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FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

Anti-oxidant enzymes during trace metal stress in alfalfa stems and their implication in cell wall dynamics

Promotor :
dr. Sacha BOHLER

Promotor :
dr. JEAN-FRANÇOIS HAUSMAN

Christophe Loix
Proefschrift ingediend tot het behalen van de graad van master in de biomedische wetenschappen

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"But beyond these, my son, be warned: there is no end to the making of many books, and much study wearies the body."

(Ecclesiastes 12:12, Holman Christian Standard Bible)

List of abbreviations

2DE:	2D electrophoresis
ACN:	Acetonenitrile
Ambic:	Ammonium bicarbonate
ANOVA:	Analysis of variance
AO:	Ascorbate oxidase
APX:	Ascorbate peroxidase
AsA:	Ascorbate
ASB-14:	Amidosulfobetaine-14
ATP:	Adenosine triphosphate
CeSa:	Cellulose synthase
CAT:	Catalase
CHAPS:	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHCA:	α-Cyano-4-hydroxycinnamic acid
Cu:	Copper
DHA:	Dehydroascrobate
DIGE:	Differential gel electrophoresis
DTE:	Dithioerythritol
DTPE:	diethylene triamine pentaacetic acid
DTT:	Dithiothreitol
EDTA:	Ethylenediaminetetraacetic acid
EGTA:	Ethylene glycol tetraacetic acid
EtOH:	Ethanol
FW:	Fresh weight
Glc-1-P:	Glucose-1-phosphate
GPI:	Glycophosphatidylinositol
GPX:	Guaiacol peroxidase
GR:	Gluthation reductase
GSSG:	Oxidized gluthation
GTP:	Guanosine triphosphate
HEEDTA:	hydroxy-2-ethylenediaminetriacetic acid
HEPES:	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
HPLC:	High performance liquid chromatography
IAA:	Iodoacetic acid
IEF:	Isoelectric focussing
IMP:	Integral membrane protein
ISO:	Inside-out
LC:	Liquid chromatography
MALDI:	Matrix-assisted laser desorption/ionisation
MeOH:	Methanol
MOPS:	3-(N-morpholino)propanesulfonic acid
mQ:	milliQ
MS:	Mass spectrometry
$NADP^+$:	Nicotinamide adenine dinucleotide phosphate
NADPH:	Reduced nicotinamide adenine dinucleotide phosphate
PAGE:	Polyacrylamide gel electrophoresis
PEG:	Poly ethylene glycol
PIC:	Protease inhibitor cocktail
PM:	Plasma membrane
PMP:	Peripheral membrane protein
PVP:	Polyvinylpyrrolidone
PVPP:	Polyvinylpolypyrrolidone
ROS:	Reactive oxygen species
RSO:	Right-side-out
SDS:	Sodium dodecyl sulfate
SOD:	Superoxida dismutase
SPX:	Syrangaldazine peroxidase
SuSy:	Sucrose synthase
TCA:	Trichloroacetic acid
TFA:	Trifluoroacetic acid
ToF:	Time-of-flight

UDP:	Uridine diphosphate
UTP:	Uridine triphosphate
Zn:	Zinc

Abstract (English)

The economic importance of plants in agriculture can be summarized in 4 words: food, feed, fiber and fuel. Of tremendous importance to each one of these are biopolymers. Biopolymers of particular interest are the components of lignocellulose, the building block of the plant cell wall. These biopolymers (cellulose, lignin and hemicellulose) have various applications and are most abundant in plant stems. Polymerization of some of these macro-molecules, such as lignin, is dependent on (anti-)oxidative enzymes, the primary role of which is to safeguard the oxidative balance of the cell. The oxidative balance can be disturbed by the excess of metal trace elements such as copper and zinc, which will first encounter the apoplast before being able to damage the protoplast. Presence of these metals might thus modify the composition of the cell wall by influencing various anti-oxidative enzymes, and thus might have agricultural and economic implications. This research aimed to investigate the activity of key enzymes and metabolites, that are involved in both the anti-oxidative defense of the plant and bio-polymerization in the cell wall in the stem tissue of *Medicago sativa* (alfalfa), exposed to excess copper and zinc. No significant effects were observed, but enzyme activity was detected.

In addition, optimization of a protocol for the extraction of the plasma membrane proteins of alfalfa stems, destined for a proteomics approach, was attempted. Proteomics is one of the more recent "-omics" approaches. This group of techniques aim to describe the composition and variation of a specific group of biological molecules in an organism, in this case proteins. It has a number of advantages on more traditional approaches such as genomics and transcriptomics, the greatest one of which is its biological relevance. The golden standard of a proteomics-experiment is 2D differential gel electrophoresis, followed by protein identification by mass-spectrometry. A proteomic investigation to plasma membrane proteins is sometimes avoided because of various problems. Plasma membrane proteins exist in a wide range of hydrophobicity. The most hydrophobic ones are notoriously hard to solubilize and their relatively low abundance in comparison with other membrane fractions can render their detection in a proteomics approach challenging. Yet their importance should not be underestimated because they are responsible for a myriad of vital cell functions, such as cell-to-cell communication, biosynthesis of the cell wall, anchoring the cytoskeleton etc. A way to cope with this abundance-problem is an enrichment step. Several techniques exist, but the polyethylene glycol/dextran aqueous two-phase separation is the best one in terms of reported enrichment and simplicity. This was found to be a challenging technique to optimize. Above all else, PEG and dextran-concentrations need to be optimal and microsomes need to be present. Instead, a protocol was optimized to extract the total membrane fraction, but results indicate PM enrichment remains necessary. Following extraction another challenge presents itself: finding a buffer that can solubilize membrane proteins whilst remaining compatible with 2D gel electrophoresis. A comparison between 4% CHAPS and ASB-14 as lysis-buffer for 2DE was made. Both buffers insufficiently solubilized the complete sample in the used concentration, yet distorted the gel considerably.

Abstract (Dutch)

Het economische belang van planten in landbouw, kan samengevat worden in 4 kernwoorden: voedsel, voeder, vezels en brandstof. Erg belangrijk in elk van deze begrippen zijn biopolymeren. Specifieke biopolymeren die er interessant zijn, zijn de componenten van lignocellulose, de bouwsteen van de de celwand. Deze biopolymeren (cellulose, lignine en hemicellulose) hebben vele toepassingen en zijn het meest aanwezig in de stam. De polymerisatie van sommige van deze macromoleculen, zoals lignine, is afhankelijk van (anti-)oxidatieve enzymes, waarvan de primaire rol het bewaren van de redox balans van de cel is. Deze oxidatieve balans kan verstoord worden door een overvloed aan metaal spoorelementen, zoals koper en zink, die eerst de apoplast moeten passeren alvorens de protoplast te kunnen beschadigen. Aanwezigheid van deze metalen zou dus zo de compositie van de celwand kunnen wijzigen door het beïnvloeden van anti-oxidatieve mechanismen en zou zo implicaties kunnen hebben op de landbouw-economie. Dit onderzoek trachtte de activiteit van een aantal sleutel enzymen en metabolieten te meten, die betrokken zijn in zowel de anti-oxidatieve verdediging van de plant en de bio-polymerisatie van de celwand in de stam van *Medicago sativa* (alfalfa), blootgesteld aan een overvloed van koper en zink. Er werden geen significante effecten gevonden, maar er werd wel enzym activiteit gedetecteerd.

Ook werd een protocol geoptimaliseerd voor de extractie van plasma membraan proteïnen uit alfalfa stammen, bedoeld voor een proteomica-experiment. Proteomica is een van de meer recente "-omica"-aanpakken. Deze groep technieken tracht de samenstelling en variatie van een specifieke groep biologische moleculen, in dit geval proteïnen, in een organisme te beschrijven. Proteomica heeft heel wat voordelen ten opzichte van de meer traditionele aanpakken, zoals genomica en transcriptomica, waarvan de grootste biologische relevantie is. De gouden standaard van een proteomica-experiment is 2D differentiële gel elektroforese, gevold door identificatie van proteïnes door massaspectrometrie. Een proteomica-experiment van plasma membraan proteïnen wordt al eens vermeden omwille van verschillende problemen. Plasma membraan proteïnen variëren sterk in hydrofobiciteit. De meest hydrofobe proteïnen zijn enorm hard op te lossen en hun relatief kleine aanwezigheid in vergelijking met andere membraanfracties kan hun detectie in een proteomica-experiment bemoeilijken. Toch moet hun belang niet onderschat worden, want ze zijn verantwoordelijk voor een heleboel vitale functies in de cel, zoals cel-tot-celcommunicatie, de biosynthese van de celwand, het verankeren van het cytoskelet, etc. Een manier om met deze lage aanwezigheid om te gaan, is het uitvoeren van een opzuiveringsstap. Hiervoor bestaan verschillende technieken, maar de polyethyleenglycol/dextran waterige twee-fase scheiding is de beste inzake behaalde opzuivering en simpliciteit volgens literatuur. Er werd vastgesteld dat dit geen eenvoudige techniek is om te optimaliseren. Het belangrijkste voor succesvolle opzuivering zijn de aanwezigheid van microsomen en optimale polyethyleenglycol en dextran-concentraties. In de plaats werd een protocol geoptimaliseerd voor de extractie van de totale membraanfractie, maar resultaten hiervan tonen aan dat verdere opzuivering van de plasma membraan proteïnen noodzakelijk is. Na extractie volgt een nieuwe uitdaging: een buffer vinden die erin slaagt membraanproteïnen oplosbaar te maken en toch compatibel is met 2D gel elektroforese. Er werd een vergelijking gemaakt tussen 4% CHAPS en ASB-14 als lysisbuffer voor 2DE. Geen van beide

buffers slaagde erin het volledige staal oplosbaar te maken en beïnvloedde toch het gelpatroon merkelijk.

1. Introduction

1.1 Food, feed, fiber and fuel

In light of a growing world population, increased pressure on the environment and depletion of affordable fossil fuels, increasing the efficiency of agriculture will more than ever be a hot topic. The economic importance of plants in agriculture can be summarized in four words: food, feed, fiber and fuel [1]. Food refers of course to plants for human consumption. Feed is material that is meant for consumption by cattle and other animals, which can in turn be destined for human consumption of meat and dairy. Plants intended mostly or specifically for feed are also referred to as fodder (harvested and fed to cattle) and forage (collected by the animals themselves). Fiber refers to the filamentous materials that can be produced by plants, and have a range of applications such as textile and building materials. Fuel refers to the possibility of generating renewable biofuels from plant material. Of particular importance for these four applications is the lignocellulosic biomass.

1.2 The cell wall and importance of lignocellulosic biomass

One of the big differences between plant and animal cells is the presence of a cell wall enveloping the cell membrane in plants. The cell wall is probably of prokaryotic origin and serves a number of purposes in the eukaryotic plant [2]. Firstly, it counter-acts the tendency of the plant cell to swell up, because of the vacuole and nature of water-transport. It also serves as the first barrier, and thus also first target for external stressors, keeping them from damaging the vulnerable protoplast. To this end a number of molecules, embedded in the lignocellulosic matrix of the cell wall, are suggested to have various defence functions against e.g. oxidative stress. Secondly, the lignocellulosic materials (and middle lamella which connects adjacent cells) give the plant much of its greater structural integrity, allowing plants to grow and remain upright, from the stems of the smallest flowers to the mighty sequoias standing over 100 meters tall. Lignocellulose is the principal component of the cell wall. The matrix formed by cellulose and hemicellulose fibrils, embedded in pectins, and lined with lignin, makes the cell wall a rigid structure. The cell walls of adjacent cells, together with their interstitial spaces, are referred to as the apoplast. Being the primary rout in and out of the cell, the apoplast is crucial to the plant cell.

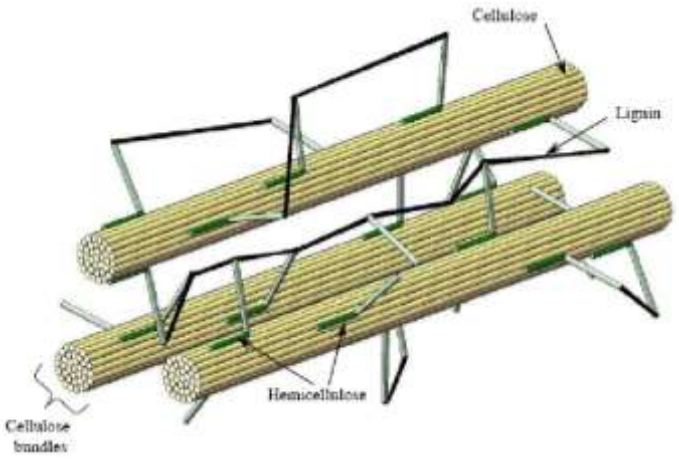


Figure 1; Lignocellulose and its components. Lignocellulose is a complex/matrix made up of polysaccharides (cellulose and hemicellulose) and lignin [102].

Lignocellulose is a matrix-like plant material composed of cellulose, hemicellulose and lignin [3]. These materials and their individual importance are discussed later, with the exception of

hemicellulose because it has little economic relevance compared to the other two. Lignocellulose is of tremendous importance to both the plant and the economy of agriculture.

The lignocellulosic biomass makes up the bulk of most plants. Because it contains the two largest carbon reservoirs on Earth (cellulose and lignin, in that order), lignocellulosic biomass is a great indicator of the economic yield of crops in agriculture. These carbon reservoirs are also the basis of what is fed to cattle or used for the creation of biofuels, since carbon is essential to creating organic material. Furthermore, the biopolymers have a myriad of applications as biofibers and composite materials [4]. More specific applications of the components of this abundant biopolymer are discussed later for its specific components.

1.3 Cellulose

Cellulose is the most abundant polysaccharide in plants and the largest fraction of the lignocellulosic biomass [3]. It consists of microfibrils made out of long chains of (1,4)-b-linked glucosyl molecules [5]. Cellulose is synthesized by a plasma membrane (PM) -associated complex made up of 36 cellulose synthase subunits (CeSa). The biosynthesis by CeSa consists of three steps: initiation, elongation, and termination of the microfibrils. Prior to this, glucose molecules need to be prepared as building blocks for cellulose synthesis by being linked to uridine diphosphate (UDP). The formation of glucose-UDP molecules is catalysed by UGPase, in which glucose-1-phosphate (Glc-1-P) and uridine triphosphate (UTP) react to form glucose-UDP and two phosphates. Another way to form glucose-UDP is a reaction which converts sucrose and UDP to glucose-UDP and fructose, catalysed by sucrose synthase (SuSy) [6].

The carbohydrate-polymer constitutes a huge carbon reservoir to e.g. be fed to cattle, and has various industrial applications ranging from making paper, cardboard and plastic to producing biofibers and biofuel [7–9]. Even though a larger complex, lignocellulose, is the basis for cellulosic ethanol, it is the polysaccharide cellulose that can actually be converted into glucose and fermented to ethanol.

1.4 Lignin

Lignin is also a polymer but not a polysaccharide [10]. On a molecular level, it serves as a hydrophobic agent, preventing water, which is being transported through the plant, from being soaked up by the highly hydrophilic polysaccharides of the cell wall. Lignin(s) consist of oxidatively radicalized and polymerized monolignols [11]. The base-molecule monolignol, the formation of which starts with the amino acid phenylalanine, is also the precursor for the production of phenylpropanoid, which forms the basis of essential oils in plants [12]. Some enzymes, thought to be involved in this oxidative coupling of monolignols, are laccase, class III peroxidases and A2 horseradisch peroxidase, making it of particular interest in this research [12–15]. Ascorbate (AsA) and ascorbate peroxidase (a class I peroxisdase) are also involved in the oxidative status of the cell wall and apoplast [16–18]. Biodegradation of lignin also happens by means of oxidative mechanisms,

catalysed by enzymes such as laccase in plants and manganese peroxidase and lignin peroxidase in other species, such as fungi [19].

Lignin has many industrial applications, e.g. it is important in the durability of wood, it can be used to produce carbon fibers, plywood, the bioplastic Arboform ('liquid wood'), adhesives and resins and is an additive to cement and paint [20]. Many of these applications are more sustainable or cleaner compared to the more conventional alternatives, because those involve fossil fuels [9]. Also interesting is its relation to the production of bioethanol. Lignin burns well and some processes have been developed that use novel approaches to attempt to turn it into biofuel [21,22]. However, when using the 'classical' methods to produce bioethanol from cellulose, lignin is a major nuisance because it makes it harder to extract cellulose from lignocellulose [23]. This is linked to the fact that it lines the polysaccharide-polymers, to counter their hydrophilic nature. In fact, much research within the field of biofuels is aimed at either genetically or enzymatically removing lignin from the plant or the lignocellulose bulk. Finally lignin can be used for the filtering of metals from water in industrial applications, thus suggesting a possible sequestration use in industry [24].

1.5 Micronutrition and oxidative stress

Zinc (Zn) and copper (Cu) are trace metals, minerals that are essential metals, present at low levels in plants and crucial to plant health and homeostasis. Zinc and/or copper are essential cofactors of various enzymes involved in both anti-oxidative defence and lignification, such as superoxide dismutase (Cu and Zn) and laccase (Cu) [25]. However, overabundance of these minerals will often have toxic effects. These toxic effects are caused by a variety of mechanisms, e.g. oxidative stress, the displacement of similar minerals on enzymes or alteration of the membrane potential. Oxidative stress is a situation that arises by a disturbance in the cellular redox balance. Variations in the ratio between reactive oxygen species (ROS) and scavenging enzymes or molecules may tip the scales of pro- and anti-oxidants in favour of the pro-oxidants [26]. These reactive oxygen species, such as superoxide, hydroxyl radicals and ions, peroxide and hydrogen peroxide can cause direct or indirect damage to various cell components, such as DNA, the lipid membranes and proteins. Zn and Cu, in large quantities, are both capable of causing oxidative stress. Cu^{2+} is redox-active and stimulates ROS-formation via the Fenton and Haber-Weiss reaction [27]. Zn^{2+} is not redox-active but disturbs the redox-balance indirectly by disrupting the cellular antioxidant pool, trough competition in uptake and translocation with other essential mineral trace elements [28]. Pollution with such metals is unfortunately an ever-growing problem in our industrialized society. In addition to historical pollution, new sources keep increasing the metal content in the soil. Examples of polluters are highway and airline traffic, smelting and high furnaces (the historical culprits in Belgium and Luxembourg), waste incineration and phosphate-fertilizers [29]. They all contribute to a problem so grave, that it is hard to find completely unpolluted soil in certain parts of Europe [30].

1.6 Oxidative stress and antioxidative defence in the cell wall

The pathways of oxidative stress and anti-oxidative defense have a number of interesting tie-ins with the cell wall composition. The polymerization of monolignols to lignin and its breakdown happen by means of an oxidative process, in which a number of antioxidative enzymes are involved. Lignin itself has been found to exhibit anti-oxidative properties[31]. Besides serving as a defense against oxidative stress, and being involved in the oxidative coupling of monolignols, a number of these enzymes also contain Cu and/or Zn, e.g. superoxide dismutase. In addition, the first injury will often occur in the apoplast occurs because the cell wall serves as first barrier against, and target of, any endogenous molecule on the cellular level. Further, ROS can also function as essential signalling molecules, e.g. in cell-to-cell communication [32]. For this use, they will often be released into the apoplast, again offering an interesting overlap between the cell wall and oxidative stress. In spite of this, relatively little is known about how micronutrition and trace metal stress influence the dynamics of lignification in the plant trough redoxenzymes.

Recent studies also suggested that cell wall polysaccharides can be actively remodelled in plants exposed to an excess of Cu and Zn and polysaccharide content can be altered, seemingly confirming suspicions about a large role for the cell wall in antioxidative defence. In *Oryza sativa* (rice) pectin and hemicellulose content increased after exposure. The cell wall being a plastic and dynamic entity, this adaptation increases the sequestrating capacity of the plant. However, similar research on cellulose has been contradictory. In *Linum usitatissimum* (flax) cellulose content increased, while in *Oryza. sativa* cellulose content decreased and was deemed not a key site for sequestration, after exposure to the toxic heavy metal cadmium [33].

1.7 Proteomics in relation to other "-omics"-approaches and the central dogma of molecular biology

Proteomics is one of the so-called "–omics" approaches, scientific approaches that aim to study a biological system by describing the composition and variation of a specific group of biological molecules in this system."-Omics"-approaches try to describe each step in the central dogma of molecular biology, the flow of information from DNA to RNA to proteins, in this way [34]. Genomics for instance, tries to grasp a complete view of the genome of a species, its sequence, evolution and its coding and non-coding sequences. Genomics is one of the oldest –omics approaches which reached its pinnacle with the publication of the human genome. However despite the ease with which genome sequences can now be determined, the function of the genome, i.e. a repository of information, makes it sub-optimal for the study of an organism in interaction with its environment. For example, organisms such as a worker bee larvae and a grown queen bee, or a caterpillar and a butterfly, have the exact same genome. To describe an organism in a changing environment or in different states (sick versus healthy, young versus old, stressed versus relaxed) the study of molecules further down the central dogma of molecular biology is needed. The reason for this is of course that not every gene is being expressed under all conditions and modifications during or after transcription and translation are the general rule. This means that the diversity increases tremendously at the mRNA and protein level. This is why one gene can account for many

transcripts and even more different proteins. One could say that genomics has a poor 'biological resolution'. The next level to study is the transcriptome and transcriptomics aims to quantify all the transcripts (mRNA's) present in a cell/organism at a certain time under specified conditions. Clearly, the transcriptome is more variable and differentiated than the genome, since it will show only the genes of the genome that are actually being expressed. However, studies indicated that the correlation between mRNA and protein levels is poor [35,36]. Differences in the number of proteins made based on a single mRNA, posttranslational modifications, protein degradation etc. imposes the study of the proteins present, in order to attain a complete view on the functioning of the cell. Contrary to most of the nucleotide-based macromolecules, proteins form the functional machinery of the cell as enzymes, structural scaffolds or signal transducers. Proteomics is the qualitative and quantitative comparison of the protein-content in two samples, to the purpose of identifying changes in protein abundance induced by different conditions. Proteomics allows to distinguish isoforms and post-translationally modified proteins, yielding information about how the plant cell is being affected by certain conditions with much greater biological resolution than genomics or transcriptomics [37]. Proteomics too has its limitations. Contrary to nucleotide-based molecules, no technique exists to amplify or duplicate proteins. Therefore a major limitation is that the possibility to study a protein depends on the matrix formed by the other proteins, with high abundant proteins often masking more important low-abundant protein-forms. In the current study this means that elaborate extraction protocols must be applied to eliminate abundant cytosolic proteins in order to be able to quantify the generally low-abundant membrane proteins, which constitute about 25% of all proteins [38]. Two main approaches exist for proteomics: gel-based and gel-free. While in the former proteins are separated and quantified as intact proteins, the latter applies separation and quantification after digestion of the proteins into peptides. Data on the quantification of peptides is then used to reconstruct and quantify the corresponding proteins. Both approaches have benefits and drawbacks as is reviewed in several publications [39–41]. Other examples of relatively new "–omics" approaches are metabolomics and epigenomics, trying to map the metabolome and epigenome.

1.8 2D gel electrophoresis: the golden standard of proteomics

2D gel electrophoresis is currently still the most frequently used technique in plant proteomics and the current golden standard 2D Differential Gel Electrophoresis (2D DIGE), eliminates some of the earlier limitations of 2D-PAGE. First, samples are differentially labelled by condition (plus an internal standard), e.g. with CyDyes. The differential labelling allows the co-seperation of two samples on the same gel. Furthermore the addition on each gel of an internal standard, a pool of an equal amount of each sample, allows cross-gel normalization, thereby increasing the reproducibility of the analysis. The pooled sample is loaded on a polyacrylamide strip with an immobilized pH-gradient and subjected to isoelectric focussing. Under the influence of an electric field, the proteins migrate to their isoelectric point, a narrow pH-point at which the net-charge of the protein is zero. This constitutes separation in the first dimension or isoelectric focussing (IEF). After isoelectric focussing, the strip is used for a classic sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in an orthogonal direction. Under the influence of an electric field, the

gel works as a sieve, allowing proteins with a low molecular weight to migrate faster than larger proteins. The use of two orthogonal parameters to separate the proteins increases the peak-capacity compared to single separations and more than 1500 spots are routinely distinguished on 2D gels [42]. The separation is visualized based on the fluorescence of the different dyes using dedicated scanners. After the analysis of the gel images with appropriate software packages and statistical analysis, the spots with a difference in abundance are generally excised and used for MS-based identification. The 2D DIGE-approach is the golden standard, yet it has its limitations, such as bias against proteins with extreme molecular weight or isoelectric point [43].

1.9 Advancements in identification through mass spectrometry

Close-to-all of the protein identifications in proteomics are done using mass spectrometry (MS) [44]. The commonly used approach for gel-based proteomics is the digestion of the protein, immobilized in the gel matrix, using a sequence specific cleavage agent and mass spectrometric analysis of the resulting mixture of peptides. For this goal, different mass spectrometers with different ionization modes can be used, but the most popular for gel-based approaches is matrix-assisted laser desorption/ionization (MALDI). The peptide mixture is mixed with a matrix solution, and spotted on a target plate. A laser is shot at the target and this results in the formation of volatilized ions that are accelerated towards a lens. MALDI is most commonly coupled with ToF analysers, in which ions move at a speed depending on the mass/charge ratio of the ion. In case of MALDI, the charge is always one, so that the speed depends only on the mass. Small ions will move faster than larger once and thus arrive at the detector sooner. This kind of measurement is called Time-of-Flight (ToF). At the arrival at the detector, the strength of the signal is recorded, and the mass for each ion is deduced after calibration of the flight time with known masses. The resulting data is represented as a mass spectrum called a Peptide-Mass-Fingerprint (PMF), on which peptides are represented as peaks. The results are compared to PMF databases and a list of potential proteins is returned as output. While this is a simple and efficient technique, its sole use as an identification tool is currently questioned, and supplemental data coming from fragmentation experiments (MS/MS) are generally required to claim the identification of a protein. During MS/MS a selected peptide is allowed to collide with neutral gas molecules (N_2 in the current study). The energy acquired during these collisions is dissipated through the fragmentation of the peptide. The fragments are further accelerated and ToF is again measured. The result is again as mass spectrum that can be compared to database entries. Apart from this database-dependent approach the sequence of the peptide can also be read from the MS/MS spectrum, either using dedicated software or manually (so called *de novo* sequencing), after which the obtained sequence can be used for homology searches [44]. The success rate of identification is primarily determined by the amount of sample present in a spot but automated approaches fail when the correct sequence is not in the database. Although the genome of ever more species is sequenced, not all sequence variants have been identified and genome annotation is not following in the same pace [45].

1.10 Plasma membrane proteins: abundance problems and enrichment

Obtaining the plasma membrane (PM) protein fraction of plants is not evident, yet enrichment is essential because the relatively low abundance of the PM-fraction compared to other fractions makes it difficult to study PM proteins [46]. The presence of the cell wall is the first problem for PM protein enrichment especially in the stem of the plant, where the cell wall is more lignified and stiffer. The cell walls of multiple cells are separated additionally by the middle lamella. [47]. After homogenisation of the tissues and isolation of the membranes, the PM-fraction has to be purified from the remainder of the membranes, such as those of the chloroplasts, mitochondria, nucleus and vacuole. Most techniques to this end require the creation of microsomes. Microsomes are lipid vesicles, formed from disrupted parts of the membranes of different organelles and cell structures. Some of these will be right-side-out (RSO), others will be inside-out (ISO) [48]. This difference is important because some of the techniques for isolating PM-proteins are based on the RSO PM-polarity. These have a slightly negative polarity, while the microsomes of other membranes have a positive polarity. ISO PM-microsomes have their negative polarity inversed with the orientation of their membrane and thus will not move to the right fraction. This can amount to loss of sample in an approach that is already limited by the amount of sample.

Three classical methods of PM-fraction enrichment are (1) continuous or discontinues sucrose gradient centrifugation, (2) free-flow electrophoresis (FEE) and (3) aqueous two-phase system partitioning. Enrichment by sucrose gradient centrifugation is based on differences in size and density of PM-membranes compared to other membranes, but can suffer from considerable contamination from the other fractions. Enrichment by FEE and aqueous two-phase system partitioning are based on the polarity of PM-microsomes. Despite recent advances FEE requires the availability of specialized material and performs no better, making the third technique the most readily used and available [49]. Aqueous two-phase systems typically only results in an isolation of 70% of the PM-microsomes but the protocol can be repeated to obtain a higher degree of enrichment. However, obtaining this optimal purification requires varying PEG, dextran and KCl concentrations depending on the samples. After the enrichment and elimination of the matrix (sucrose, percol, iodixanol, or polyethylene glycol (PEG) in the case of two-phase partitioning while FEE is matrix-free) we are ideally left with the PM microsomes, containing lipids and the PM proteins, which differ in abundance, molecular weight and physiochemical properties.

1.11 Plasma membrane proteins: variation and solubilization

Plasma membrane proteins are proteins that are temporarily or permanently part of the cell plasma membrane. Numerous proteins, responsible for vital processes at the cell/environment interface, comply with this definition. The most obvious functions covered by PM-proteins are signal exchange across the plasma membrane, import and export molecules in and out of the cells, cell adhesion and cell-cell interaction [49,50]. Another function, one of particular importance in the project where this study is part of, is the development of the cell wall. All membrane proteins (PM or otherwise) can be divided into different categories, which also need to be taken into account in a proteomic investigation, namely peripheral and integral PM proteins [49]. Integral PM proteins (IMP) are

proteins that are 'permanently' part of the membrane. Peripheral PM proteins (PMP) on the other hand, only temporarily associate with the membrane e.g. trough binding IMP's, binding lipids, GPI anchors etc. Integral membrane proteins will tend to be more hydrophobic and firmly embedded in the lipid membrane (mostly by transmembrane domains), in order to stay permanently anchored. Examples of IMP's are the cellulose synthase super family and aquaporins [51,52]. Peripheral membrane proteins tend to be more hydrophilic and dissociate more rapidly from the lipid membrane. Well researched examples of PMP's are various disease resistance proteins [53]. The difference between IMP's and PMP's matters in the solubilisation of the proteins in a lab setting, as several techniques will tend to favour the solubilisation of hydrophilic PMP's, while leaving IMP's behind. On the other hand, techniques used to solubilise the strongly bound IMP's will be chemically of physically harsh and could damage the proteins. One way to deal with this, is by first purifying the soluble PMP's and depleting the sample thereof, and only later extract the IMP's [54]. Indeed, little over a decade ago, it was still considered near impossible to effectively solubilize eukaryotic integral membrane proteins for 2D gel electrophoresis due to their highly hydrophobic nature and the drawbacks of available solubilisation methods [55]. Various treatments did and do exist to break down lipid bilayers and solubilize proteins, ranging from salt treatment to the use of organic solvents and detergents, but options become more limited if the goal is a proteomic experiment involving 2D gel electrophoresis [56]. Ionic detergents, such as sodium dodecyl sulphate (SDS), are generally avoided because they mask the native charge of the proteins in favour of their own charge and thereby interfere with the first dimension of 2D gel electrophoresis. Therefor the use non-ionic (e.g. Trition X-100) or zwitter ionic detergents (e.g. Amidosulfobetaine-14 (ASB-14) and 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)) is advisable. Higher concentrations of zwitter- or non-ionic detergents can create problems too, such as interference on the gel [57].

1.12 Choice of organism: alfalfa

The organism of focus in this research was the crop alfalfa (*Medicago sativa*), also called lucerne. Alfalfa is a viable source for the production of bioethanol from lignocellulose and the most important forage crop (feed) [28]. In addition, it is an excellent soil enhancer in crop rotation due to its symbiosis with *Sinorhizobium meliloti* and its nitrogen-fixing ability [58,59]. The latter trait also drastically cuts the need for nitrogen fertilizer, which makes alfalfa even more interesting for sustainable agriculture. It's a perennial legume and can on average be cut 4 times a year by either grazing cattle or harvesting, and will regrow from the 'crown' [60]. Though technically not a fibercrop, it contains a substantial amount of lignocellulosic fibers. Apart from this, it is also fairly rich in proteins, adding to its strengths as forage crop [61]. Most studies on the subject of apoplastic and non-apoplastic anti-oxidants, in relation to cell wall remodelling or biopolymerization, as well as plasma membrane proteomics experiments, have been done on the roots and leaves of organisms such as pea (*Pisum sativum L*) seedlings [62]. Data on primary lignifying tissues, such as the stem, of actual crops in which such data may bear significant economic and agricultural relevance is still scarce.

1.13 Objectives

This research will focus on alfalfa stem tissue, because it contains substantial amounts of lignocellulosic fibers, cell wall polysaccharides and is the primary site for lignification [63]. Furthermore, no research could be found on anti-oxidative enzyme activities and metabolites in alfalfa stems. The possible link between oxidative stress, the redox-balance in the cell wall and apoplast and its effects on biopolymerization have also not been investigated that much. This gap of knowledge leads to the aim of this research: to investigate the specific enzymatic activity and presence of metabolites. We will focus on enzymes and metabolites which offer interesting overlaps between biopolymerization, presence in and around the cell wall, and oxidative stress. In addition, we look at these in the primary lignifying tissue of alfalfa, the stem tissue and expose plants to an excess of Cu and/or Zn to assess the influence of trace metal stress. In the second part of this project, an attempt was made to optimize a protocol for the extraction and enrichment of plasma membrane proteins of alfalfa stem tissue, as well as comparison between the buffers ASB-14 and CHAPS for solubilisation and 2DE.

2. Materials and Methods

2.1 Plant materials

2.1.1 Plant materials for enzyme activities and metabolites

Soil obtained from Musson, Belgium (BE-6750), was first dried and mixed, before it was filled into 192 pots (421,875 cm^3; 7,5 cm x 7,5 cm x 7,5 cm). The depth of the pots approached the level of root growth normally observed in alfalfa during the first week. The pots were divided into 4 groups of 48 pots. One group was used as control, the other groups were treated with either Zn, Cu, or both. Treatment consisted of applying 25 ml of water containing commercially available fertilization solutions CHELAL®Zn (171 µg/l) and/or CHELAL®Cu (60 µg/l) (BMS Micro-Nutrient NV, Bornem, Belgium)) to amount to concentrations of 7.5 kg/ha Zn and/or 2.5 kg/ha Cu in the soil. Such concentrations are tenfold the dose recommended by the manufacturer. Chelating agents are diethylene triamine pentaacetic acid (DTPA), ethylenediaminetetraacetic acid (EDTA) and hydroxy-2-ethylenediaminetriacetic acid (HEEDTA).

Seeds of *Medicago sativa* (Giulia) were inoculated with their symbiont *Sinorhizobium meliloti* by mixing 10 g of seeds, 80 µg of peat based inoculant and 20 µl milliQ water. After treatment of the soils, 3 seeds were planted per pot, in the first week of February 2014. Pots were placed into incubators (Fitotron SGC 120, Weiss Technik UK, Leicestershire, UK) at 22°C, on a 13h/11h day/night cycle, at 60% humidity. The pots remained covered for the first 3 days to allow the seeds to germinate in the dark. Weeds were routinely removed, and during the first week deionized water was added regularly to certain pots in quantities of 10-20 ml to homogenize the humidity of the soils. Once humidity was stable, 40 ml or 80 ml of deionized water was added on a regular basis, depending on the humidity of the soil. Plants were removed and repotted in the third and fourth week, to allow two strong looking plants per pot.

Sampling for all tests was done 2 months after sowing, giving careful consideration to randomization of incubators and plant size. The primary stems were divided into 3 equally long parts: apical, medial and basal. The stems were weighed, put in 2 ml Eppendorf tubes and frozen in liquid nitrogen. They were transported on dry ice and stored in the freezer (-70°C) until further use.

2.1.2 Plant materials for proteomics optimization

Plant materials for the optimization of (plasma) membrane proteomics consisted of greenhouse and field-grown alfalfa stems. Plants differed in age, but had all been cut several times and were several seasons old. Plants were watered regularly with tap water but humidity and light were not controlled because samples were meant merely for optimization. The majority of plants were unexposed.

2.2 Enzyme extractions and measurements (syrangaldazine peroxidase, guaiacol peroxidase, superoxide dismutase, catalase, glutathione reductase)

2.2.1 Enzyme extractions

Approximately 200 mg of samples were ground together with some sand in liquid NO_2 by use of cooled mortar and pestle, after which the powdered samples were stored in the freezer (-70°C). Enzymes were extracted by adding 2 ml of enzyme extraction buffer (5 mM EDTA, 1% polyvinylplopyrolidone (PVPP) K30, 5 mM 1,4-dithioerythritol (DTE), 1% nonidet P40 in 0,1 M Tris-HCl (pH 7,8)) to the powder and mixing the sample for 30 minutes in a rotating mixer (4°C). Next, the samples were transferred to Corning tubes and centrifuged (48 000 x g, 25 min, 4°C) to pellet debris. A volume of 1 800 µl of supernatant was transferred to a tube with 0,437 g $(NH_4)_2SO_4$ and shaken again under the same conditions. Because different proteins have different solubility in the presence of various salt concentrations, the aim of this step is to selectively precipitate the target enzymes by use of different salt concentrations. Again the samples were transferred to corning tubes and centrifuged (48 000 x g, 25 min, 4°C). A volume of 1700 µl of supernatant was transferred to a tube with 0,485 g $(NH_4)_2SO_4$, shaken, transferred to Corning tubes and centrifuged again under the same conditions. Raising the salt concentration in this manner would precipitate the target enzymes. The pellet was resolubilized in 1750 µl of 25 mM Tris-HCl (ph 7,8). The enzyme extracts were desalted using PD-10 desalting columns (GE healthcare, Little Chalfont, UK). This was done by loading the extracts onto the column and centrifuging briefly (1 600 x g, 2 min, 4°C). Subsequently the enzyme extracts were aliquoted, frozen in liquid nitrogen and stored in the freezer (-70°C). Samples for each condition constituted 4 biological replicates.

2.2.2 Measuring enzyme activity

All enzyme activities were measured optically with a spectrophotometer, based on a change of absorption induced by the changing concentrations of either substrate or products due to enzymatic activity. This substrate differed for each of the enzymes. Laccase was measured by use of syringaldazine. Although it may not be exclusively oxidized by plant laccase, there are strong indications that the cell wall peroxidases that can use syringaldazine as substrate (often referred to as syringaldazine oxidase or SPX) are involved in lignification [64]. Class III peroxidases, which are PM bound peroxidases and thus might be important for the cell wall dynamics, were measured using guaiacol and are referred to as guaiacol peroxidase (GPX) [65]. The activity of superoxide dismutase (SOD) was assessed by measuring its capacity to prevent the reduction of cytochrome C by superoxide radicals, which are generated by xanthine oxidase (XO) [66]. An additional technical replicate was measured for SOD and all SOD measurements were corrected for a blank sample. Glutathione reductase (GR) activity was measured by the decreasing absorbance that results from the oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) to nicotinamide adenine dinucleotide phosphate ($NADP^+$), on which the reduction of gluththione disulphide (GSSG) by GR is dependent. Catalase (CAT) was measured using the gradually decreasing absorbance of H_2O_2.

All buffers were prepared beforehand and stored in the fridge, the cytochrome C and xanthine solutions were stored in the freezer at -20°C. The rest of the solutions were prepared fresh each day. When measuring, the buffers were kept at 25°C, the rest of the solutions are kept on ice. The concentrations of the prepared solutions and final concentration in the cuvettes (1 ml) are shown in table 1. Solutions were added to the cuvette in the order presented in the table and the absorbance over the course of 2 minutes was measured with a spectrophotometer (UV-1600, Shimadzu, Kyoto, Japan) To calculate the activity, only the linear ranges were used.

Table 1; Concentrations of solutions for measuring enzyme activities. Solutions in blue are kept on ice, solutions in red are kept at 25°C.

Enzyme	Cuvette	Wavelength	Product	Conc. solution	Conc. cuvette
SOD	Plastic	550 nm	KH_2PO_4 (pH 7,8)	50 mM	31,5 mM
			Cytochrome C	0,1 mM	10 nM
			Xanthine	0,5 mM	50 nM
			EDTA	1 mM	0,1 mM
			extract		5%
			Xanthine oxidase	1/20	0,10%
SPX	UV	530 nm	Tris-HCl (ph 7,5)	0,1 M	85 mM
			H_2O_2	10mM	1 mM
			extract		3,00%
			SAZ (3,6mg in 1ml MeOh, 2ml Dioxane)		1,7%
GPX	Plastic	436 nm	KH_2PO_4 (pH 7)	0,1 M	75 mM
			H_2O_2	8 mM	0,8 mM
			Extract		5%
			guaiacol	18 mM	1,8 mM
GR	plastic	340 nm	Tris-HCl (pH8)	0,1 M	89 mM
			EDTA	1 M	0,89 M
			GSGG	81,6 mM	1,4 mM
			NAPDH	6,7 mM	118 µM
			Extract		10%
CAT	UV	240 nm	KH_2PO_4 (pH 7)	0,1 M	73 mM
			H_2O_2	5 mM	0,85 mM
			Extract		10%

2.3 Extraction and measurement of acsorbate peroxidase activity

2.3.1 Ascorbate peroxidase extraction

Approximately 50 mg of stem material was ground with some sand and PVPP in 500 µl ice-cold APX extraction buffer (0,1 M Tris-HCl (pH 7,8), 1 mM DDT, 1 mM EDTA, 10 mM AsA) using cooled mortars and pestles. The samples were centrifuged and the supernatants transferred to new tubes.

After this the measurements were done immediately. Samples for each condition constituted 4 biological replicates.

2.3.1 Ascorbate peroxidase activity measurement

Ascorbate peroxidase (APX) was measured by the absorbance of H_2O_2, which is neutralized by APX in the presence of ascorbate. The HEPES-EDTA buffer was prepared beforehand, stored in the fridge and kept at 25°C for measurements. H_2O_2-solutions were prepared daily and kept on ice. The concentrations of the solutions and final concentrations in the cuvette are shown in table 2. Solutions were added to the cuvette in the order presented in the table and measured with a UV-1600 spectrophotometer for 2 minutes. Activities were calculated using the slope of the linear part of the absorbance profile.

Table 2; Concentrations of solutions for measuring ascorbate peroxidase activity. Solutions in blue are kept on ice, solutions in red are kept at 25°C.

Enzyme	Cuvette	Wavelength	Product	Conc. solution	Conc. cuvette
APX	UV	298 nm	HEPES (pH 7)	0,1M	86,7 mM
			EDTA	1mM	0,867 mM
			H_2O_2	20 mM	0,66 mM
			Extract		10,00%

2.4 Extraction and measurement of total and reduced ascorbate

Approximately 120 mg of stem material was ground in liquid nitrogen by use of mortar and pestle, after which 800 µl of 200 mM HCl acidic AsA extraction buffer was added and the sample was crushed further. After thawing, the sample was transferred to a tube and centrifuged (16 000 x g, 10 min, 4°C). Three hundred µl of the supernatant was saved in a new tube. To adjust the sample pH, 30 µl of 200 mM NaH_2PO_4 (pH 5,6), followed by an adequate volume of 200 mM NaOH were added to obtain pH 4,5. Samples were divided into two fractions, one of which was used to measure reduced ascorbate.

The second fraction was used to measure AsA, for which oxidized AsA first had to be reduced by adding 140 µl of 120 mM NaH_2PO_4 (pH 7,5) and 10 µl 25 mM DTT to 100 µl extract. Samples were then vortexed and incubated for 15 min at 20°C. Finally, the pH was adjusted to 5,5, using adequate volumes of 200 mM HCl..

For both reduced and total AsA, buffer and samples were pipetted on a UV micro plate and absorbance of the blank was measured. Next, ascorbate oxidase (AO) was added and absorbance was measured again. Ascorbate oxidase oxidizes all AsA to non-absorbing dehydroascorbate (DHA). All solutions were kept on ice.

The concentrations of stock solutions and in the wells of the UV microplate are shown in table 3. Solutions were pipetted in the order presented in the table into a 96-well plate and measured in the plate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany). Four biological replicates were measured in 3 technical replicates.

Table 3; Concentrations of solutions for measuring reduced and total ascorbate. All solutions were kept on ice.

Metabolite	Microplate	Wavelength	Product	Conc. solution	Conc. cuvette
Reduced AsA	96 well, UV	256 nm	NaH_2PO_4 (pH 5,6)	71 mM	60,3 mM
			extract		10,00%
			AO	40 U/ml	2U/ml
Total AsA	96 well, UV	256 nm	NaH_2PO_4 (pH 5,6)	71 mM	53,2 mM
			Incubated extract		20,00%
			AO	40 U/ml	2U/ml

2.5 Early attempts at membrane protein extraction and PM enrichment

An attempt was made to optimize a protocol for the extraction and enrichment of cell membrane proteins from alfalfa stem tissue by two-phase partitioning, starting from a protocol by Nouri et al. (2010) [67].

2.5.1 Extraction of microsomes

Twenty grams of plant stems were ground with mortar and pestle in liquid nitrogen in duplicate. Samples were divided in 8 x 50 ml-tubes with approximately 5 g of ground plant material, and stored at -24°C for later use. Plant material was rehomogenized in 200 ml microsome extraction buffer (0.4 M sucrose, 75 mM 3-(N-morpholino)propanesulfonic acid (MOPS)/KOH (pH 7,6), 5 mM EDTA/KOH (pH 7,5), 5 mM ethylene glycol tetraacetic acid (EGTA)/KOH (pH 8,2), 10 mM KF, 1 mM DTT and 2% polyvinylpolypyrrolidone-40 (PVP-40), filtered through 3 layers of Miracloth and centrifuged (4 500 x g, 15 min, 4°C). The supernatant was ultracentrifuged (235 000 x g, 45 min, 4°C) (Optima^{TM} L-90k Ultracentrifuge, Beckman Coulter, Brea, CA, US), after which the pellets were re-suspended in 3 ml microsome suspension buffer (0,33 M sucrose, 5 mM KH_2PO_4/K_2HPO_4 buffer (pH 7,8) and 3 mM KCl) and subsequently diluted to 9 ml of the same buffer.

2.5.2 PM-enrichment using PEG/dextran aqueous two-phase seperation

The PM-fraction enrichment was attempted by polyethylene glycol (PEG) 3350 and dextransulphate two-phase separation. Dextran is denser and will form a hydrophilic lower phase, while PEG will form a hydrophobic upper phase. Because of the polarity of RSO PM-microsomes, they will collect in the PEG upper phase, while other microsomes collect in the lower phase.[49] A 6,2% PEG/dextran sulphate solution was prepared in microsome suspension buffer and 9 ml of microsome suspension was loaded onto 27 ml of the phase system, followed by centrifugation (2 450 x g, 4 min, 4°C).

Additionally, 27 ml of the phase system without sample was centrifuged under the same conditions to obtain fresh upper and lower phases for repeating the enrichment step. However, no phase separation was observed in the loaded system or in the unloaded system.

2.5.3 Precipitation of proteins

An attempt was made to precipitate any protein still in the phase system with a 20% w/v trichloric acid (TCA) in icecold acetone, followed by centrifugation (13 000 x g, 30 min, 4°C). Subsequently pellets were washed 3 times with ice cold acetone and centrifuged (13 000 x g, 30 min, 4°C). Samples were solubilized in 100 µl membrane buffer (20 mM Tris-HCl (pH 7,5), 0.25 M sucrose).

2.5.4 Automatic sample blending

The protocol was repeated a second time using a blender for both homogenization steps. Because of the fibers in alfalfa stem tissue, this was not successful. Careful attention was given so as not to pipet the sunken PVP-40 in the sample preparation onto the phase-system. Again no phase separation was observed.

2.6 Extraction of membrane and soluble protein fractions

A protocol was attempted, based on Song et al. (2011), to attempt to extract the entire membrane proteome (non-enriched) [68].

2.6.1 Extraction and solubilization of microsomes to obtain total membrane protein fraction

Fifteen g of fresh field grown alfalfa stems were ground in duplicate by use of mortar and pestle in liquid NO_2, with 1% w/w polyvinylpolypyrrolidone to bind phenolic compounds. The resulting powder was homogenized in 75 ml microsome extraction buffer (0,5 M Tris-HCl (pH 8,5), 0,7 M sucrose, 0,1 M KCl, 0,05 M EDTA, 2% β-mercaptoethanol, 0,1% PIC (protease inhibitor cocktail)) , centrifuged (12 000 x g , 4 min , 4°C) and filtered through a Duran nr.3 filter funnel (16-40 µm) with the help of a Büchner flask. The filtrate was diluted with an equal volume of cold milliQ water and ultracentrifuged (150 000 x g , 30 min , 4°C). The resulting microsomal pellet was washed carefully three times with cold milliQ water. Instead of re-suspending the pellet as previously described for enrichment by aqueous two-phase separation, the pellet was immediately solubilized in 5 ml SDS-buffer (0,5 M Tris-HCl (pH 8,5), 2 % β-mercaptoethanol, 30 % glycerol, 4 % SDS) and heated for 5 min to 80°C to improve solubilization.

2.6.2 Purification, precipitation and solubilization of proteins

Any remaining insoluble debris was pelleted by centrifugation (12 000 x g , 30 min , ambient temp.) and the supernatant was further purified. An equal volume of Tris-buffered phenol was

added to the sample, and an equal volume of microsme extraction buffer was added to this mixture. The mixture was vortexed thoroughly and centrifuged (2 000 x g , 5 min , 4°C). The resulting upper phase (containing phenols and proteins) was saved, diluted once again with an equal volume of microsome extraction buffer and centrifuged. This purification step was repeated three times. The final upper phase was diluted in 5 x volume methanol/0,1 M ammonium acetate and placed in the freezer overnight to precipitate the proteins. Next, the samples were centrifuged (12 000 x g , 10 min , 4°C) and the resulting pellet washed three times with 90% methanol and once with 90% aceton. The pellet was dried at ambient temperature and re-suspended in 200 µl ASB-14 buffer (20 mM Tris-HCl (pH 8,5) 2% ASB-14, 7 M Urea, 2 M Thiourea, 100 mM DTT).

2.6.3 Extraction of soluble fraction proteins

Alternatively, the protocol based on Song et al. was applied on the supernatant after ultracentrifugation, rather than the pellet, to determine whether membrane proteins remain in the supernatant, that primarily contains the soluble proteins. Of the 250 ml collected supernatant (125 per duplicate), 3 times 20 ml was added to 30 ml 20% w/v TCA in ice-cold acetone and left in the freezer overnight to precipitate the proteins. The samples were then centrifuged (4 850 x g , 30 min , 4°C) to pellet the proteins. The pellet was washed by adding cold acetone, vortexed and centrifuged (4 850 x g , 30 min , 4°C) three times. The final pellet was solubilized in 200 µl of the same ASB-14-buffer.

2.6.4 Separation on 1D SDS-PAGE and analysis of proteins

Membrane and soluble proteins were separated by SDS-PAGE and stained with InstantBlue (Expedeon Ltd., Cambridgeshire, UK) (see 7.1 Protein separation by 1D SDS-PAGE; 7.3 Staining gels with InstantBlue and Lavapurple). Five protein bands were cut from the gel with membrane fraction and digested for analysis by the Orbitrap LC/MS (see 7.4 Digesting bands/spots for analysis with MALDI TOF/TOF MS/MS or Orbitrap LC/MS) (Thermo Scientific OrbiTrap, Thermo Fisher Scientific, Waltham, MA, US) (Dionex Ultimate 3000 nano HPLC, Thermo Fisher Scientific). The Orbitrap LC/MS is another system for analyzing proteins based on mass spectra. In Orbitrap LC/MS, samples first get separated by high performance liquid chromatography (HPLC) in a liquid or "mobile" phase. Subsequently they get injected by electrospray-ionization into the Orbitrap, where their axial oscillations around and back and forth up an electrode (a balance between centrifugal forces and their electrostatic attraction to the electrode, which are dependent on mass-to-charge ratio) are detected [69].

2.7 Membrane protein extraction and PM enrichment using 6,4% PEG/dextran phase separation

The protocol based on Song et al (2001) was repeated a third time, adding the PEG/dextran enrichment after obtaining the microsomal pellet (see 2.6 Extraction of membrane and soluble protein fractions).

2.7.1 Extraction and re-suspension of microsomes

The microsomes were extracted conforming to the protocol described earlier. The final solubilization step was omitted. Instead, re-suspended in 9 g microsome suspension buffer (5 mM KH_2PO_4 (pH 7,8), 330 mM Sucrose, 2 mM DTT, 10 mM NaF) immediately after washing. In order not to overload the phase system, the equivalent of 20-40 g of fresh weight can be resuspended in 9 g microsomal suspension buffer. (see 2.6.1 Extraction and solubilization of microsomes to obtain membrane protein fraction)

2.7.2 PM enrichment using 6,4% PEG/dextran phase separation

The enrichment with 6,4% PEG-3350 and 6,4% Dextran T-500 was based on an extensive protocol by Santoni et al. (2006) [70]. Microsomal pellets were re-suspended in microsome suspension buffer to make 9 g of microsome suspension from 30 g of fresh weight plant material. The composition of the two-phase system is shown in table 4.

Table 4; Composition of the PEG/dextran aqueous two-phase system for PM enrichment. All components were prepared separately, stored in the fridge and mixed right before centrifugation in the cold room.

	Product	Conc. Stock	Mix	Final conc.
27 g phase system	Dextran T-500	20% (w/w)	11,82 g	6,40%
	PEG-3350	40% (w/w)	5,76 g	6,40%
	K_2HPO_4	0,2 M (pH 7,8)	0,9 ml	5 mM
	KCl	2 M	0,09 ml	5 mM
	Sucrose	1,6 M	6,74 ml	300 mM
	H2O	add to 27 g		
loaded	Sample	9 g (equivalent to 20 - 40 g fresh weight)		
unloaded	microsome suspension buffer	9 g		

Two 27 g phase systems were prepared. To one ("designated as loaded") 9 g of sample in the form of microsome suspension was added and to the other (designated as "unloaded"), 9 g of microsome suspension buffer was added. The tubes were shaken vigorously and centrifuged (2 450 x g , 4 min , 4°C), resulting in two distinct phases. The loaded phases were separated and fresh unloaded lower and upper phases were added to further purify the fractions. These fractions were purified a third time and pooled. Both fractions of both steps were saved and pooled, resulting in 30 ml PEG-upper phase with PM microsomes and 30 ml Dextran lower phase with the remaining microsomes. Each pooled fraction was diluted with 110 ml milliQ water and was

ultracentrifuged (38 800 x g , 1 h , 4°C) to pellet the microsomes. Subsequently, pellets were dissolved in 10 ml SDS-buffer and heated for 10 min to 70°C to augment solubilization.

2.7.3 Purification, precipitation and solubilization of proteins

Purification of the samples from PEG and other inorganic contaminants was done using a Tris-buffered phenolic extraction. Subsequently protein samples were precipitated and solubilized in 200 µl ASB-14 buffer. (see 2.6.2 Purification and precipitation of proteins)

2.7.4 Separation on 1D SDS-PAGE and analysis of proteins

Proteins were separated by SDS-PAGE and stained with LavaPurple (Gel Company, San Fransisco, CA, US) (see 7.1 Protein separation by 1D SDS-PAGE). Subsequently the gel was scanned with Typhoon FLA 9000 (GE Healthcare) and analyzed with ImageQuant (GE Healthcare) (see 7.3 Staining gels with InstantBlue and Lavapurple).

2.8 Membrane protein extraction and PM enrichment using 6,0% PEG/dextran phase separation

The protocol was repeated a second time with a 6,0% PEG/dextran system, as it was reported that the concentrations of PEG and Dextran can alter the degree of enrichment, and concentrations between 5,4% and 6,4% were reported to be used in other research.

2.8.1 Extraction and re-suspension of microsomes

The microsomes were extracted conforming to the protocol described earlier. The final solubilization step was omitted. Instead, pettets were washed and subsequently re-suspended in 9 g microsome suspension buffer (see 2.6.1 Extraction and solubilization of microsomes to obtain membrane protein fraction).

2.8.2 PM enrichment using 6,4% PEG/dextran phase separation

Again a 20% Dextran-solution was made from 11 g Dextran T-500 and 39 g milliQ water. This way, a water content of 5% is estimated and taken into calculation because of the hygroscopic nature of Dextran. From this solution, 5 g was carefully diluted in 25 ml water and its optical rotation analyzed by use of a polarimeter. An optical rotation of 7,25° was measured. The calculated true concentration from this was 18,2%, meaning the hygroscopic character of Dextran greatly influences the preparation of solutions on basis of weight (1,2% of 20% constitutes an error of 9%, on top of the 5% that is already assumed) [71]. Thus, the use of a polarimeter is advised, in addition to preparing a large batch of 20% Dextran for real experiments. Of this dextran solution, 11,86 g was mixed with 5,4 g of the 40 % PEG solution in the 27 g phase system. The volumes and

weights of the rest of the products in the phase-system remain the same, since milliQ water is added at the end to make up 27 g (see Table 4).

2.8.3 Purification, precipitation and solubilization of proteins

Purification of the samples from PEG and other inorganic contaminants was done using a Tris-buffered phenolic extraction. Subsequently protein samples were precipitated and solubilized in 200 µl ASB-14 buffer. (see 2.6.2 Purification and precipitation of proteins)

2.8.4 Separation on 2D-PAGE and analysis of proteins

The rest of the protocol was identical to the previous one. This time, a 2D gel was run for greater resolution (see 7.2 Protein separation by 2D gel electrophoresis). Gels were stained with LavaPurple and scanned with Typhoon FLA 9000 (GE Healthcare) (see 7.3 Staining gels with InstantBlue and Lavapurple). The analysis was done with DeCyder (GE Healthcare).

2.9 Comparison membrane protein extraction and solubilization zwitterionic detergents CHAPS and ASB-14

The protocol used to obtain the entire membrane fraction based on Song et al. (2011) was repeated and a comparison was made between the zwitterionic detergents CHAPS and ASB-14. The duplicates of the samples were therefor finally solubilized in different buffers.

2.9.1 Extraction and solubilization of microsomes to obtain total membrane protein fraction

The microsomes were extracted and solubilized conform the protocol described earlier (see 2.6.1 Extraction and solubilization of microsomes to obtain membrane protein fraction).

2.8.3 Purification, precipitation and solubilization of proteins

Purification of the samples from PEG and other inorganic contaminants was done using a Tris-buffered phenolic extraction. Subsequently protein samples were precipitated conform the protocol described earlier(see 2.6.2 Purification and precipitation of proteins). To make a comparison, proteins were solubilized in 200 µl of 4% CHAPS (plus 7 M Urea, 2 M Thiourea, 100 mM DTT) or 4% ASB-14 (plus 7 M Urea, 2 M Thiourea, 100 mM DTT)

2.8.4 Quantification, separation on 2D-PAGE and analysis of proteins

Protein concentrations were measured by use of 2D Quant Kit (GE Healthcare). Because the samples appeared to be dirty, they were centrifuged (16 000 x g, 10 min, 4°C) and the supernatant and pellet were separated. This was done because what appeared to be a remaining

insoluble fraction would create interference on the 2D gel and possibly with the 2D Quant Kit. Protein quantification using 2D Quant kit was carried out on the supernatant, and only the supernatant was used for loading on a 2DE.

2D gels were run and stained with Lavapurple (see 7.2 Protein separation by 2D gel electrophoresis; 7.3 Staining gels with InstantBlue and Lavapurple; 7.4 Digesting bands/spots for analysis with MALDI TOF/TOF MS/MS or Orbitrap LC/MS). Proteins of interest were identified by MALDI TOF/TOF MS/MS using peptide mass fingerprinting and fragmentation (10 peaks per spectrum).

2.10 Protein quantifications

Protein quantifications were done by various methods to various ends.

2.10.1 Protein quantifications of enzyme extracts using Bio-Rad Protein Assay

Protein content of enzyme extracts was determined as a possible normalization factor for enzyme extracts by use of the Bio-Rad Protein Assay, based on the Bradford method [72]. Bradford Dye Reagent (Bio-Rad Laboratories, Hercules, CA, US) was diluted 1/5 times and stored in the dark on ice. Eight BSA standards of concentrations ranging from 0 to 250 µg/µl BSA were prepared from a 250 ng/µl BSA stock solution. Twenty µl of standard or sample were pipetted into a flat-bottom 96-well plate and 180 µl of dye reagent was added. Standards and samples were pipetted in triplicates. After shaking, absorbance at 595 nm was measured in a plate. Only concentrations that fell in the linear range of the standard curve were accepted. If not, samples were diluted in their original buffer and measured again.

2.10.2 Protein quantifications of proteomics experiments using 2D Quant Kit

Protein concentrations of the proteomics experiments were originally also done with Bio-Rad Protein Assay, but this assay was later found to be incompatible with the buffers used to solubilize proteins for SDS-PAGE or 2D-PAGE. Solubilization buffers contained urea, thiourea and DTT at concentrations slightly higher than the tolerance of the Bio-Rad Protein assay, and the concentrations of detergents required to solubilize membrane proteins (up to 4% ASB-14 and CHAPS) severely affected the estimated protein concentrations. These quantifications are omitted from the results in this dissertation.

Thus, proteins concentrations were later quantified with the alternative 2D Quant Kit (GE Healthcare), based on the Lowry method [73]. The 2D Quant Kit does not suffer the same limitations as the Bradford method, since the buffer is completely eliminated from the sample. This is done by precipitating and re-suspending the proteins prior to the coloration, effectively eliminating most if not all possible interfering components. Samples were prepared and absorbance was measured at

480 nm with a spectrophotometer (DU® 800, Beckman Coulter) in accordance with the protocol provided by the manufacturer.

2.11 Statistics

Statistical analysis was done with Minitab® 16.2.3 (Minitab, State College, PA, US) and GraphPad (GraphPad Software, La Jolla, CA, US). Outliers were detected with the Grubb's test (95% CI) in GraphPad and eliminated from the population. Subsequently, normality was tested with an Anderson-Darling test (95% CI) and homoscedasticity with Bartlett's (95% CI) and Levene's tests (95% CI). Data which was both normal and homoscedastic was analyzed with a parametric one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Data which failed to meet the normality or homoscedasticity criteria, was analyzed with non-parametric Kruskall-Wallis' and Mood's Median test. An attempt was made to normalize data which failed the criteria of normality and homoscedasticity by a log-transformation, enabling the use of parametric tests, but results were not found to be superior to untransformed data.

3. Results

3.1 Enzyme activities

The activity measured is the total capacity of the enzymes, since an excess of substrate is added. Enzymatic activity was calculated using the law of Lambert-Beer : $\frac{\Delta c}{\Delta t} = \frac{\Delta E_\lambda}{\Delta t} . \frac{1}{\varepsilon_\lambda . d}$ ($\frac{\Delta c}{\Delta t}$ = the change in concentration of the detectable reaction product, $\frac{\Delta E_\lambda}{\Delta t}$ = the change in measured absorption over time, ε = the reaction product-specific molar absorption coefficient, d = the length of the path of the light). No significant effects were observed due to low effects and high standard deviations. Correcting activity for fresh weight gave overall lower standard deviations than correcting for protein concentration. For catalase, no good measurements could be obtained.

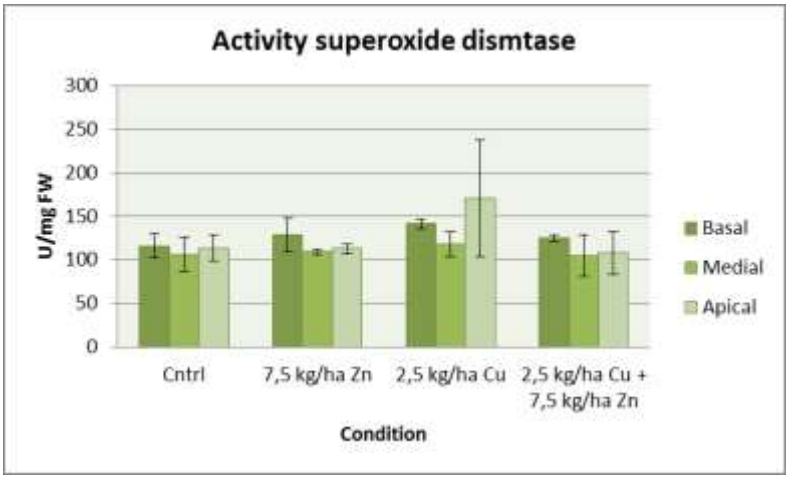


Figure 2; Activity of superoxide dismutase in three parts of the stem exposed to excess 7,5 kg/ha Zn and/or 2,5 kg/ha Cu and a control. No significant effects were observed.

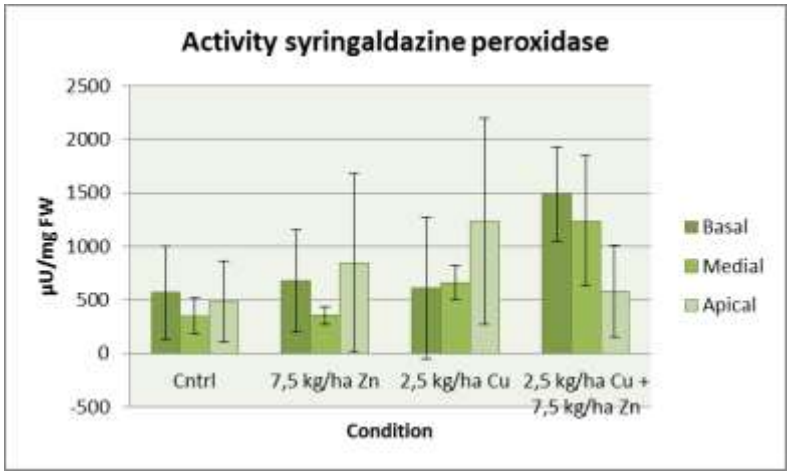


Figure 3; Activity syringaldazine peroxidase in three parts of the stem exposed to excess 7,5 kg/ha Zn and/or 2,5 kg/ha Cu and a control. No significant effects were observed.

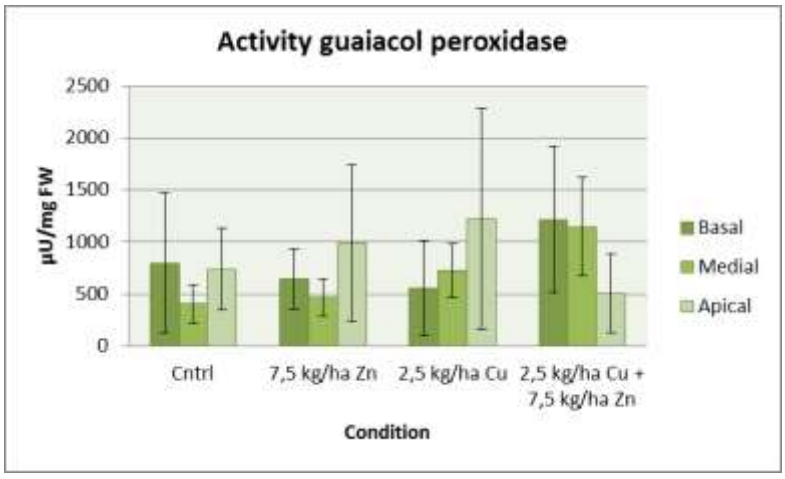


Figure 5; Activity of guaiacol peroxidase in three parts of the stem exposed to excess 7,5 kg/ha Zn and/or 2,5 kg/ha Cu and a control. No significant effects were observed.

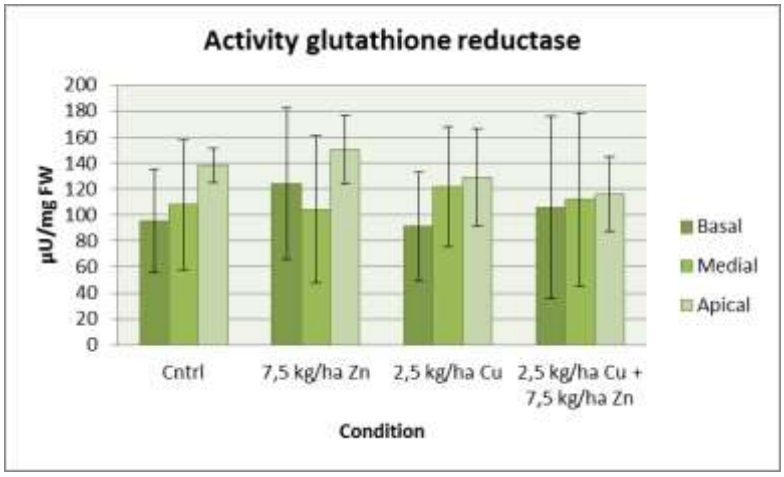


Figure 4; Activity of glutathione reductase in three parts of the stem exposed to excess 7,5 kg/ha Zn and/or 2,5 kg/ha Cu and a control. No significant effects were observed.

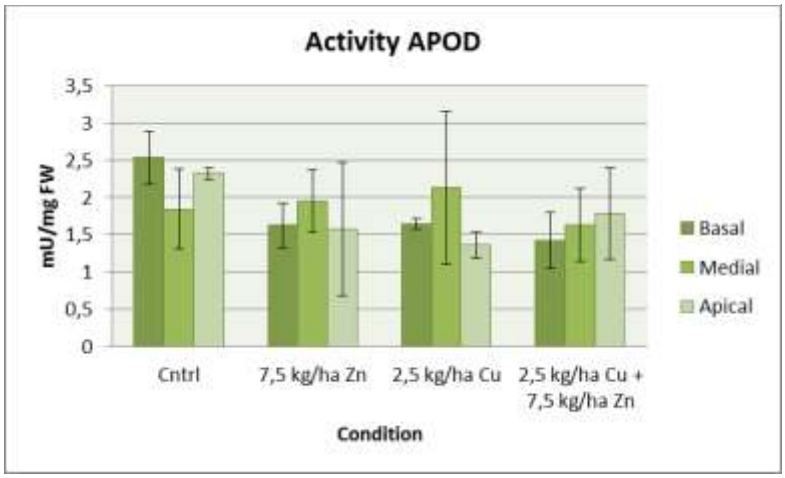


Figure 7; Activity of syrangaldazhine peroxidase in three parts of the stem exposed to excess 7,5 kg/ha Zn and/or 2,5 kg/ha Cu and a control. No significant effects were observed.

3.2 Total ascorbate determination

Ascorbate concentration is calculated using the law of Lambert-Beer (see 3.1 Enzyme activities). Reduced AsA could not be measured consistently.

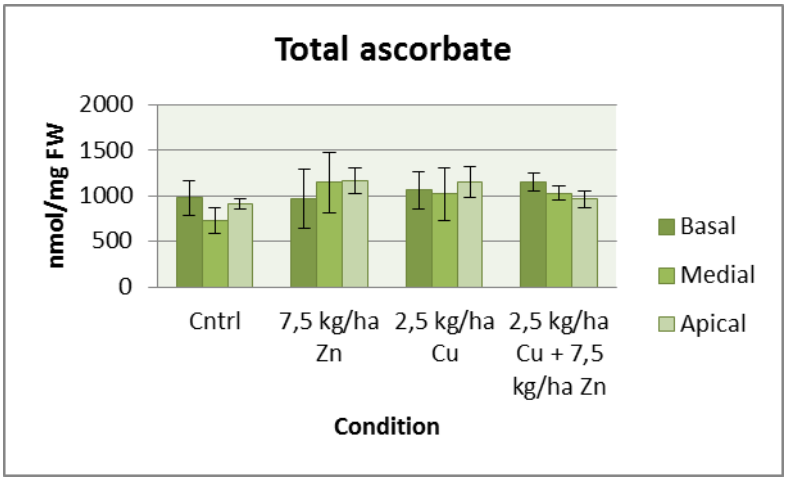


Figure 8; Total ascorbate content in three parts of the stem exposed to excess 7,5 kg/ha Zn and/or 2,5 kg/ha Cu and a control. No significant effects were observed.

3.3 Failure of phase separation in PEG/dextran sulphate phase system

Following the protocol of Nouri et al. (2010), no phase separation was observed and despite efforts to precipitate proteins from the loaded unseparated phase systems no results worth reporting were found.

3.4 Extraction of membrane and soluble protein fractions

3.4.1 Separation of membrane and soluble proteins by 1D SDS-PAGE

Two Criterion TM XT 1D gel 12% Bis-Tris precast gels (Bio-Rad) were run to separate proteins of both the membrane and soluble fraction.

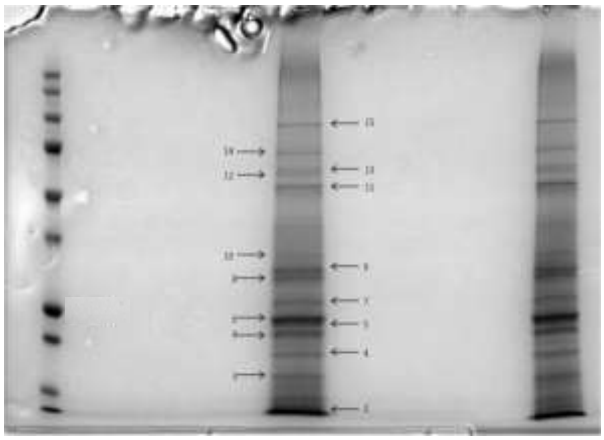


Figure 8; 1D gel of membrane protein extracts according to the protocol based on Song et al (2001), stained with InstantBlue. The molecular ladder is Precision Plus Protein™ Unstained Standard.

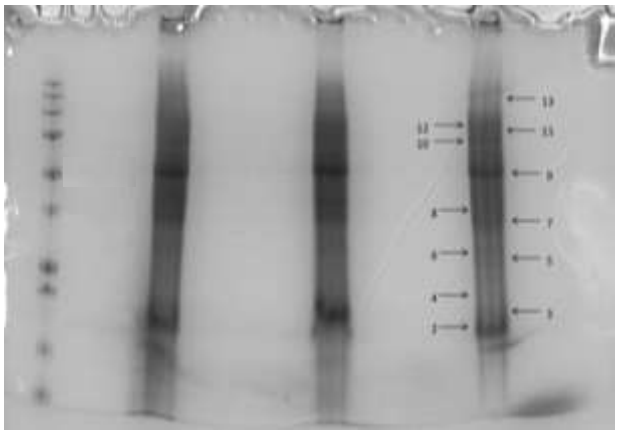


Figure 9; 1D gel of soluble protein extracts according to the protocol based of Song et al (2001), stained with InstantBlue. The molecular ladder is Precision Plus Protein™ Unstained Standard.

3.4.2 Analysis of membrane fraction proteins

For the membrane fraction gel, 5 of 15 bands were cut out and analyzed with Ortbitrap LC/MS to have some idea of the nature of the proteins extracted by this protocol (see Figure 8). The complete list of identified proteins can be found in the supplemental information. Of the 72 identified proteins, 83% were membrane proteins and 17 % were cytoplasmic or of unknown location. The sub-cellular location of the identified membrane proteins is shown in Figure 10. The families of identified proteins (membrane and non-membrane) are shown in Figure 11. Annotation was done primarily with Thermo ProteomeDiscoverer (Thermo Fisher Scientific) supported by freeware online tools, the foremost of which was InterProScan 5 (EBML-EBI, Cambridge, UK). Most membrane proteins found are part of the chloroplastic and mitochondrial membranes, and their functions are part of the chloroplastic or mitochondrial electron transport chain complexes and ATPase complex (see Figures 10 and 11). The plasma membrane proteins that are found are associated with the fusion of endosomes , namely small GTPase, and the anchoring of the cytoskeleton, actin-related protein.

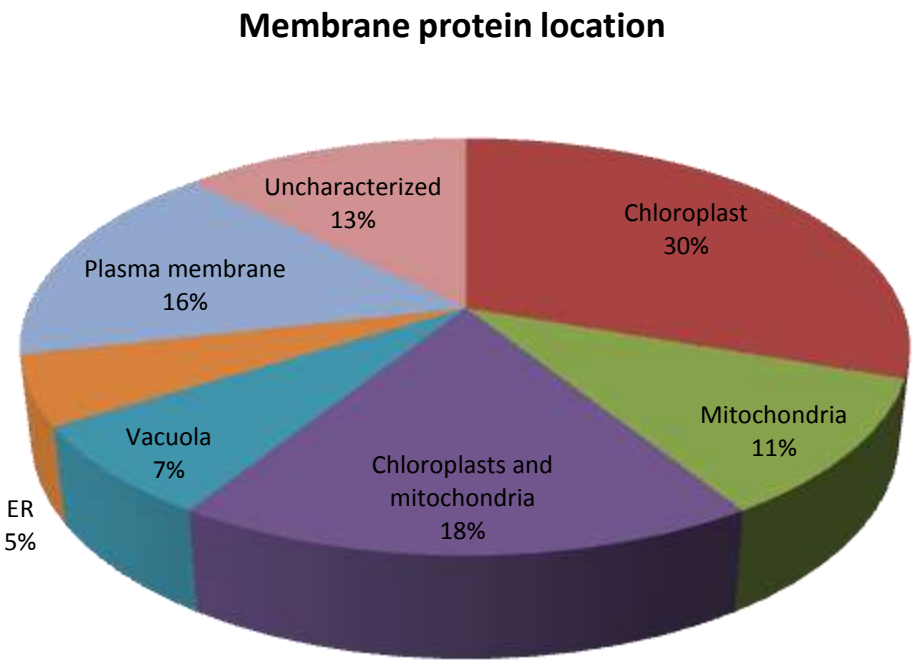


Figure 10; Sub-cellular location of membrane proteins. Most membrane proteins are chloroplastic and/or mitochondrial.

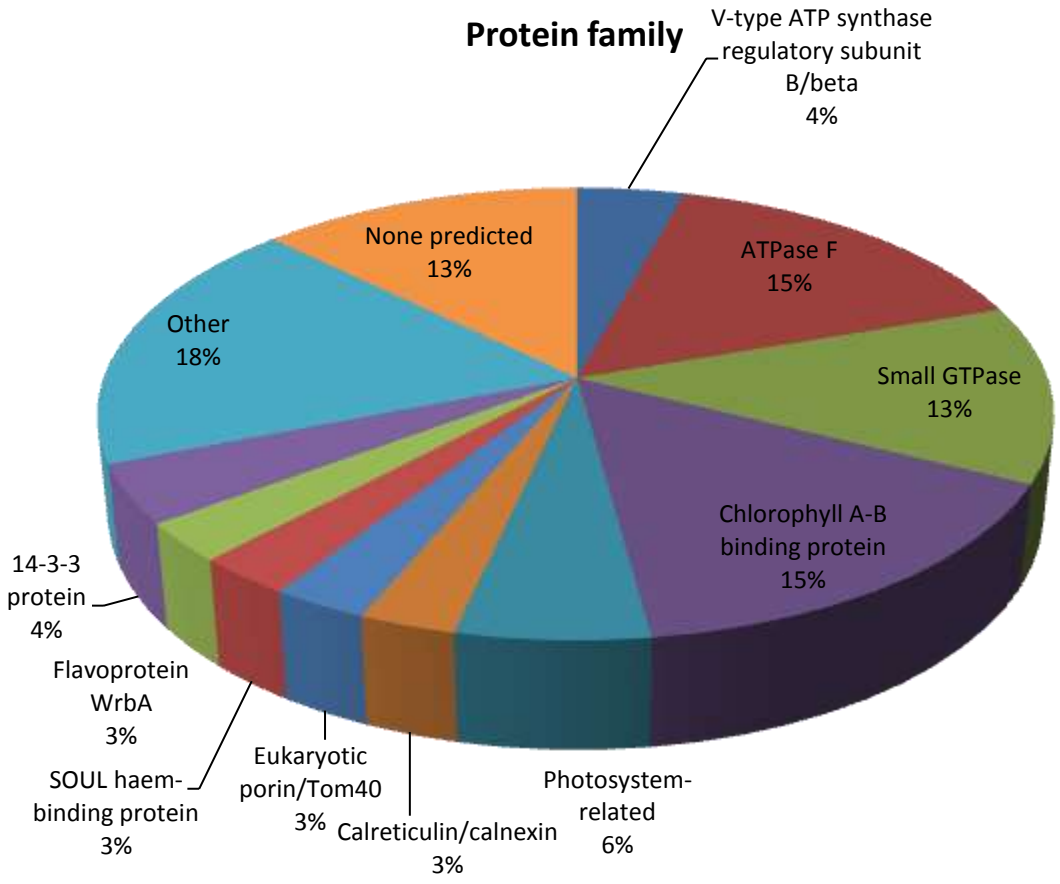


Figure 11; Protein families of the identified proteins. Most proteins are involved in photosynthesis and the electron transport chain.

3.5 PM enrichment by 6,4% PEG/dextran phase separation on 1D PAGE

The first successful phase separation was achieved with a 6,4% PEG-dextran phase system. Samples were quantified and separated on gel.

3.5.1 Quantification of protein concentrations

The PEG-fraction contained 16,14 µg protein / g fresh weight and the dextran fraction contained 47,83 µg protein / g fresh weight.

3.5.2 Separation by 1D SDS-PAGE and analysis

A Criterion TM XT 1D 12% Bis-Tris precast gel was run with the enriched PEG- and dextran-fraction in duplicate, plus a molecular ladder. The gel was stained with LavaPurple and scanned with the Typhoon FLA 9000. It is shown in Figure 3. Patterns were matched in ImageQuant and found to be identical for the PEG- and dextran-fraction. Since the density of the PEG-phase is the main determinant in the successful enrichment of PM-membranes subsequent trials were done, changing the percentage dextran, to optimize the enrichment.

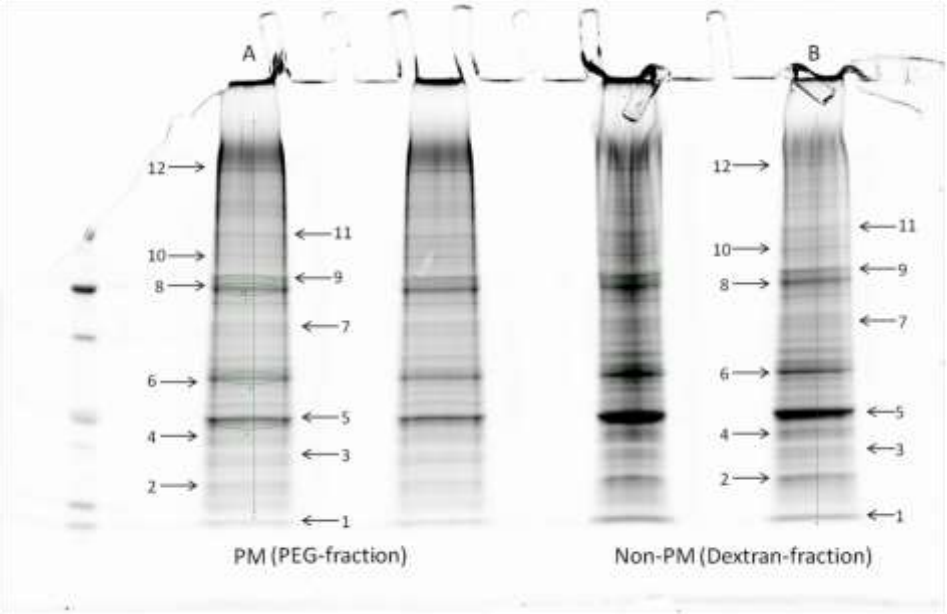


Figure 12; 1D gel of membrane protein extracts according to the protocol based of Song et al (2001) and Santoni et al. (2006), stained with LavaPurple. The molecular ladder is Precision Plus Protein™ Prestained Standard. Similar patterns are observed in both fractions.

3.6 PM enrichment by 6,0% PEG/dextran phase separation on 2D-PAGE

The previous protocol was essentially repeated with lower PEG/Dextran concentrations, followed by a 2D gel for greater resolution.

3.6.1 Quantification of protein concentrations

The PEG-fraction contained 37,9 µg protein / g fresh weight and the dextran fraction contained 46,92 µg protein / g fresh weight. Lowering the PEG/dextran concentrations elevated the protein concentration after phase separation in the PEG-fraction, but not in the dextran fraction.

3.6.2 Separation by 2DE and analysis

The 2D gels, stained with LavaPurple and scanned with the Typhoon FLA 9000 are shown in Figure 6 and 7. Using DeCyder, 110 spots were matched. The umatched spots of the PEG-fraction were in such a position that their absence on the gel of the dextran-fraction is probably due to a problem with the strip loaded with this fraction, which started heating by the end of the first dimension. Consequently it was removed from the IEF 2 hours before the end of the IEF program.

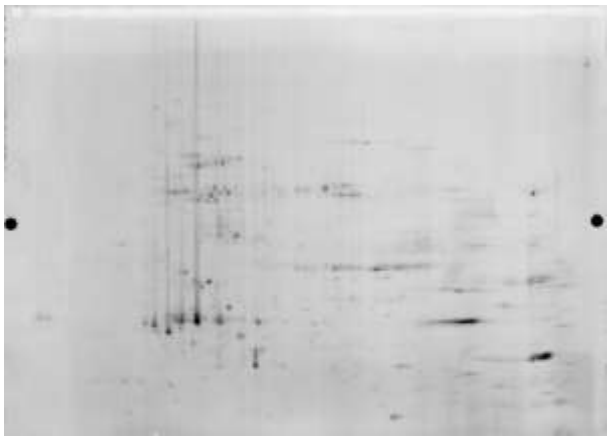


Figure 13; 2D gel of the PEG-fraction, according to the protocol based of Song et al (2001) and Santoni et al. (2006), stained with LavaPurple.

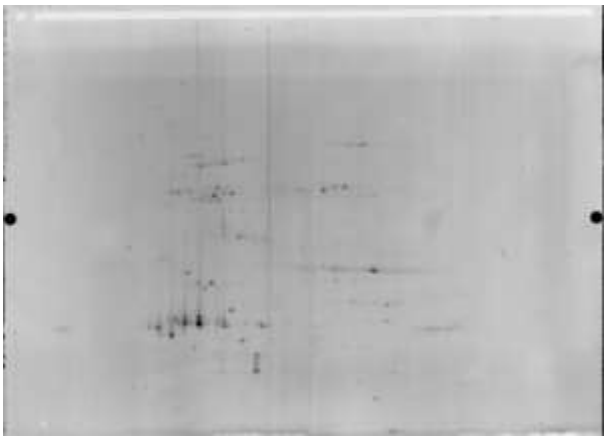


Figure 14; 2D gel of the dextran-fraction, according to the protocol based of Song et al (2001) and Santoni et al. (2006), stained with LavaPurple. Notice the blank area due to problems with the 1st dimension.

Several other optimization steps were performed but the results were not superior to those described here. Given the fact that little time remained within the frame of this study it was decided to restart working with the initial protocol. While the use of this protocol does not allow the enrichment in PM-proteins, the results obtained indicate that a fraction significantly enriched in membrane proteins was obtained (see 7.5 Complete list of annotated proteins). A literature survey indicated that the detergent used for solubilizing the extracted membrane proteins significantly affects the number and identity of the proteins that can be separated. The detergents most generally cited for this are CHAPS and ASB-14, both were used in a side-by-side comparison.

3.7 Comparison membrane protein extraction and solubilization with zwitterionic detergents CHAPS and ASB-14

3.7.1 Quantification of protein concentrations

The protein yield was 226 µg protein / g fresh weight for the extract solubilized in buffer containing ASB-14 and 220 µg protein / g fresh weight for the extract solubilized in CHAPS-containing buffer. However, because of concerns of interference of what appeared to be an insoluble fraction with the 2D Quant Kit and on gel, samples were centrifuged and the 2D Quant Kit was repeated on the supernatant. With the supernatant, protein yield was 32 µg protein / g fresh weight in the ASB-14-solubilized extract and 43 µg protein / g fresh weight in the CHAPS-solubilized extract. A concern however is that what is in the pellet may be the harder to solubilize membrane fraction , while the supernatant may consist primarily of easy to solubilize remnant cytoplasmic proteins.

3.7.2 Separation by 2DE and analysis

Two 2D-gels were run with the supernatant of the extracts (Figurs 15 and 16). Gels were matched in DeCyder and showed a high similarity. Some streaking is observed, as well as distortion of the gel. The gel using CHAPS as lysis buffer appears to be less distorted than the gel using ASB-14.

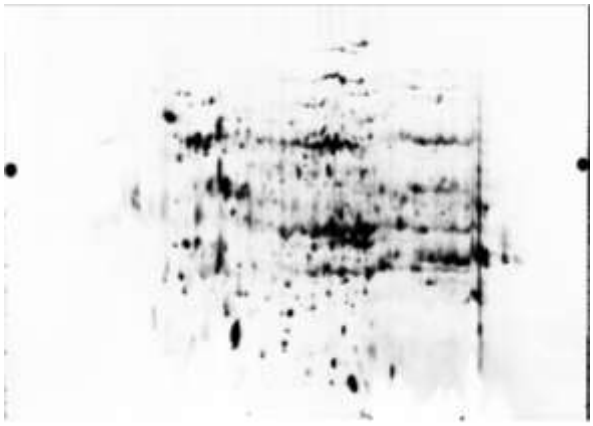


Figure 15; 2D gel using 4% ASB-14 lysis buffer, stained with Lavapurple

Figure 16; 2D gel using 4% CHAPS lysis buffer, stained with lavapurple

Forty-eight matched spots were picked from both gels, and 96 additional spots from the CHAPS-gel and analyzed by MADLI ToF/ToF MS/MS. As suspected, most proteins from the supernatant appeared to be soluble. Like the spots on the gel, identifications were identical for both detergents. Results from the re-solubilized pellets are still being analyzed.

4. Discussion

4.1 Enzymes and metabolites

4.1.1 Lack of statistically significant effects

No significant effects were observed in any of the enzymes. This is due to two problems: (1) high standard deviations and (2) low effects.

Extreme differences in activity were observed within the 4 biological replicates of some conditions. This leads to high standard deviations. Additional technical and biological replicates may solve this problem of variation, e.g. in the GPX and SPX assays.

Measurements that seemed to be biologically (and technically) reproducible often showed little to no effect, such was the case with SOD. This may be due to a high stress-level of the plants because of growth conditions. It was observed that growth of plants in the new incubators was not very optimal, in comparison with previous experiments in the greenhouse and in the field. Plants showed a lot of variation within conditions, but not between, and were overall very small, thin and had a reddish color (possibly due to elevated anthocyanin, which can be caused by environmental stress) [74]. It might therefor be that plants experienced such a level of stress, that many stress-responses, including anti-oxidative defense, already reached their maximum capacity.

However, if there really is no difference between the treatments that cannot be blamed on by high basal stress levels, it may indicate the commercially available fertilizer, which was given at tenfold the recommended dose, is ineffective.

4.1.2 Differences in plant tissue and the need for further optimization

The protocols utilized were optimized on *Arabidopsis thaliana* (thale cress) leaves and roots, which do not feature the same fiber contents and dead structures such as the sclerenchyma of alfalfa stems [75]. This may have contributed to the difficulties experienced when performing the assays. In addition, interfering compounds may be present that are not so prevalent in Arabidopsis. It was observed that ascorbate extracts during enzymatic extraction colored red, which might indicate a strong presence of phenolic compounds, something which is usually not observed when performing the extraction on *Arabidopsis* tissue. Indeed, the absorbance of phenols in the UV-range, around 280 nm and varying, close to the absorbance of H_2O_2 (substrate for CAT and APX assay) and AsA used in the assays [76]. A solution to this might be the use of PVPP in the AsA-extraction. However, in the enzymatic assays, PVPP was already used, and phenolic compounds should have been eliminated. Another interesting possibility for further optimization, though it most likely will not help with interference of phenolic compounds, is to use techniques that focus on obtaining the apoplastic or extracellular washing fluid. This will also add to the resolution of our enzymatic assays, if our goal is to gain information about the apoplastic redox-balance and its components.

4.1.3 Enzyme activity detected in stem tissue

It should be noted that even though the protocols were optimized for *Arabidopsis thaliana* leaves and roots, extracts could be used in similar or lower concentrations for most enzyme measurements, starting from similar fresh weights. The only enzyme for which no activity measurement could be obtained was catalase. This is important, because it indicates that in spite of a very different make-up from *Arabidopsis* leaves and roots, there is a lot of enzymatic activity in the stems.

4.1.4 Enzyme activities and metabolites in relation to Cu/Zn stress and lignification in other research.

While none of the data gathered was statistically significant due to reasons mentioned earlier, an inquiry is made into other plant research where enzymatic activities were measured. The relation to Cu and Zn is of particular interest as well as the implications they might have on lignification.

Cell wall peroxidases use the oxidant H_2O_2 to form monolignol radicals from monolignol precursors [77]. These monolignols are polymerized to lignin by oxidative mechanisms, and it is clear anti-oxidative pathways, enzymes and metabolites can play central roles in this process, especially if they are present in the apoplast and their reaction product or substrate is H_2O_2.

Cu, Zn-SOD is one of several SOD's and has both chloroplastic and cytosolic iso-enzymes, but there is also research pointing to a possible apoplastic location for SOD's [78]. It has been hypothesized that it might thusly play a part in lignification, though little further research into this hypothesis has been done [79]. A possible mechanism for this is the following: SOD catalyzes the reduction of the superoxide ion (O_2^-) to the less reactive H_2O_2, which is the substrate of lignifying enzymes such as SPX and GPX. It is also the substrate for the important apoplastic anti-oxidative enzyme APx, which will be discussed later in relation to lignification. In Cu-accumulator *Elsholtzia haichowensis* exposed to excess Cu (100 µM) , H_2O_2 accumulated in the apoplast of leaves due to the actions of Cu, Zn-SOD [80]. At the same time, apoplastic GPX and APX activities increased. The relation of Cu, Zn-SOD to Cu and Zn micro nutrition and excess remains interesting as well. In the *Brassica napus* (rapeseed), a plant used for feed like alfalfa, SOD activity decreased after Zn-exposure (0,07–1,12 mM) [81]. Furthermore, Zn and Cu have been reported to have complicated interactions. In rice seedlings, Zn (50 mM) ameliorated Cu-induced (200 and 500 µM) oxidative stress by increasing the activity of enzymes such as SOD, CAT and GPX [82].

Like SOD, laccase uses Cu (but unlike SOD, not Zn) as an essential cofactor [25]. In Cu-exposed alfalfa roots, GPX activity increased with Cu (10-100 µM) concentration (no data for stems was gathered) [83]. In *Helianthus annuus* (sunflower) seedlings, activity of lignifying enzymes referred to as SPX increased after treatment with Cu (50 µM) [84]. In the same plant, GPX and superoxide dismutase activity were also raised in both stems and leaves in response to Cu excess (50 µM), a similar response to the one observed in rice and *Elsholtzia haichowensis* mentioned earlier [80,82,85].

Even though the research was done on seedlings, this may have interesting implications, as sunflower is also a fodder crop and lignin concentration has been negatively correlated with digestibility for foraging [86]. Research on enzyme activities in *Phaseolus vulgaris* (bean) found that an Zn-treatment (50 µM) could activate GPX in higher parts of the plant, and Cu (50 µM) could activate both SPX and GPX in the roots [87]. Other research confirmed the activation of GPX by Zn (100 µM) in the stem, but not in other parts of the plant [88]. Thus, there are strong implications that excess Cu and Zn can activate lignifying enzymes. A role for lignin itself in the anti-oxidative defense has been suggested, but if lignification is an active part of this defense or just a secondary consequence of the activation of other anti-oxidative pathways, remains to be investigated [31].

Ascorbate is probably the most important anti-oxidant in the apoplast [89]. Ascorbate can inhibit lignification by scavenging H_2O_2 and monolignol radicals [77]. It has been suggested that the redox balance between ascorbate and H_2O_2 in the cell wall controls the extent of lignification. Research comparing the AsA pool in lignifying and adult *Picea abies* (Norway spruce) needles has found the AsA pool was bigger and less oxidized in the latter, which seems to support this theory [90]. Further research in Norway spruce lignifying cell suspensions, though arguably less representative, also pointed towards a complex role of ascorbate in modulating the apoplastic availability of H_2O_2 and thereby, influence lignification itself [91].

Ascorbate is also the cofactor of a few apoplastic redox enzymes, namely APX and AO. Research in *Pisum sativum* (bean) roots has indicated that ascorbate redox enzymes change during cell differentiation and the implication has been made that ascorbate and related anti-oxidants are involved in lignification [62]. Especially the balance between APX and AO is of interest in this matter. In the case of AsA and related enzymes, the theory is that lignifying class III peroxidases (GPX) compete with APX for H_2O_2 as oxidizing substrate [13]. AO on the other hand, competes with APX for its substrate, AsA, which AO will oxidize to DHA. Research in alfalfa roots indicated APX increased in plants exposed to low concentrations of Cu (< 30 µM) and decreased in plants exposed to higher concentrations (≥ 30 µm) [83]. In *Arabidopsis*, GPX and SPX activities increased after exposure to Zn (0-500 µM), while APX activity was not increased [92]. Excess Cu however decreased APX activity in roots (2 and 5 µM) [93].

Not much has been hypothesized or researched about GR's involvement in this cascade of anti-oxidants and lignification. However, the involvement of GR, if it exists, would seem to be quite straight-forward: its link to AsA. GR is an essential part of the ascorbate-gluthation cycle, through which DHA is recycled to AsA. This is done by DHA reductase, which oxidizes GSH to GSSG in the process. GSSG is then recycled back to GSH again by GR, which oxidizes NADPH to $NADP^+$ to catalyze this reduction [94]. The reason it's not directly implicated, is its cytosolic location. Research in Cu-exposed alfalfa roots mentioned in relation to APX and GPX, found that GR followed a similar trend to APX: an increase at low concentrations (< 30 µM) and a decrease at higher concentrations (≥ 30 µm) [83]. In Arabidopsis leaves, it was observed that Cu excess (50µM) temporarily increased GR activity in initial stages of oxidative stress. This was followed by a second increase after Cu-accumulated in the leaves, which was accompanied by an increase in APX-activity [95]. This suggest

an intricate, but clearly related response of GR in relation to Cu excess and the ascorbate-gluthation cycle, and thereby might also play an indirect role in lignification. Zn (50 µM) on the other hand decreased both APX and GR activity in bean [87]. No research could be found on GR activity in stem tissue of either alfalfa or other plants.

Catalase, which we were not able to measure, also detoxifies H_2O_2, by converting it to H_2O and O_2. There is still no definite clarity on the subcellular location of catalase, but mention has been made in research of apoplastic catalase (as well as a major peroxisomal component, a cytosolic and a chloroplastic) [96,97]. If catalase has a significant apoplastic presence, it's another competitor for H_2O_2 as substrate, competing with the lignifying peroxidases. In addition, some catalases, referred to as Class II catalases, are highly expressed in vascular tissue, the primary site for lignification within the stem [98]. In spite of this, no recent research could be found linking catalase and lignification. Micro nutrition and metal excess in relation to catalase is however a much researched subject. In rice shoots, Cu excess (200 and 500 µM) alone induced no change in CAT activity, but Cu in combination with Zn (50 mM) increased activity [82]. In bean roots and leaves, CAT activity decreased after exposure to excess Zn (100 µM), but interestingly, not in stems [88]. The decrease of CAT-activity in leaves after exposure to Cu was confirmed in sunflower [99]. In alfalfa roots however, Cu-exposure (10-100 µM) increased CAT activity [83].

To summarize, in spite of our lack of significant effects, it is clear from literature that Cu and Zn excess cause oxidative stress and can activate anti oxidative enzymes in stems. Many of these are directly or indirectly involved or suspected to be involved in lignification. The key to redox enzymes' and metabolites' involvement in lignification seems to be apoplastic location and H_2O_2 metabolism. Combined exposure to Cu and Zn can give rise to complicated effects, e.g. Zn has been reported to alleviate Cu-induced oxidative stress. The subtle differences between research depending on plant parts, plant organs, trace metal concentrations and methods, stress the need for further research, if conclusions with regards to specific crops are to be made. Such specific research may be useful with regards to the importance of lignin in agriculture (e.g. its undesirability for fodder or bioethanol).

4.2 The need for specific dextran-products in aqueous two-phase separation.

In the first experiments, no phase separation was observed in the loaded and unloaded systems. This was due to the fact that the dextran required in this technique should be Dextran T-500 and not a dextran salt such as dextran sulphate, even if the molecular weight (i.e. 500 000 g/mol) of the two is comparable. Another problem was that the phase system was prepared by simply adding 6,2% w/v of dextran and PEG 3350 to one volume of microsome suspension buffer. Instead separate dextran and PEG solutions of higher concentrations should be prepared and these should be mixed just before centrifugation. In addition, it is better to work by w/w percentages in every step of the phase separation protocol [70].

4.3 A protocol for the extraction of the total membrane fraction

The protocol was successful in the extraction of membrane proteins. A large amount of membrane proteins were found from only 5 bands of a 1D gel, totaling 72 proteins from a redundant database.

The second gel (Figure 9), containing the supposed soluble fraction, showed a very different pattern from that of the membrane fraction (Figure 8). The fact that there appear to be few proteins and a lot of smearing on the second gel probably stems from the fact that the non-membrane bound proteins were regarded as waste during the extraction procedure and no precautions were taken to maintain them intact. Furthermore, the final step (resulting in the separation illustrated in the Figures 8 and 9) results in a large volume for the soluble proteins. Together with the interference of the detergents used with the Bradford Protein Assay, this resulted in a a sample load on the gel in Figure 9 which is probably much lower than estimated.

While this data indicates that a significant enrichment of membrane proteins can be attained, it also illustrates that for the study of cell wall development additional enrichment steps need to be included. Plasma membrane proteins, involved in the synthesis of the cell wall, only represent a very minor fraction of the membrane proteins identified (Figure 3) [46]. Given the known higher abundance of chloroplastic and mitochondrial membrane proteins this is unlikely to be very different after full analysis of the obtained fraction, be it with 2D-PAGE and/or LC-MS/MS. Therefore the possibility to enrich PM-proteins was further explored, and a two-phase aqueous approach was selected to do this. At the moment no success was attained in this approach.

4.4 Possible factors in the failure of PM enrichment by PEG/dextran aqueous two-phase separation

Several protocols, identical to or based on the protocols described by Song et al. (2001) and Santoni et al. (2006), were tested, in which phase separation was attained with 6,4% and 6,0% PEG/dextran aqueous two-phase systems. The resulting fractions were visualized and studied using 1D and 2D gel electrophoresis. The gel-images were analyzed with ImageQuant and the Decyder software, indicating that the PEG- and dextran-fractions on both 1D (Figure12) and 2D-gels (Figure 13 and 14) had identical band/spot patterns. Therefore, enrichment was not successful. Furthermore, in Figure 12, the enrichment-step simply seemed to have lowered the protein concentration in the PEG-fraction, as indicated by less intense bands, in comparison to the dextran-fraction.

4.4.1 Possible problems in obtaining a microsomal fraction

Obtaining a plasma membrane protein enriched fraction of alfalfa stem material by use of the PEG/dextran aqueous two-phase system turned out to be difficult. Although two distinct phases were obtained relatively easyly when using the correct products, the difficulty lies in having the PM and non-PM fraction migrate to the specific phases. Although literature reports indicate that a

70%-30% enrichment can be obtained in the best case scenario (in a single step), no enrichment was observed in this study.

This can be due to a number of causes. It's crucial to obtain microsomes, small vesicles made up of PM and non-PM membranes, to separate in an aqueous two-phase system. Non-microsomal cell material such as cell wall debris, soluble proteins etc. may favor one of both phases and migrate in the phase system, but will not be separated correctly into PM and non-PM-fractions.

The microsomes, if present, should be pelleted in the first ultracentrifugation-step of all attempted protocols. There are four steps in preparing microsomes for enrichment wherein the problem might conceivably lay: (1) homogenization, (2) filtration, (3) ultracentrifugation and (4) re-suspension.

Homogenization was done by use of mortar and pestle and extra care was given to obtaining a homogenous powder. Early in the optimization, homogenization by use of several lab mixers was attempted, but unsuccessful due to the tough fibers of alfalfa stem material. A possibility to augment homogenization is using a small amount of sand during grinding, as was done for the enzyme extractions. An argument against grinding being the problem is that, even though no enrichment was observed, a fair amount of membrane proteins were extracted and identified in several protocols, suggesting that the cells and their membranes are being sufficiently broken.

Filtration was done by use of a number 3 glass filter funnel or 2 layers of Miracloth. A Duran number 3 glass filter funnel has a maximum pore size of 16-40 µm, while the typical pore size of Millipore Miracloth is 22-25 µm. Thus, pore size overlaps and both techniques have been successfully used in other research.

The third step is the ultracentrifugation where the microsomes were pelleted while soluble proteins remain in the microsome extraction buffer. A pellet was observed every time in this step and unlikely to consist of merely debris, since most debris should have been removed in the prior filtration and centrifugation step.

The next step, re-suspending the pelleted microsomes, was often more problematic. It was found that it was extremely difficult to re-suspend the microsomal pellet in short timeframe. One possibility would be to place the sample overnight on the shaker, however, this might influence the results considerably because of the temperature concerns in proteomics due to protease-activity. Another possibility is finding a more appropriate buffer.

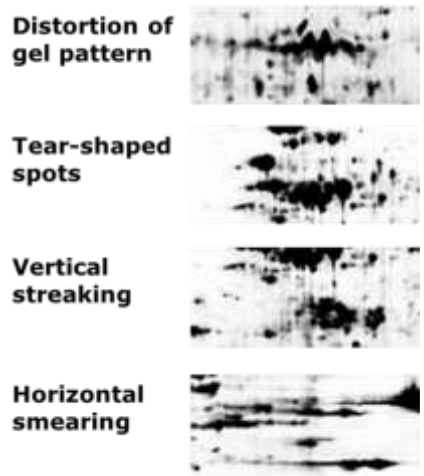
4.4.2 Optimal PEG, dextran and KCl concentrations influence enrichment

Secondly, less-than-optimal concentrations of PEG and dextran will influence enrichment. This is why the use of a polarimeter is suggested in many protocols, as it is claimed to be of importance for successful enrichment, but seems to be of less importance for simply obtaining two distinct phases. This reaffirms that phase separation by itself does not necessarily mean the enrichment

was successful. Concentrations between 5,4% and 6,4% are reportedly used for enrichment of PM from other plant species and based on this, the protocol was carried out with 6,4% and 6,0% phase systems. There appears to be no way to calculate or estimate optimal concentrations except trial-and-error. As already mentioned, no enrichment was observed, even with protocols that employed three phase separation steps to augment enrichment. However, one would assume that even with less-than-optimal concentrations, some enrichment would be observed if microsomes are present.

4.5 Finding a 2DE-compatible buffer to solubilize membrane proteins

Concentrations of 4% CHAPS and especially 4% ASB-14 are too high for 2DE. Deforming of the 2D pattern is especially clear with ASB-14. Gels also show streaking and smearing. Examples of interference are shown in Figure 17. Streaking, smearing and distortion can be due to high salt concentrations as well. Streaking can also be due to the presence of insufficiently solubilized proteins. Horizontal patterns of interference are usually due to problems with IEF, the first dimension, while vertical patterns are due to problems in the second dimension. However, the centrifugation of the samples should have pelleted insoluble proteins and salt concentrations conformed to most 2D protocols (7M Urea, 2M Thiourea). The tear-like shape of some spots has been reported by manufacturers to be caused by overloading. A sample load of 200 µg of proteins is high, but can ease identification and is only 1/4th of the advised maximum sample load. Even though it was possible to match the distorted gels trough software functions such as "warping", the distortion raises concerns. Likewise, DeCyder can often still identify spots quite clearly in what appears to be a continuous smear to the naked eye (or contrary, a blank area).



Figuur 17; Examples of interference with the 2DE pattern.

There is a lot of contradictory information in literature about which buffer in what concentration and in the presence of which chaotropes is best for solubilizing membrane proteins. A paper comparing the capacity of detergents to solubilize the IMP "human histamine H2 receptor" showed ASB-14 to be a superior detergent to CHAPS [100]. The results from this research contradict that finding at first glance. This may be due to the fact this solubilization was a lot less controlled, dealing with an only slightly enriched protein extract. From this, the range and area of proteins separated on gel were also much greater. However, only cytosolic fraction proteins were solubilized, while membrane proteins may have remained in the insoluble fraction of the sample. Cytosollic proteins are a lot easier to solubilize than membrane proteins. Though similar capacities to solubilize cytosolic proteins were observed and more distortion on the ASB-14 gel, it may thus still be possible that CHAPS is a superior detergent for solubilizing membrane proteins and separating them by 2DE. A possible further optimization step might also be to prepare a combination of both detergents in lower concentrations. In research involving brain membrane

proteins (also notoriously hard to solubilize), it was reported that a buffer containing 4% CHAPS and 2% ASB-14 was best to solubilize both membrane and cytosolic proteins [101].

It is often a reported problem that membrane proteins are hard to solubilize, and indeed it was observed that there appeared to be an insoluble fraction present in the partially solubilized sample. Yet the concentrations that fail to solubilize membrane proteins already cause noticeable distortion on gel.

In addition, proteins from this solubilized supernatant turned out to be mostly cytosolic/soluble fraction proteins. Starting from 15 g fresh weight, membrane protein concentration may be too high to solubilize in 200 µl 4% CHAPS or ASB-14. However, the solubilized fraction of both buffers contained approximately the same concentration of proteins, suggesting a similar solubilization capacity.

From the results of the 2D Quant Kit, protein concentrations were high enough to make it possible to lower the fresh weight considerably with the goal of performing 2DE. Another option would be to increase the volume of the lysis buffer while lowering its concentration. However, if PM-enrichment by two-phase separation is intended, some considerably loss of sample should be anticipated.

5. Conclusion

5.1 Low reproducibility and small effects

Some enzymatic assays showed low reproducibility while others showed no effects. It is possible that the assays require further optimization and might have to be specifically adapted to alfalfa stem tissue. Difficulties that may have contributed to difficult measurements are tough fibers and high presence of phenolic compounds. However, in spite of expectations, there appears to be a lot of enzymatic activity in the stem.

5.2 Total membrane protein extraction and the need for PM enrichment

A protocol was optimized which was effective in extracting membrane proteins, based on a paper by Song et al. (2011). Protein concentrations were relatively high, in part due to the high amounts of fresh weights that were started from to compensate for loss of material in the aqueous two-phase separation (which was left out in our adaptation of the protocol). However, the low abundance of PM proteins compared to the other fractions, stresses the need for enrichment when performing a proteomics experiment where the primary interests are PM proteins. The reason for the failure of PM enrichment by aqueous two-phase separation remains unknown, though most likely it's a problem with the creation or adequate suspension of the microsomes.

5.3 The paradox of detergents for solubilization and 2D-PAGE of membrane proteins

Concentrations of 4% ASB-14 and CHAPS appeared to fail in solubilizing the complete protein extracts. Quantifications of the solubilized fractions showed that both detergents solubilize a comparable amount of proteins, but a volume of 200 µl for 15 g fresh weight solubilizes mostly cytosolic/soluble fraction proteins. Both detergents caused some distortion of the 2D gel, but 4% ASB-14 seems less compatible with 2D-PAGE than 4% CHAPS. This draws attention to an interesting paradox. Detergent concentrations required to solubilize membrane proteins can be so high that they influence the 2D-PAGE.

5.4 A proposed future experiment and final remarks

To repeat this experiment and obtain more statistical power as well as biological relevance, more biological replicates could be done and a technical replicate can be included. To verify the results, especially the activities with small effects, and provide additional information, a proteomics experiment would be ideal. Because of the nature of proteomics as opposed to a gene experiment such as qPCR, which ignores post-translational modifications, the differential abundance of proteins might more directly be correlated with their activity. This could give some indication whether the enzymatic assays need further adaptation. If a repeated proteomics experiments seemingly confirm the results of both these and the repeated enzymatic assays, it obviously requires inquiry as to why there is no effect. If not due to the incubators or other such external factors, than perhaps the treatment in ineffective, and if this is the case, it casts doubt on the usefulness of the

commercially available fertilizers which were given at 10 times the recommended dosage. It should be noted that the soil was not characterized but there was no reason to assume it was already low in trace metals. However, it may be interesting to measure metal content in both the soil and different parts of the stem and attempt to link any differences in the parts of the stem to the abundance and activity of the proteins. Knowing the metal content of the soil will make it easier to compare results with other research and the conditions thereof. In addition, research in other plants has found Zn accumulates in higher plant parts, while Cu initially collects in below ground parts and this difference impacts anti-oxidative defense [81,87]. Through this, it may also impact lignification, in a manner which may be time- and organ-dependent, as the anti)-oxidative response seems to be. In addition, it may be useful to include techniques focusing on apoplastic or extracellular washing fluid.

In conclusion, much has been hypothesized and implied on the subject of lignification in relation to the redox-balance and all its components and safe-guards, but research is still relatively scarce, especially in more agriculturally relevant plants and their primary lignifying tissues. With the obvious need for agricultural efficiency and unfortunate problems of pollution, it may be time to give this interesting subject the attention it is due.

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7. Supplements

7.1 Protein separation by 1D SDS-PAGE

Use was made of Criterion TM XT precast 1D gels 12% Bis-Tris 12 + 2 wells, comb, 45µl 1,0 mm. A loading mix is prepared for the equivalent of 20-25 µg protein. The mix was prepared as such:

V sample	H_2O mQ	V_{tot}	XT Sample Buffer	XT Reducing Agent
for 20 -25 µg	V_{tot}-V	45 µl or less	V_{tot} / 4	V_{tot} / 20

The mixture was vortexed, centrifuged briefly and denaturated for 10 min. at 70°C. The electrophoresis tank was filled with1x XT MOPS buffer and the samples were loaded, together with 5 µl of the molecular ladder Precision Plus Protein™ Unstained Standard (Bio-Rad) if colored with InstantBlue, or Precision Plus Protein™ Prestained Standard (Bio-Rad) if colored with Lavapurple. for coloring with Lavapurple. The gel is run at constant 200V for approximately 4 hours, depending on how fast the proteins migrate on gel. The migration is stopped when the samples have reached the edge of the gel.

7.2 Protein separation by 2D gel electrophoresis

7.2.1 Passive rehydration and isoelectric focusing

For the first dimension of 2DE, samples were loaded on strip by passive rehydration and focused by IEF.

For passive rehydration of the Immobiline DryStrip pH 3-10 NL, 24 cm (GE Healthcare), the equivalent of up to 200 µg of protein sample is adjusted to 450 µl with lysisbuffer (7M Urea, 2M Thiourea, 4% ASB-14 or CHAPS with bromophenol blue). Nine µl of ampholytes and 2,7 µl of Destreak reagent were added to the adjusted sample. The samples were centrifuged and pipetted into the rehydration tray. The strips were placed on the sample, gel-side down and covered with Drystrip cover fluid to rehydrate for at least 12 h.

Table 5; Program for IEF

Step	U (V)	Time
1	100	5 h
2	1000	6 h
3	1000	6 h
4	10000	6 h
5	10000	7 h
I/strip	75 µA	
Temp	20°C	

The isoelectric focusing was done a IEF system (Ettan IPGphor 3 IEF system, GE Healthcare). The strips were places gel-side up in the manifold tray and paper wicks (wetted with distilled water for the cathode, lysis buffer for the anode) were placed on the ends of the strip. Electrodes were attached and the strips were covered with Drystrip cover fluid. The program for the isoelectric focusing is presented in the table on the left. Strips can be saved in the freezer at -20°C until the second dimension is run.

7.2.2 Second dimension on gel

The second dimension of 2D gel electrophoresis experiments was run on 2D HPE Large GEL NF – 12,5% gels kit (SERVA Electrophoresis GmbH, Heidelberg, Germany) in a HPE FlatTop Tower (Gel Company). A volume of 4,5 ml of cooling contact fluid was applied to the ceramic surface and the gel was places gel-side up on the surface. The proteins on the strip were reduced (15 min in 680 mM Urea / 50 mM DTT in equilibration buffer) and alkylated (15 min in 680 mM Urea / 130 mM IAA in equilibration buffer) and the strip was placed gel-side down in the intended slot on the gel. The fibre wicks were wetted with 45ml of the appropriate buffer according to the instructions of the kit and placed between the gel and the electrodes. The program for the 2nd dimension is shown in the table on the right.

Table 6; Program for the 2nd dimension of 2D gel electrophoresis

Step	Max U (V)	Max I (mA)	Max P (W)	Time
1	100	7	1	30 min
2	200	13	3	30 min
3	300	20	5	10 min
	remove the IPG-strip			
4	1000	40	30	3 h 50 min
5	1500	45	40	40 min

Temp 15°C

7.3 Staining gels with InstantBlue and Lavapurple

The protocol to stain and fixate gels with InstantBlue or Lavapurple is presented in the table below. InstantBlue is an easy, quick and visible stain. Lavapurple requires a longer procedure, including fixation and is only visible under the scanner at 524 nm, but is more sensitive.

Table 7; Coloring 1D and 2D gels with Lavapurple and InstantBlue.

Lavapurple		InstantBlue	
Product	**Time**	**Product**	**Time**
Fixing		**Staining**	
15% EtOH / citric acid 1%	+ 45 min	Instant Blue solution	+ 15 min
Prebuffering		**Washing**	
Old LavaPurple	20 min	H2O mQ	+ 1 h
Staining		*repeat until gel is clear*	
LavaPurple (1/200)	1 h 30 min	*Save gel in H2O mQ*	
Washing			
15% EtOH	15 min		
Acidify			
15% EtOH / citric acid 1%	+ 10 min		
Save gel in H2O mQ			

7.4 Digesting bands/spots for analysis with MALDI TOF/TOF MS/MS or Orbitrap LC/MS

The protocol for preparing spots for analysis by mass spectrometry is presented in the table below. It can be adapted, depending on various factors such as the dye which was used or the nature of the gel which was run.

Table 8; Protocol for destaining, reducing, alkylating , extracting and spotting peptides from excised spots or bands. Destaining steps should only be repeated with a visible dye, e.g. InstantBlue. Reduction and alkylation are not necessary here if they were already part of the gel electrophoresis e.g. with a 2D gel. The last steps, involving the target plate are solely for analysis by MALDI, not for analysis by Orbitrap LC/MS.

			Time	Temp
1. Destaining				
2x	100 µl 50 mM Ambic / MeOH 50%		20 min	ambient
	100 µl ACN 100%		20 min	ambient
	Repeat until gel plugs are no longer blue			
	Dry			
2. Reduction				
	100 µl 100mM Ambic / 10 mM DTT		30 min	56°C
3. Alkylation				
	100 µl Ambic 100 mM / IAA 55 mM	dark!	20 min	ambient
	100 µl ACN 100%		20 min	ambient
	Dry			
4. Digestion				
	8 µl Trypsin 5 ng / Ambic 50mM		30 min	on ice
	10 µl 50 mM Ambic		over night	37°C
5. Extraction				
	Spin tubes			
	30 µl 50% ACN / TFA 0,1 %		20 min	37°C
	Transfer supernatant to a second tube			
	30 µl 50% ACN / TFA 0,1 % *in first tube*		20 min	37°C
	Dry second tube in speedvac			
	Store			- 20°C
6. Spotting (for MALDI)				
	2 µl 50% ACN / TFA 0,1 % *in tube*			
	0,7 µl sample *to target plate*			
	0,7 µl CHCA 7mg/ml 50% ACN / TFA 0,1% *to target plate*			
	Dry			

7.5 Complete list of annotated proteins

Presented below is a complete list of annotated proteins from the experiment discussed in 2.6 Extraction of membrane and soluble protein fractions. Proteins that are part of the membrane fraction are designated by a letter representing the membrane fraction in question (C: chloroplastic, ER: endoplasmatic reticulum, PM:plasma membrane, M: mitochondrion).

spot	*number*	*species*	*protein*	*accesion number*	*length (aa)*	*protein family (interProscan)*	*membrane type*	*Gene Ontology (interProScan)*		
								cellular component	*molecular function*	*biological process*
5	1	Cucumis sativus	**PREDICTED: photosystem I reaction center subunit II, chloroplastic-like**	XP_004134141.1	207	Photosystem I PsaD	C	photosystem I photosystem I reaction center	None predicted.	photosynthesis
	2	Medicago truncatula	**unknown**	ACJ84031.1	202	Small GTPase superfamily, Rab-type	PM	None predicted.	GTP binding	small GTPase mediated signal transduction protein transport
	3	Medicago truncatula	**unknown**	ACJ86051.1	203	Flavoprotein WrbA		membrane	FMN binding oxidoreductase activity	negative regulation of transcription, DNA-templated
	4	Medicago truncatula	**unknown**	AFK39740.1	205	Flavoprotein WrbA		membrane	FMN binding oxidoreductase activity	negative regulation of transcription, DNA-templated
	5	Medicago	**ATP synthase subunit**	XP_003591976.1	144	ATPase, F0	M	mitochondrial	hydrogen ion	ATP synthesis

		truncatula	**d**			complex, subunit D, mitochondrial		proton-transporting ATP synthase complex, coupling factor F(o)	transmembrane transporter activity	coupled proton transport
	6	Arachis hypogaea	**threonine endopeptidase**	ACF74359.1	161	Proteasome, subunit alpha/beta		proteasome core complex	endopeptidase activity threonine-type endopeptidase activity	proteolysis involved in cellular protein catabolic process
	7	Medicago truncatula	**Tetratricopeptide repeat protein**	XP_003627353.1	300	None predicted.	C, M, ER	None predicted.	protein binding	None predicted.
	8	Cicer arietinum	**PREDICTED: oxygen-evolving enhancer protein 2, chloroplastic-like**	XP_004499534.1	265	Photosystem II PsbP, oxygen evolving complex	C	photosystem II photosystem II oxygen evolving complex extrinsic component of membrane	calcium ion binding	photosynthesis
	9	Medicago truncatula	**Outer membrane lipoprotein blc**	XP_003610371.1	184	Lipocalin, ApoD type		None predicted.	transporter activity	None predicted.
6	1	Amaranthus hypochondriacus	**Chloropyll a/b binding protein**	CAA52749.1	186	Chlorophyll A-B binding protein	C	membrane	None predicted.	photosynthesis, light harvesting
	2	Amaranthus tricolor	**type I chlorophyll a/b-binding protein a**	BAB41190.1	154	Chlorophyll A-B binding protein	C	membrane	None predicted.	photosynthesis, light harvesting
	3	Oxytropis arctobia	**chloroplast photosystem II light harvesting complex protein type I, partial**	AEV59638.1	212	Chlorophyll A-B binding protein	C	membrane	None predicted.	photosynthesis, light harvesting

4	Medicago truncatula	**unknown**	AFK39586.1	265	Chlorophyll A-B binding protein	C	membrane	None predicted.	photosynthesis, light harvesting
5	Pseudotsuga menziesii	**light-harvesting chlorophyll a/b binding protein of photosystem II**	CAA89823.1	234	Chlorophyll A-B binding protein	C	membrane	None predicted.	photosynthesis, light harvesting
6	Vitis vinifera	**unnamed protein product**	CBI20177.3	171	Small GTPase superfamily	PM	None predicted.	GTP binding	small GTPase mediated signal transduction protein transport
7	Medicago truncatula	**GTP-binding protein ras-like protein**	XP_003593685.1	217	Small GTPase superfamily	PM	None predicted.	GTP binding	small GTPase mediated signal transduction protein transport
8	Medicago truncatula	**unknown**	AFK33953.1	215	Small GTPase superfamily	PM	None predicted.	GTP binding	small GTPase mediated signal transduction protein transport
9	Medicago truncatula	**Ras-related protein Rab-11A**	XP_003596706.1	218	Small GTPase superfamily	PM	None predicted.	GTP binding	small GTPase mediated signal transduction protein transport
10	Medicago truncatula	**vacuolar H+-ATPase B subunit**	ABO61030.1	489	V-type ATP synthase regulatory subunit B/beta	V	proton-transporting two-sector ATPase complex, catalytic domain proton-transporting V-type ATPase, V1 domain	ATP binding hydrolase activity, acting on acid anhydrides, catalyzing	ATP hydrolysis coupled proton transport proton transport

								transmembrane movement of substances ATP metabolic process	
11	Medicago truncatula	**Ras-like protein**	XP_003590522.1	216	Small GTPase superfamily	PM	None predicted.	GTP binding	small GTPase mediated signal transduction protein transport
12	Medicago truncatula	**unknown**	AFK41349.1	214	Small GTPase superfamily	PM	None predicted.	GTP binding	small GTPase mediated signal transduction protein transport
13	Medicago truncatula	**unknown**	AFK46231.1	215	Small GTPase superfamily	PM	None predicted.	GTP binding	small GTPase mediated signal transduction protein transport
14	Medicago truncatula	**unknown**	AFK45661.1	277	Chlorophyll A-B binding protein	C	membrane	None predicted.	photosynthesis, light harvesting
15	Oxytropis maydelliana	**chloroplast photosystem II light harvesting complex protein type III, partial**	AEV59649.1	186	Chlorophyll A-B binding protein	C	membrane	None predicted.	photosynthesis, light harvesting
16	Medicago truncatula	**unknown**	ACJ86147.1	193	Ribosomal protein L6		intracellular ribosome	structural constituent of ribosome rRNA binding	translation
17	Lotus japonicus	**unknown**	AFK41222.1	173	Ribosomal protein L6		intracellular ribosome	structural constituent of ribosome rRNA binding	translation
18	Zea mays	**hypothetical protein**	AFW60045.1	157	Small GTPase	M	None	GTP binding	small GTPase

			ZEAMMB73_972006			superfamily		predicted.		mediated signal transduction protein transport
	19	Medicago truncatula	**unknown**	AFK42921.1	253	None predicted.		None predicted.	None predicted.	None predicted.
	20	Arabidopsis thaliana	**D-ribulose-5-phosphate-3-epimerase**	NP_200949.1	281	Ribulose-phosphate 3-epimerase-like	C	None predicted.	catalytic activity racemase and epimerase activity, acting on carbohydrates and derivatives	carbohydrate metabolic process metabolic process
	21	Oryza sativa Indica Group	**chlorophyll a-b binding protein**	AAB82142.1	263	Chlorophyll A-B binding protein	C	membrane	None predicted.	photosynthesis, light harvesting
	22	Fragaria vesca subsp. vesca	**PREDICTED: chlorophyll a-b binding protein 40, chloroplastic-like**	XP_004293579.1	263	Chlorophyll A-B binding protein	C	membrane	None predicted.	photosynthesis, light harvesting
	23	Aegilops tauschii	**Chlorophyll a-b binding protein, chloroplastic**	EMT02412.1	261	Chlorophyll A-B binding protein	C	membrane	None predicted.	photosynthesis, light harvesting
	24	Medicago truncatula	**Heme-binding-like protein**	XP_003604820.1	201	SOUL haem-binding protein		None predicted.	None predicted.	None predicted.
	25	Medicago truncatula	**Heme-binding-like protein**	XP_003604821.1	202	SOUL haem-binding protein		None predicted.	None predicted.	None predicted.
	26	Medicago truncatula	**hypothetical protein MTR_5g008750**	XP_003610942.1	272	None predicted.	C	None predicted.	oxidoreductase activity 2 iron, 2 sulfur cluster binding	oxidation-reduction process
	27	Medicago truncatula	**unknown**	AFK42007.1	183	ETC complex I subunit	M	mitochondrial inner membrane	oxidoreductase activity, acting on NAD(P)H	respiratory electron transport chain
8	1	Medicago	**Oxygen-evolving**	XP_003636084.1	329	Photosystem II	C	cell outer	calcium ion binding	photosynthesis

		truncatula	**enhancer protein**			PsbO, manganese-stabilising		membrane photosystem II photosystem II oxygen evolving complex integral component of membrane extrinsic component of membrane		photosystem II stabilization
	2	Raphanus raphanistrum	**photosystem II D1 protein**	ABR08703.1	101	Photosynthetic reaction centre, L/M	C	None predicted.	electron transporter, transferring electrons within the cyclic electron transport pathway of photosynthesis activity	photosynthetic electron transport in photosystem II photosynthesis, light reaction
	3	Medicago truncatula	**unknown**	ACJ85279.1	276	Eukaryotic porin/Tom40	M	mitochondrial outer membrane	voltage-gated anion channel activity	anion transport regulation of anion transport transmembrane transport
	4	Medicago truncatula	**14-3-3 protein**	XP_003615160.1	260	14-3-3 protein		None predicted.	protein domain specific binding	None predicted.
	5	Medicago truncatula	**14-3-3-like protein**	XP_003602824.1		14-3-3 protein		None predicted.	protein domain specific binding	None predicted.
	6	Musa AAB Group	**actin**	AEQ16454.1		Actin-related protein	PM	None predicted.	None predicted.	None predicted.

7	Medicago truncatula	**unknown**	ACJ85133.1	276	None predicted		None predicted.	None predicted.	None predicted.
8	Medicago truncatula	**unknown**	ACJ85076.1	267	Cytochrome c1	M	None predicted.	iron ion binding electron carrier activity heme binding	None predicted.
9	Medicago truncatula	**Adenylate kinase B**	XP_003608862.1	240	Adenylate kinase/UMP-CMP kinase		None predicted.	adenylate kinase activity ATP binding phosphotransferase activity, phosphate group as acceptor nucleobase-containing compound kinase activity	nucleobase-containing compound metabolic process
10	Medicago truncatula	**unknown**	ACJ86038.1	214	ATPase, V1/A1 complex, subunit E	V	proton-transporting two-sector ATPase complex, catalytic domain	proton-transporting ATPase activity, rotational mechanism	ATP hydrolysis coupled proton transport
11	Medicago truncatula	**Mitochondrial outer membrane protein porin**	XP_003611587.1	276	Eukaryotic porin/Tom40	M	mitochondrial outer membrane	None predicted.	transmembrane transport
12	Medicago truncatula	**Ascorbate peroxidase**	XP_003607199.1	287	Haem peroxidase		response to oxidative stress oxidation-reduction process	peroxidase activity heme binding	None predicted.

	13	Medicago truncatula	**Disease resistance response protein**	XP_003604505.1	192	Plant disease resistance response protein		None predicted.	None predicted.	None predicted.
	14	Cicer arietinum	**PREDICTED: chlorophyll a-b binding protein AB80, chloroplastic-like**	XP_004512704.1	138	Chlorophyll A-B binding protein	C	membrane	None predicted.	photosynthesis, light harvesting
	15	Ricinus communis	**conserved hypothetical protein**	XP_002532180.1	243	None predicted.		None predicted.	None predicted.	None predicted.
	16	Medicago truncatula	**Mitochondrial carnitine/acylcarnitine carrier-like protein**	XP_003612918.1	297	None predicted.		None predicted.	None predicted.	None predicted.
	17	Cucumis sativus	**PREDICTED: 14-3-3-like protein-like**	XP_004152627.1	261	14-3-3 protein		None predicted.	protein domain specific binding	None predicted.
	18	Cicer arietinum	**PREDICTED: gamma carbonic anhydrase 1, mitochondrial-like**	XP_004512067.1	269	None predicted.		None predicted.	None predicted.	None predicted.
	19	Medicago truncatula	**Cysteine proteinase**	XP_003589136.1	295	Peptidase C1A, papain		None predicted.	cysteine-type peptidase activity	proteolysis
11	1	Medicago truncatula	**ATP synthase subunit beta**	XP_003592582.1	1129	ATPase, F1 complex, beta subunit	M, C	mitochondrial proton-transporting ATP synthase complex, catalytic core F(1) proton-transporting two-sector ATPase complex, catalytic domain	nucleotide binding ATP binding hydrolase activity, acting on acid anhydrides, catalyzing transmembrane	ATP catabolic process ATP biosynthetic process ATP synthesis coupled proton transport

									movement of substances	
									ATPase activity	ATP hydrolysis coupled proton transport
									nucleoside-triphosphatase activity	proton transport
									proton-transporting ATP synthase activity, rotational mechanism	ATP metabolic process
	2	Cicer arietinum	**PREDICTED: uncharacterized protein LOC101500213**	XP_004497004.1	1128	ATPase, F1 complex, beta subunit	M, C	mitochondrial proton-transporting ATP synthase complex, catalytic core F(1)	nucleotide binding	ATP catabolic process
								proton-transporting two-sector ATPase complex, catalytic domain	ATP binding	ATP biosynthetic process
									hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances	ATP synthesis coupled proton transport
									ATPase activity	ATP hydrolysis coupled proton transport
									nucleoside-triphosphatase activity	proton transport

									proton-transporting ATP synthase activity, rotational mechanism	ATP metabolic process
	3	Medicago truncatula	**vacuolar H+-ATPase B subunit**	ABO61030.1	490	V-type ATP synthase regulatory subunit B/beta	V	proton-transporting two-sector ATPase complex, catalytic domain proton-transporting V-type ATPase, V1 domain	ATP binding hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances	ATP hydrolysis coupled proton transport proton transport ATP metabolic process
	4	Medicago truncatula	**V-type ATP synthase regulatory subunit B/beta**	XP_003624258.1	488	V-type ATP synthase regulatory subunit B/beta	V	proton-transporting two-sector ATPase complex, catalytic domain proton-transporting V-type ATPase, V1 domain	ATP binding hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances	ATP hydrolysis coupled proton transport proton transport ATP metabolic process
	5	Medicago truncatula	**ATP synthase F1 sector subunit beta**	Q9TKI7.1	490	ATPase, F1 complex, beta subunit	M, C	proton-transporting two-sector ATPase	nucleotide binding	ATP synthesis coupled proton transport

								complex, catalytic domain	ATP binding hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances proton-transporting ATP synthase activity, rotational mechanism	ATP hydrolysis coupled proton transport ATP metabolic process
	6	Bombax ceiba	**H(+)-transporting ATP synthase**	CAB43987.1	471	ATPase, F1 complex, beta subunit	M, C	proton-transporting two-sector ATPase complex, catalytic domain	nucleotide binding ATP binding hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances nucleoside-triphosphatase activity proton-transporting ATP synthase activity, rotational	ATP synthesis coupled proton transport ATP hydrolysis coupled proton transport proton transport ATP metabolic process

									mechanism	
	7	Zanthoxylum sp.	**ATP synthase beta subunit**	AFQ41194.1	472	ATPase, F1 complex, beta subunit	M, C	proton-transporting two-sector ATPase complex, catalytic domain	nucleotide binding ATP binding hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances nucleoside-triphosphatase activity proton-transporting ATP synthase activity, rotational mechanism	ATP synthesis coupled proton transport ATP hydrolysis coupled proton transport proton transport ATP metabolic process
	8	Metzgeria consanguinea	**ATP synthase beta subunit**	BAJ65649.1	350	ATPase, F1 complex, beta subunit	M, C	proton-transporting two-sector ATPase complex, catalytic domain	nucleotide binding ATP binding hydrolase activity, acting on acid anhydrides, catalyzing	ATP synthesis coupled proton transport ATP hydrolysis coupled proton transport ATP metabolic process

								transmembrane movement of substances proton-transporting ATP synthase activity, rotational mechanism	
9	Cyananthus incanus subsp. incanus	**ATP synthase beta subunit, partial (chloroplast)**	AGL95059.1	350	ATPase, F1 complex, beta subunit	M, C	None predicted.	nucleotide binding ATP binding hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances proton-transporting ATP synthase activity, rotational mechanism	ATP synthesis coupled proton transport ATP binding nucleoside-triphosphatase activity proton-transporting ATP synthase activity, rotational mechanism
10	Schima superba	**ATP synthase beta subunit, partial (chloroplast)**	AAM17945.1	350	ATPase, F1 complex, beta subunit	M, C	None predicted.	nucleotide binding ATP binding hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances proton-transporting ATP synthase activity, rotational	ATP synthesis coupled proton transport proton transport ATP metabolic process

									mechanism	
	11	Medicago truncatula	**Calreticulin**	XP_003591212.1	424	Calreticulin/calnexin	ER	endoplasmic reticulum	calcium ion binding protein binding unfolded protein binding	protein folding
	12	Lotus japonicus	**ATPase subunit 1 (mitochondrion)**	YP_005090487.1	505	ATPase, F1 complex, alpha subunit	M, C	proton-transporting two-sector ATPase complex, catalytic domain proton-transporting ATP synthase complex, catalytic core F(1)	ATP binding hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances proton-transporting ATP synthase activity, rotational mechanism proton-transporting ATPase activity, rotational mechanism	ATP synthesis coupled proton transport ATP hydrolysis coupled proton transport proton transport ATP metabolic process
	13	Hordeum vulgare subsp. vulgare	**predicted protein**	BAJ97146.1	495	ATPase, F1 complex, beta subunit	M, C	proton-transporting two-sector ATPase complex, catalytic domain	nucleotide binding ATP binding	ATP synthesis coupled proton transport ATP hydrolysis coupled proton transport

									hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances nucleoside-triphosphatase activity proton-transporting ATP synthase activity, rotational mechanism	proton transport ATP metabolic process
	14	Medicago truncatula	**unknown**	AFK41442.1	447	None predicted.		None predicted.	None predicted.	None predicted.
	15	Medicago sativa	**group 3 LEA protein**	ACD14089.1	436	None predicted.		None predicted.	None predicted.	None predicted.
13	1	Medicago truncatula	**unknown**	ACJ85683.1	542	Calreticulin/calnexin	ER	endoplasmic reticulum	calcium ion binding protein binding unfolded protein binding	protein folding
	2	Medicago sativa	**putative endomembrane protein precursor**	AAA32662.1	512	Protein disulphide isomerase	ER	endoplasmic reticulum	protein disulfide oxidoreductase activity isomerase activity	glycerol ether metabolic process cell redox homeostasis

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Loix, Christophe

Datum: **10/06/2014**