, 2004). The abundance of PL-like sequences in plant genomes strongly suggests the important role of these enzymes in various developmental processes in plant. While the functions for PL in pollen include the initial loosening of the pollen cell wall, allow the emergence of pollen tube and breaking down of the cell wall of transmitting tissue in the style to ease pollen penetration.

4.2.1.6.2 Group 5 allergen

Amb a 5 is one of the minor allergen (5 kDa) only limited to 10-15% of ragweed pollen allergic patients (Hauser et al, 2013). Two isoforms (Amb a 5 and Amb a 5.0101) have been identified and showed small difference in their IgE binding patterns (Roebber et al, 1982). Further studies of purified Amb a 5 from pollen showed that four disulfide bonds responsible for stabilizeing its fold structure (Metzler et al, 1992). The Amb a 5 was produced as recombinant protein and characterized immunologically by ELISA inhibition (Hauser et al, 2013). Although the recombinant Amb a 5 have a lower IgE binding activity contrasted to natural Amb a 5 (Metzler et al, 1992).

4.2.1.6.3 Non-specific lipid transfer protein (nsLTP)

Non-specific lipid transfer protein (nsLTP) represent in ragweed pollen by Amb a 6 (10 kDa) (Hauser et al, 2013). At least four closely related isoform have been identified (Hiller et al, 1998; Lubahn & Klapper, 1993). The nsLTP (Amb a 6) is considered a minor allergen, with a 21% sensitization prevalence among ragweed sensitized individuals (Roebber et al, 1983). Recently, the Amb a 6 molecule has been recombinant in E. coli (Wopfner et al, 2008a).

4.2.1.6.4 Profilin

Amb a 8 one of the minor allergen that belong to profilin family. The cloning of AMb a 8 from ragweed pollen revealed two isoforms Amb a 8.01 and 8.02 (Wopfner et al, 2008b).

Sequence searches with Amb a 8.01 revealed profilins from mugwort pollen, Art v 4 (89% identity), sunflower, Hel a 2 (81% identity) and timothy grass, Phl p 12 (79% identity) (Asturias et al, 1998; Asturias et al, 1997; Wopfner et al, 2002).

The IgE cross-reactivity of mugwort and ragweed profilin could be explained by their strong sequence identity (Wopfner et al, 2008a). So in case of weed-pollen profilins, either Art v 4 or Amb a 8 could be used as a diagnostic marker for profilin sensitization (Wopfner et al, 2008b). Profilins function in regulating the dynamics of actin polymerization during processes such as cell movement, cytokinesis, and signalling (Witke, 2004).

4.2.1.6.5 Cystatin protein inhibitor (Amb a CPI)

The cystatin superfamily has been divided into three animal families and one plant cystatin family (phytocystatins) (Abrahamson, 1994). Nearly the phytocystatins ranged between 12-14 kDa in size and showed significant amino acid sequence similarity to the cystatin families of animal origin (Nagata et al, 2008).The phytocystatins allergens are found in short ragweed pollen (Rogers et al, 1993). kiwifruit, and gold kiwifruit (Bublin et al, 2004)

4.2.1.6.6 Calcium binding protein (polcalcins CBP)

Amb a 9 and Amb a 10 that are minor allergens and belongs to 2EF-hand calcium-binding proteins (CBP), they share a conserved domain consisting of a 12 residue calcium-binding loop flanked on both sides by α-helices of 12 residues in length (Ledesma et al, 2006).

The isolated calcium-binding proteins from mugwort and ragweed encode two homologous proteins designated as Art v 5 and Amb a 9 with a sequence identity of 82% (Wopfner et al, 2008b).

The BLAST searches of Amb a 9 and Art v 5 revealed the polcalcins CBP from birch Bet v 4 (71% and 68%), Ole e 3 from olive (71% and 65%) and Aln g 4 from alder (68% and 65%), respectively (Batanero et al, 1996; Engel et al, 1997; Hayek et al, 1998). While the Amb a 10, a ragweed pollen 3EF-hand CBP, possessed highest sequence similarities to Ole e 8 and Jun o 4, which are the 4EF-hand CBPs from olive and juniper respectively (Wopfner et al, 2008b). Nearly 10% of the patients that are suffering from pollen-allergic react with CBPs from various sources like trees, grasses or weeds (Asero et al, 2006; Niederberger et al, 1999). The CBP function can be divided into two classes: signaling (calmodulin and troponin) and calcium buffering or transport.

Allergen patterns can changed in response to climate change (especially specific weather conditions) and air pollution which can modify the allergenic potential of pollen (D'Amato et al, 2013). The allergic symptoms of pollen are induced in a dose-response manner (Singer et al, 2005). Although the concentration of pollen can be used as an indicator of the potential dose, the underlying mechanism of allergenicity depends on the specific protein allergens in the pollen (Ahlholm et al, 1998; Beggs, 2010). Recent studies have examined the effect of environmental variables (such as temperature and precipitation) and air pollutants (such as CO_2) on the concentration of various proteins (mainly allergenic proteins) in pollen or the overall allergenicity of the pollen (Reid & Gamble, 2009).

In ragweed pollen, the concentration of the allergen Amb a 1 is positively increased in response to rising CO_2 concentrations (Singer et al, 2005). Moreover, the IgE-immunoblotting responses in human sera are stronger for individuals exposed to pollen grown at higher temperatures compared to that of individuals exposed to pollen grown at a lower temperature (Ahlholm et al, 1998). Reid and Gamble (2009) suggested that even if the pollen count remained unaltered - higher CO_2 concentrations may increase the allergenicity of pollen by increasing the levels of allergenic proteins in pollen. Finally, increasing CO_2 concentrations and ultraviolet light levels may exacerbate the increased allergenicity of pollen induced by air pollution (Frenguelli, 2002).

Overall, there is sufficient evidence to suggest a probable link between rising temperatures and/or CO_2 levels, and increasing levels or temporal shifts in the aeroallergen production and allergenicity of plants (Ziska et al, 2008).

The role of drought stress in enhancing the allergenic content of pollen is well characterized. The plant hormone abscisic acid (ABA) plays a critical role in regulating the water status in plants by inducing the expression of genes that encode the enzymes and other proteins involved in cellular dehydration tolerance (Luan, 2002; Zhu, 2002). It has been shown that ABA and higher temperatures enhance the transcription of birch allergens by activating the Bet v 1a promoter in developing pollen. Therefore, either ABA or temperature-responsive and other stress-related elements may interact with tissue-specific transcription factors (Busk & Pages, 1998). Tashpulatov et al., (2004) suggested that ABA-responsive elements could play a significant role in the activation of the Bet v 1a promoter during pollen desiccation

These results may contribute to the structural analysis of other plant allergen gene promoters by providing a reference for the functionality of a given motif (Tashpulatov et al, 2004). The high expression of the *Bet v 1a* gene in pollen during development is a trait common to other pollen allergens, e.g. profilin (Mittermann et al, 1995). Thus the knowledge gained regarding *Bet v 1a* gene expression could be applied to the study of other pollen genes which encode allergens (Tashpulatov et al, 2004)

Expansins are also implicated in the drought response in maize seedlings (Wu et al, 1996). It may be possible to engineer enhanced drought tolerance into crop plants by manipulating the gene expression of expansins (Cosgrove, 2000). Expansins have distant sequence homology with a group of grass pollen allergens called group-1 allergens (Smith et al, 1996); these pollen proteins have been identified as the main causative agents of hay fever and seasonal asthma induced by grass pollen (Cosgrove, 2000; Knox & Suphioglu, 1996).

Song et al. (2013) found that the expression of GmPOI which encodes the allergen Pollen_Ole_e_I was induced by drought, cold, and salt stress in wild-type soybean.

Plants overexpressing GmPOI displayed a higher tolerance to drought stress than wild-type plants; therefore, Song et al. (2013) concluded that GmPOI may be a novel gene involved in the response to various stresses in soybean.

Several reviews have indicated that cell stress can induce protein modifications that lead to increased expression of the cross-linker transglutaminase (which plays a role in allergy-associated inflammation) in/on pollen (Kay et al, 2008), or enhance the allergenicity of pollen by activating secretory phospholipase A, which is responsible for the inflammatory response in humans exposed to pollen (Brunner et al, 2002; Del Duca & Serafini-Fracassini, 2005).

4.2.1.7 Functional annotation and categorization of SuperSAGE tags

The process of sexual reproduction in angiosperms involves regulation of the cellular interactions between the male gametophyte (pollen) and the female gametophyte (embryo sac) (Boavida et al, 2011). Our first approach aimed to identify stage-specific and/or stage-enriched transcription in ragweed pollen exposed to elevated CO_2, drought, and CO_2 plus drought.

In each test condition, the expression profiles are likely to include transcripts which are abundantly expressed in mature pollen and function to support and modulate pollen tube growth. Similarly, the expression profiles of pollen exposed to elevated CO_2 showed enrichment of transcripts also expressed in the pollen exposed to drought; these transcripts may be equally important for pollen tube growth modulation or guidance.

The transcriptome of the expressed pollen transcripts in different functional categories were broadly identical for the three different treatments (Fig. 32). However, pollen exposed to elevated CO_2 contained a much higher number of transcripts compared to the pollen exposed drought or elevated CO_2 plus drought.

At a biological level, genes associated with carbon utilization or pigmentation were proportionately underrepresented in the ragweed pollen libraries, while genes associated with signaling, the response to stimuli, reproduction, and cellular processes were proportionately over-represented. The latter categories are involved in regulation of the tip growth of pollen tubes upon germination and along the female reproductive tract (Hepler et al, 2001).

Several define (GO) terms representing the stress response, and pollen and seed development classes, were commonly over-represented in the pollen-enriched transcripts from the elevated CO_2, elevated CO_2 plus drought, and drought samples (Tab. 25, 26, 27). A similar expression pattern of expression was previously described (Durand & Durand, 1991; Taylor & Hepler, 1997).

The Venn diagrams (Fig. 33) of the up-regulated tags associated with ESTs in biological processes linked to water deprivation and oxidative stress, reveal a group of genes that play central roles in enhancing the pollen developmental process. In the water deprivation upregulated transcript, the *Populus* EST from severe drought-stressed leaves was very common transcript. The present findings seem to be consistent with other research which found that the abundance of *Populus* EST from severe drought-stressed leaves transcript underpins cellular and whole plant-level responses that enable plants to better contend with a water deficit (Bogeat-Triboulot et al, 2007; Caruso et al, 2008; Street et al, 2006; Wilkins et al, 2009a).

Furthermore, a high degree of homology was observed between ragweed pollen and genes from families in *Arabidopsis thaliana* that are known to play roles in water balance, stress response, and photosynthesis, including responsive to ABA 18 (RAB18) (Mantyla et al, 1995) and responsive to dehydration 22 (RD22) (Wilkins et al, 2009b). The second effective expressed gene in the water deprivation Venn diagram was UDP-glucose: protein transglucosylase-like. UDP-glucose and ADP glucose are known to play an important role in glucan biosynthesis (Taliercio & Kloth, 2004). ADP-glucose provides a substrate for starch biosynthesis in both maize and potato (Hannah & Nelson, 1976; Muller-Rober et al, 1992), and UDP-glucose is the preferred substrate for cellulose biosynthesis (Amor et al, 1995; Haigler et al, 2001; Kawagoe & Delmer, 1997). The increased expression of UDP-glucose in pollen under drought stress is in accordance with Taliercio and Kloth (2004), who reported that motifs (stresses) directed the expression of UDP-glucose during heat shock and in response to light stress. Additionally, the findings that UDP-glucose: protein transglucosylase-like was over expressed in response to drought stress are consistent with Marino et al. (2009) who investigated drought tolerance in maize by transcriptional profiling and mapping.

4.2.2 Global DNA methylation

As it well known that the genetic information within a cell is encoded by DNA, which is packaged into chromatin (Law & Jacobsen, 2010). Epigenetic modifications of DNA and histones, the main components of chromatin, comprise an extra layer of information that affects the expression of the underlying genes (Law & Jacobsen, 2010). DNA methylation (addition of a methyl group to a cytosine base) plays an important role in regulating plant development (Finnegan et al, 2000). Recently several evidence proved that genome-wide demethylation has a pleiotropic effect on plant morphology, including homeotic transformations in floral organs and changed flowering time (Burn et al, 1993; Ronemus et al, 1996; Vongs et al, 1993).

DNA methylation could also be necessary in genome management (Finnegan et al, 2000). Preventing large scale disruption of the genome by the methylation of transposable elements or invading DNA represses transcription of the elements and decreases recombination between identical, nonallelic elements (Bennetzen et al, 1994). Also, the silencing of transgenes is normally one of the methylation strategy.

In a specific system that look awry, this may be due to methylation of endogenous genes and its inactivation (Cubas et al, 1999; Melquist et al, 1999). The global DNA methylation of ragweed pollen in the first and the second generation with different treatment were quantified by using Imprint Methylated DNA Quantification technology, which provides a rapid and reliable method to measure global DNA methylation shifts. One of the important traits in the flowering plants that the epigenetic modification that occuerd can be inherited for many generations and have dramatic developmental consequences (Cubas et al, 1999). In plant the epigenetic inheritance of transposon involves DNA methylation (Martienssen & Baron, 1994; Schmitz et al, 2011). Generally the imprinted genes tend to be sided by transposable elements, whose methylation can effects on their expression (Radford et al, 2011). While, imprinting in plants is only limited to the extraembryonic endosperm (differentiated tissue in seed), so that imprinted chromatin and DNA modifications don't need to be removed once they are formed (Feng et al, 2010; Raissig et al, 2011).

The study analyses of DNA methylation pattern dynamics in relation to drought stress and elevated CO_2 during pollen development, showed that only the drought that is epigenetically regulated.

Differences in global DNA methylation levels between the two pollen generations (first generation and second generation pollen) are being observed. High levels of global DNA methylation were characteristic of first generation pollen, whereas low levels of global methylation were found in the second generation ragweed pollen within different treatments. Several studies have described an increase on global DNA methylation levels in plant subjected to different abiotic stresses (Wang et al, 2011), resulting in specific expression of 31 stress response related genes (Wada et al, 2004).

Previous reports showed that environmental factors such as drought, salt, heavy metals, and cold stress can induce the demethylation of genomic DNA (Choi & Sano, 2007; Zhong et al, 2009). In this study, drought induced the highest genome-wide changes in the DNA methylation status of ragweed pollen. Little research has investigated the effect of DNA methylation alterations on pollen allergenicity. Valdivia et al., (2007) found that group A grass pollen allergens are highly methylated; therefore, high levels of methylation may promote the expression of allergen genes in pollen.

4.3 Proteomic analysis

However the analysis of global gene expression is advantageous for selecting candidates for further functional studies, the absence or presence of a certain transcript does not necessarily have predictive power for the absence or presence of the protein it encodes (Noir et al, 2005). For example, low levels of a transcript may be enough for adequate accumulation of the specific protein, while the regulation of post-transcriptional and/or post-translational could negatively affect accumulation of other proteins encoded by high level of transcripts (Noir et al, 2005). Thus, the transcriptome analysis alone is not precisely demonstrating protein expression, so in a given tissue it is important to confirm the transcriptomic part with a proteomic approach. The two-dimensional gel electrophoresis (2-DE) has confirmed its excellence and singularity in achieving such goal, and already been applied to study the differential protein synthesis and protein phosphorylation patterns of *Brassica napus* microspores during microspore embryogenesis (Cordewener et al, 2000) and to comparing pollen coat proteins of self-incompatible and self-fertile *Festuca pratensis* (Kalinowski et al, 2001). Moreover, drawing the whole picture of the proteins involved at different stages of pollen development (Imin et al, 2001; Kerim et al, 2003a). In the 2D-DIEG, according to isoelectric point in a pH gradient in first dimension and molecular mass in second dimension, thus the separated spots are typically detected, quantified by image analysis, and analyzed by LCMS/MS mass spectrometry, identifying the components by comparing the results with databases (Langen et al, 2000).

4.3.1 Functional Categories of Mature Pollen Proteins

Proteome analysis of the first and second generation spots in ragweed pollen grain of all analyzed treatments showen that, they assigned in the following functional categories; metabolism and energy generation, protein processing, cytoskeleton dynamics and cell-wall remodeling, cellular transport and signaling, defense and stress response, unknown function and allergenic proteins.

4.3.1.1 Metabolism and energy generation.

As mentioned previously, the task of the pollen grain in plant reproduction includes the production of a tube to deliver the sperm cells to the embryo sac (Noir, 2008).

Therefore it seems normal that the pollen grain saves proteins required for pollen tube germination and early growth, as well as proteins for energy metabolism (Mascarenhas, 1993). These results are consistent with those of other studies and they suggest that the majority of the *Arabidopsis* and *Oryza sativa* pollen polypeptides are involved in metabolism (42%) (Dai et al, 2006; Noir et al, 2005). Moreover, high percentages of proteins (at least 17%) required for energy generation were identified (e.g., ATP-synthase, enolase, or GAPDH).

4.3.1.2 Protein processing.

Interestingly, in the first and second generation pollen more than 9% (Tab. 19, 20) of the identified proteins were assigned to protein processing functions (protein fate). It was hypothesized by Honys and Twell (2003) that mature pollen is charged with a preformed translational apparatus enabling fast activation upon hydration and germination. Noir and Dia (2006; 2005) respectively found that major proteins involved in protein synthesis such as ribosomal proteins or translational initiation and EFs have been identified. Other proteins more related to protein fate functions, such as chaperone (heat shock proteins), ubiquitin, were identified. Finally, these collective data provide molecular proof at the proteomic level that mature pollen in plants anther pre-synthesizes the vital components of the translation machinery to initiate protein synthesis and destination upon entry into germination (Dai et al, 2006).

4.3.1.3 Cytoskeleton dynamics and cell-wall remodeling.

Pollen germination demands polarization by cytoplasmic and cytoskeletal reorganization; after that pollen tube growth reached the maximum tip region, which demands a continuous deposition of new cell wall and plasma membrane (Mascarenhas, 1993). Considering the first and second generation of ragweed pollen spot annotation, a significant proportion of identified proteins were categorized as related to cytoskeleton dynamics and cell-wall remodeling functions (>9%) (Tab. 19, 20). Different isoforms of actin, tubulin, and also actin-binding proteins, namely profilin and actin-depolimerizing factors, which regulate the dynamics of the actin cytoskeleton in the growing pollen tube (Chen et al, 2002a), have been identified in mature pollen proteomes. The main function of these cytoskeletal proteins is their contribution in the deposition of cell-wall components such as pectin, cellulose, and callose (Cai et al, 2005). Furthermore, in *Arabidopsis* and

rice pollen several proteins were identified that involved in cell wall dynamics (Noir, 2008). It includes both proteins involved in degradation of cell-wall such as cellulase, polygalacturonase, pectinesterase, and other proteins involved in active wall synthesis (e.g., cellulose synthase, reversibly glycosylated polypeptide). The presence of these pre-synthesized components suggests complex machinery available in mature pollen to intercede reorganization leading to pollen germination and rapid tube elongation and orientation.

4.3.1.4 Cellular transport and signaling

In view of the vast physiological and metabolic events that took place in the mature pollen grain, so it is logically to detected the signaling molecules in ragweed mature pollen proteomes. Actually, considering the different annotations, this function was assigned to 7% to 10% of the identified proteins (Tab. 19, 20). Many of the proteins involved in signal transduction are calcium sensors (calreticulin), kinases, and proteins mediating phosphorylation/dephosphorylation activity or proteins implicated in GTP-mediated signaling (Noir, 2008).

4.3.1.5 Defense and stress response.

A clear portion of stress related proteins were also identified in the ragweed pollen proteome analyses (~5%, Tab. 19, 20). The identification of proteins such as bZIP, SOD, TOR 1 suggests a broad spectrum tools of the mature pollen grain to protect itself against biotic and abiotic stresses (Noir, 2008). As mentioned by Dai et al. (2006), the pollen, as a highly compact tri-cellular organism, must have gain the ability during evolution to deal with extracellular stresses after release from the anther and intracellular stresses resulted by the active metabolism of germinating pollen and its interaction with cells of the stigma and the style (Noir, 2008).

4.3.1.6 Unknown function.

Finally, in both first and second generation of ragweed pollen a significant number (>8%) (Tab. 19, 20) of the identified polypeptides are of unknown function.By analogy, this set of proteins may be considered as yet un-identified cellular processes, some of them might be specific for the male gametophyte (Noir, 2008).

4.3.1.7 Allergenic proteins

Inhalative allergies are mainly triggered by pollen of various plant species (Noir, 2008). Recently, proteome and immunochemical approaches have been extensively used to analyze the allergen content of pollen grains (Corti et al, 2005; Kao et al, 2005; Kerim et al, 2003b). So far, most of the described pollen allergens are water-soluble proteins or glycoproteins of molecular weight from 10 to 70 kDa (Puc, 2003). They have been identified in diverse plant species and grouped to various protein families. For example, major pollen allergens−such as the pectate lyases, which are effective cell-wall-degrading enzymes (Wing et al, 1990) are implicated in cell-wall dynamics. Likewise, profilins, which play an active role in the regulation of actin polymerization (Valenta et al, 1991), or members of pathogenesis related (PR) protein families (namely, TLP) have been reported as allergens in many plants (Breiteneder & Radauer, 2004).
The results of this study showed that in the first generation, the drought pollen possesed fold change higher than the CO_2 and CO_2 with drought (Amb a 1.2,1.3,1.4).

Only Amb a 1.1 and Amb a 2 (Amb a 1.5) in the CO_2 and CO_2 with drought having higher fold change than drought. In the second generation the elevated CO_2 and CO_2 plus drought comprise the higher fold change than the drought for Amb a 1.2, Amb a 1.3, and Amb a 2 (Amb a 1.5).

Ragweed pollen grains have been studied for allergen proteins in a number of species because of their allergenic activity toward humans (Mohapatra et al, 2008; Radauer et al, 2008). A number of proteins in ragweed pollen, such as pectate lyase (Amb a 1), profilins (Amb a 8), and some of the calcium-binding proteins (Amb a 9) discussed earlier, are also known to act as allergens. Allergenic proteins were also reported in rice and *Arabidopsis* pollen (Dai et al, 2006; Noir et al, 2005).

This study produced results which corroborate the findings of a great deal of the previous work in this field. 2D-PAGE and mass spectroscopy of ragweed pollen proved the presence of all 5 known Amb a 1 isoallergens, Amb a 1.01 represents the dominant form and additionally all other ragweed allergens known by sequence (Amb a 3, Amb a 4, Amb a 5, Amb a 6, Amb a 8, Amb a 9, Amb a 10) were also identified. But Amb a 1 is the most abundant Amb a 1 isoallergen, and presumably the most important ragweed allergen (Augustin et al, 2012). In ragweed pollen subjected to elevated CO_2, although total pollen

protein remained unchanged, but significant increases in Amb a 1 allergen were observed (1.8 fold) (Singer et al, 2005). Jagadish et al., (2011) found that pollen allergens and beta expansin in rice on the proteome level, were highly up-regulated with water deficit but were at normal levels under combined stress of heat and drought stress.

5. Conclusion

This dissertation has investigated and reported, for the first time, on genome wide transcriptome analysis in a non-model weed. To this point, an improved SAGE protocol, SuperSAGE, was successfully applied on ragweed pollen, thereby characterizing the expression under different environmental condition (elevated CO_2, drought, elevated CO_2 + drought). Also the proteomic studies presented here addressed and confirmed the response of ragweed pollen to such condition that has been mentioned above.

The purpose of the current study was to analysis the impact of elevated CO_2, drought and elevated CO_2 plus drought on transcriptome and proteome of ragweed pollen, and investigates the induction of the components that have an allergenic potential.

The most obvious finding to emerge from this study is that the Amb a 1 isoallegens showed to be the most prominent major allergen in the ragweed pollen under different environmental condition, this were observed in the superSAGE libraries, RT-qPCR and 2D-DIEG.

The second major finding was that both the elevated CO_2 and/or drought increased the allergenic potential on the transcriptomic, proteomic and enzymatic level. The evidence from this study suggests that the drought proved to possess the highest level of fold change of allergenic genes in the RT-qPCR and in 2D-DIEG. Combination of elevated CO_2 plus drought found to be the second after the drought as the causative pollen its allergenic potential. Finally the elevated CO_2, that also increases the allergens more than the control in the transcriptome and proteome level but less than that of drought.

This study is pioneer in investigating the effect of drought and elevated CO_2 with drought, simulating the future and what will be the situation of pollen from the allergenic potential view against this new environmental condition. It was speculated that the drought might acquire the ragweed pollen to be more virulence than the elevated CO_2 with the atopic patient and causing more allergenic reaction. But to confirm this fact, allergenic potential then, has to be tested in a mouse model or by prick tests.

In conclusion, the present study confirmed the previous findings and contributed additional evidence that suggested that the ragweed pollen under global change scenarios, its transcriptome and proteome will be altered indicating a changed allergenic potential of that pollen.

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ACKNOWLEDGEMENTS

I would sincerely like to thank all those people who made this thesis possible and who guide me and help me from the initial to the final stage of my work. Without their support and encouragement, this thesis would not have been possible.

I owe my deepest gratitude to my principal supervisor, Dr habil Dieter Ernst who gave me the opportunity to carry out the exciting and challenging work presented in this thesis. I would also like to thank my co-supervisors, Prof. Dr. Jörg Durner, for their support and advice.

I would like to thank my present and past colleagues from Helmholtz Zentrum, specially Feng Zhao, Ulrike Kanter, Kuruthukulangarakoola Gitto, Wittek Finni, Dr. Heller Werner for sharing your expertise and for all the scientific discussions we made Special thanks to Evi Bieber, Barbara Gross, Susanne Stich, Claudia Knappe for their technical assistance. In particular I would like to give special thanks to Elke Gerstner for always making time to assist me whenever I was in need and for her endless patience. Thanks for EUS staff Dr Barbro Winkler, Peter Kary, Dagmar Schneider for helping concerning the greenhouse during the experiment.

Millions thanks to my parents dad and mam, and my wife Lougin and my daughter Hla for their unlimited support, care, encouragement, unconditional help, understanding, and patience.

I acknowledge the thesis reviewers Prof. Dr. Jörg Durner and Prof. Dr. Gerhard Müller-Starck and Prof. Dr Rainer Matyssek. The study was financially supported by Christine Kühne - Center for Allergy Research and Education (CK-CARE), Helmholtz Zentrum München Deutsches Forschungszentrum für Gesundheit und Umwelt (GmbH), GERLS (German Egyption long-term research scholarship).

"Anyone who has never made a mistake has never tried anything new."

"Science is a wonderful thing if one does not have to earn one's living at it."

Albert Einstein

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