PLANT SUSPENSION CULTURE DERIVED EXTRACELLULAR VESICLES

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ABSTRACT

PLANT SUSPENSION CULTURE DERIVED EXTRACELLULAR VESICLES

Although extracellular vesicles (EVs) are an ever-present part of life, research of non-mammalian EVs have been severely limited compared to the studies of human EVs. Initial studies of plant EVs already report promising results, such as therapeutic effects for various types of cancers, colitis, immunomodulation, and wound healing.

However, there are still many unknowns in plant EV research. A large majority of the articles published in the field fail in adhering to the standards and definitions set in international guidelines of EV research. The abundant contaminants created due to the harsh isolation procedures, lack of adequate controls, and incomplete characterization of the isolated EVs makes these findings questionable. The issues are exacerbated due to a lack of a plant EV isolation method or biomarkers agreed upon by the scientific community.

This dissertation attempts to address this issue by providing a complete workflow for the isolation and characterization of true plant EVs using plant cell suspension cultures. Using this workflow, we have isolated and characterized EVs from *Nicotiana tabacum*, *Vitis vinifera* and *Stevia rebaudiana* cell cultures. Proteomic analysis of *N. tabacum* sEVs revealed the presence of eight proteins suggested as plant EV biomarker candidates. Furthermore, we ran an ortholog protein discovery analysis using proteomes of *N. tabacum* and seven other plant EV proteomes against top human EV proteins. We identified 116 human proteins with plant orthologues, with 24 of these proteins being present in more than five or more plants.

Our findings prove that plant cell suspension cultures can be used for the production of highly pure plant EVs. Plant EVs have considerable potential as therapeutic agents or drug delivery devices, which can be easily, and efficiently produced in a large scale manner. Finally, the biomarkers identified in this study can pave the way for future studies in the field of plant EV research.

ÖZET

BİTKİ SÜSPANSİYON KÜLTÜRÜ KAYNAKLI EKSTRASELÜLER VEZİKÜLLER

Her ne kadar ekstraselüler veziküller (EVler) her canlı hücrede gözlemleniyor olsa da, memeli olmayan canlıların EVleri üzerindeki çalışmalar, insan EV çalışmalarına göre çok daha kısıtlı kalmıştır. Günümüzde bitkisel EVler üzerine yapılan ilk çalışmalar kanser, kolit, bağışıklık sistemi hastalıkları ve yara kapanması gibi alanlarda umut vadedi sonuçlar göstermektedir.

Fakat, bitkisel EV alanında hala çok sayıda bilgi eksikliği bulunmaktadır. Alandaki yayınların büyük bir kısmı uluslararası EV araştırma standartları ve tanımlarına uymamaktadır. Çalışmaların bulguları, kullanılan kaba izolasyon metotlarından dolayı örneklerinde bulunan çok sayıdaki kirlilik, yetersiz kontroller, ve eksik karakterizasyonlardan dolayı şüphelidir. Bu sorunlar, bitkisel EVler için bilimsel camia tarafından kararlaştırılmış bir izolasyon veya biyomarker olmadığı için daha şiddetli hissedilmektedir.

Bu tezle bu sorunlara çözüm olarak, bitki hücre süspansiyon kültürleri ile gerçek bitkisel EVlerin izolasyon ve karakterizasyonunu sağlayan bir yöntem geliştirilmiştir. Bu yöntem ile from *Nicotiana tabacum, Vitis vinifera* ve *Stevia rebaudiana* hücrelerinden EVler izole ve karakterize edildi. *N. tabacum* EVleri üzerinde yapılan proteomiks çalışma ile yedi bitkisel EV biyomarker adayı tanımlandı. Ayrıca, *N. tabacum* ve yedi ayrı bitkisel EV proteomu, insan EVlerinde en sık görünen proteinler ile ortolog keşfi için karşılaştırıldı. 116 insan ortholoğu olan bikti protein tanımlandı, bu proteinlerin 24'ü en az beş bitkide gözlemlendi.

Bulgularımız bitki hücre süspansyon kültürlerinin yüksek saflıkta bitkisel EV üretimi için kullanılabileceklerini kanıtlamaktadır. Bitkisel EVlerin kolay ve efektif şekilde üretilen terapötik ajanlar veya ilaç taşıyıcılar olarak kullanımları yüksek potansiyel taşımaktadır. Ayrıca bu çalışmada tanımlanan biyomarkerler bitkisel EV alanında başka çalışmaların yolunu açabilir.

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LIST OF SYMBOLS/ABBREVIATIONS

BLAST	Basic local alignment search tool
CELLO	Subcellular localization
CHOL	Cholesterol
ENOA	Enolase 1
ESCRT	Endosomal sorting complex required for transport
EV	Extracellular vesicle
DLS	Dynamic light scattering
GO	Gene ontology
HPLC	High performance liquid chromatography
ILV	Intraluminal vesicle
ISEV	International society of extracellular vesicles
KEGG	Kyoto encyclopedia of genes and genomes
lEV	Large extracellular vesicle
MISEV	Minimal information for studies of extracellular vesicles
MVB	Multivesicular body
MS/MS	Tandem mass spectrometry
NP	Nanoparticle
NTA	Nanoparticle tracking analysis
PANTHER	Protein analysis through evolutionary relationships
PC	Phosphatidylcholine
PDNP	Plant derived nanoparticle
PI	Phosphatidylinositol
PMB	Paramural bodies
PPIA	Peptidyl-prolyl cis-trans isomerase A
PPIB	Peptidyl-prolyl cis-trans isomerase B
PS	Phosphatidylserine
SAHH	Adenosylhomocysteinase
sEV	Small extracellular vesicle
SM	Sphingomyelin

1. INTRODUCTION

Extracellular vesicles (EVs) are nano-sized messengers secreted by all cell types. They are a ubiquitous presence of life, having been observed in all organisms and cell types studied to date [1]. Despite their universal presence, studies on EVs remain mostly limited to the study of mammalian EVs, with the research of EVs in non-mammalian organisms severely lacking compared to their mammalian counterparts.

Extracellular vesicles of plants are a particularly promising subject for further research. Plants produce a majority of therapeutic compounds used in medicine [2], which indicates that their EVs can have therapeutic properties as well. Studies report the therapeutic effects of EVs from plants such as lemon [3], ginseng [4], grape [5], grapefruit [6,7], *Dendropanax morbifera* [8] against diseases such as cancer or colitis in *in vitro* and *in vivo* models Other studies have successfully used plant EVs as a delivery vehicle to deliver miRNA therapeutics across the blood-brain barrier [9], or to deliver functional, exogenous proteins human cells [10].

However, the lack of information and standardization is a major obstacle for the study of plant EVs. Unlike mammalian EVs, there are no generally accepted guidelines for the isolation and characterization of plant EVs. This in turn has led to inconsistencies between the nomenclature used to describe plant and mammalian EVs [11]. For instance, MISEV2018 states that to ensure an EV isolation is free of intracellular vesicles, there should be minimal disruption of the cells producing EVs before the collection of their conditioned media [1]. However, earlier studies in the plant-EV field used plants acquired from non-standardized sources such as local markets, and employed physically disruptive methods such as the use of blenders and juicing [3,6,7,12]. Such disruptive methods may disrupt the integrity of plant cells, contaminating the EV isolate with internal vesicles. Additionally, some isolation methods, such as differential ultracentrifugation, may be inadequate in dealing with soluble contaminants, such as plant phytochemicals, which may cause biological effects that could be falsely attributed to plant EVs.

Only recently the methodologies of earlier studies started to be questioned by the small but diligent field of plant EV researchers [11,13]. These papers raise the point that, currently,

there are only a handful of studies that isolated plant EVs that are in accordance with the MISEV2018 guidelines - protoplast derived EVs [14], and the apoplastic fluid method [15]. However, the former method is difficult to implement for continuous production, while the latter is limited to the EVs present in the apoplastic space of the plants.

A method similar to the *in vitro* production of mammalian vesicles using cell cultures is necessary for further developments in the field of plant EV research. Easy production of efficient and pure plant EVs could lead to major developments in plant EV research, and provide an alternative to mammalian EVs in clinical applications. Furthermore, standardized characterization methods with biochemical markers agreed by the plant EV community is necessary for the rigour required in EV research.

To address these issues, we developed a workflow that uses plant cell suspension cultures as the source of plant EVs. Isolating EVs from the conditioned media of cell suspensions avoided the disruption of cellular material, allowing the isolation of pure plant EVs. Extracellular vesicles from conditioned media of three such cultures, *Nicotiana tabacum, Stevia rebaudiana,* and *Vitis vinifera* were isolated with this protocol. Using density cushion ultracentrifugation to ensure the purity of the samples, the physical properties of large EVs (lEVs, pelleted at 10,000 x g) and small EVs (sEVs, pelleted at 100,000 x g) were characterized from these plants. To determine biochemical markers for plant EVs a conducted a proteomic analysis of *N. tabacum* sEVs.and seven other previously published plant EVs were conducted.

A total of 135 proteins were identified in *N. tabacum* sEVs. Plant EV biomarker candidates, reported in other plant EV proteomic studies were also identified in *N. tabacum* sEVs, such as HSP70, Tubilin, Glycerophosphodiester phosphodiesterase, lipid transfer proteins, Blue copper protein, glucan endo-1,3-beta-glucosidase, and malate dehydrogenase [11,19]. Gene ontology enrichment study of the *N. tabacum* sEV proteome shows that they fit the patterns of human EVs, are enriched in enzymes and catalytic proteins, and carries proteins associated with exosomes.

Conducting an orthologue discovery study for the 7 plant proteomes available, I have identified a total of 271 plant EV proteins from 6 plants, which are orthologous to 117 human EV proteins – 39 percent of the human EV proteins studied. 20 *N. tabacum* sEV proteins

were orthologous to 14 human proteins from the top 300 human EV proteins. 24 human EV proteins had plant orthologues in at least five out of the eight plants studied.

Our findings support that sEVs can be produced and isolated from plant tissue suspension cultures. Using bioreactors, large scale and cost-effective production of highly pure plant EVs can be achieved. The high degree of homology between plant and human EV proteins not only suggests the evolutionarily conserved nature of EV secretion, but also suggests that our knowledge in human EVs can be safely applied to plant EVs. Applications left to human EVs, such as carrying therapeutic agents, can also be done with plant EVs in an easier, cost-effective manner, and without ethical concerns.

1.1. HISTORY OF EV RESEARCH

Initial studies of mammalian EVs were functional studies, where researchers observed that the biological effects of some biological fluids, such as plasma, changed after high-speed centrifugation. The 1946 study by Erwin Chargaff and Randolph West [20] is commonly attributed to being the first paper that observed the effects of EVs, however, in truth, their paper itself was based on a short report by Macfarlane, Trevan, and Attwood in 1941. In their paper, Macfarlane et al. reported that centrifuging human or horse plasma at high speeds removed its ability to coagulate. Their claim of 'some of the accelerating substance is found in the deposit from the centrifugation of the plasma as well as in the supernatant fatty layer,' could be considered the first observation of a biological function by EVs [21]. Building on these foundations, Chargaff and West used high-speed centrifugation on human plasma to prove the existence of a circulating anticoagulant. They found that centrifuging plasma at 31,000 x g for 150 minutes made it incapable of coagulation, and the plasma only regained its ability to coagulate after the centrifugation pellet is reintroduced to it [20]. The findings of Chargaff and West were further articulated by the study of Peter Wolf, who was the first person to employ differential ultracentrifugation to isolate EVs [22]. Other functions of EVs were soon revealed when H. Clarke Anderson and Ermanno Bonucci independently discovered the roles of EVs during the calcification of the bone matrix [23,24].

Investigations into the biogenesis of EVs and their roles in biological systems as a whole started during the 1980s. Stegmayr and Ronquist described the effects of extracellular vesicles of semen on sperm motility [25], while two independent teams discovered that

maturating reticulocytes shed their transferrin receptor through extracellular vesicle secretion [26,27]. Further interest in the reticulocyte vesicles leads to the discovery of the endocytosis-to-multivesicular body biogenesis pathway of exosomes [28–30]. However, the field did not attract widespread attention until the discovery of immune-cell derived extracellular vesicles and their biological activity.

Raposo et al. reported that B-lymphocytes secreted extracellular vesicles that carried MHCII receptors with bound antigens, which were capable of antigen presentation and raising an immune response [31]. Soon after, Zitvogel et al. reported that extracellular vesicles of dendritic cells pulsed with tumour peptides could suppress tumour growth [32]. The next breakthrough happened during the 2000s, through the discovery of functional mRNA [33] and miRNA [34] transfer by extracellular vesicles. Discovering that extracellular vesicles not only carried proteins that raise short-term responses, but also mRNA and miRNA -which can mediate long term changes- resulted in a rush for the research and discovery of extracellular vesicles from all biological sources. Currently, the field of EV research consists of more than 25,000 scientific papers, with an influx of more than 4,500 papers last year.

1.2. APPLICATIONS OF EXTRACELLULAR VESICLES

As an element of cellular communications, EVs take part in virtually all biological functions. This has led to the study of EV functions in different processes of the human body, such as the immune system [35], wound healing [36], and regeneration [37], as well as the pathophysiology of diseases such as cancer [38], autoimmune diseases [39], or neurodegenerative disorders [40]. In addition to discovering the functions of EVs in biological processes, studies started on their potential as tools for the diagnosis and treatment of diseases. Isolation of EVs from a patient's blood sample can allow for a non-invasive and thorough diagnosis of their malignancies, such as cancer [41]. Microfluidics based systems are being developed that can isolate and analyse EVs on a chip using various methods [42], such as fluorescence [43], colourimetric [44], nuclear miniaturized nuclear magnetic resonance [45], or electro-kinetics [46]. Due to the wealth of biological information, an EV carries in the form of protein, lipids, miRNA, mRNA, and DNA fragments, studies have started to discover their potential al liquid biopsy tools in diseases such as cancer [47] or Alzheimer's [48].

Along with the diagnostic applications of EVs, they have also been successfully used in the treatment of diseases. Mesenchymal stem cell EVs have been used as a treatment for inflammatory and autoimmune diseases [49], and as a regenerative medicine [50]. EVs of the immune system have been used as a tool for immunotherapy against diseases such as cancer [32], and immune-related diseases [51].

In addition to acting as therapeutics, EVs can also be used as highly effective drug delivery devices. Their native function as transporters grants EVs many beneficial traits as a platform to carry therapeutic compounds for research and clinical applications [52]. For instance, EVs induce little to no toxicity or immune response from the administered body, as they are already present in all living systems [53]. Even the administration of non-autologous EVs, or EVs from other species, appears to induce little to no negative response, opening up the possibility of cheap, high-throughput production of clinical-grade EVs from sources such as bovine milk [54]. EVs remain in circulation longer compared to synthetic vehicles, possibly due to the surface proteins such as CD47 [55]. Furthermore, it has been speculated that EVs more readily avoid lysogenic pathways during cellular uptake, improving the chances of delivering fragile molecules such as miRNA in a functional form [56]. Finally, it has been speculated that the cellular uptake of EVs is a semi-selective process. Based on the composition of surface markers, EVs secreted by a particular cell are more likely to be internalized by cells of the type [57], or by cells under specific conditions, such as injury [58].

1.3. STRUCTURE AND PROPERTIES OF EXTRACELLULAR VESICLES

International Society of Extracellular Vesicles (ISEV) defines EVs as ‘particles naturally released from the cell that is delimited by a lipid bilayer and cannot replicate’ [1]. EVs range between 30 nm to up to several micrometres in diameter [59]. They consist of double-layered lipid membranes that enclose proteins, lipids, nucleic acids and other biomolecules.

The primary function of EVs is the transfer of information. Through the secretion of EVs, cells can communicate biological information, such as their responses to stimuli, to other cells in their vicinity [60]. Information carried by EVs takes the form of the proteins, nucleic acids and lipids that make up the EV. Transfer of information by EVs is not limited to the cargo enclosed within them - the lipids and membrane proteins that form the EV are capable

of initiating response by attaching to surface receptors of the recipient cells [61]. The messages carried by EVs are highly plastic and depend on the physiological state of the cell during the time of their biogenesis [62]. This behaviour allows cells to use EVs to relay their physiological responses to a biological stimulus and affect their surrounding cells - this behaviour is further supported by findings of increased EV secretion as a part of many biological responses. This 'stress-response' behaviour of EVs allows them to act as signalling cascades, where the initial cells that are directly affected by the stimulus produce EVs specially tailored towards raising a similar or related response to other cells they interact with. Passing a signal through EVs instead of cell-to-cell interactions both increases the range of which the cell can affect others, and amplifies the response, as millions of vesicles are released. The exact composition of the biomolecules that make up an EV changes from one secreting cell to another, or different physiological conditions for a particular cell. However, some biomolecules are more likely to be secreted via EVs, leading to their enrichment.

1.3.1. Proteins of Extracellular Vesicles

EVs carry both transmembrane and cytosolic proteins in their functional forms. These proteins are often used as biochemical markers for the characterization of an EV isolation, as well as to ensure purity from non-EV bound, co-isolating contaminants such as serum albumin. Tetraspanins (CD9, CD63, CD81), heat shock proteins (HSP70, HSP90), Tsg101, and elements of the Endosomal Sorting Complex Required for Transport (ESCRT) are some of the more commonly used protein markers for EVs [1]. Protein secretion through EVs allows an alternative to the conventional endoplasmic reticulum-to-Golgi protein secretion pathway, allowing signal peptide independent protein secretion [63]. Amongst the top 100 proteins identified in EVs, 80 percent of them carried no signal peptides. Proteins found in EVs are enriched in domains such as the ADP Ribosylation Factors family domain, which has functions in the biogenesis of cellular vesicles, and intracellular trafficking [64].

EVs are not limited to being passive transporters of proteins they carry within their membrane. As membrane-bound vesicles, it is unsurprising to see that more than 40 percent of proteins identified in EVs have at least one transmembrane domain [65]. These transmembrane proteins allow EVs to act as a proxy to their cell-of-origin for cell-to-cell

interactions. The best examples of this phenomenon can be seen in the EVs of the immune system. Antigen-presenting cells such as B-lymphocytes can secrete extracellular vesicles with functional, antigen bound MHC proteins, capable of initiating adaptive immune responses [31,32,66]. Furthermore, these EVs can 'attach' antigen-bound MHC proteins to target cells through membrane fusion, or through the internalization and recycling of their MHC proteins, allowing a transfer of antigens between antigen-presenting cells [67]. This event can happen to cells that traditionally do not express MHC proteins – such as follicular dendritic cells and MHC-II proteins – allowing them to present antigens [68].

Protein secretion by EVs could also be a major pathophysiological element of diseases involving misfolded and aggregated proteins. Findings of Parkinson's related α-synuclein in EVs not only suggests that EVs could be transporting the toxic protein between different sections of the brain [69] but also suggests that the initiation of the disease could be through EVs of α-synuclein carrying erythrocytes [70]. Similarly, β-amyloid proteins were observed also observed in EVs [71]. Whether or not EVs have a major function in the pathology of these diseases is still unclear. However, there are certain indications of the promising potential of EVs as an early diagnostic tool for such diseases [72].

1.3.2. Nucleic Acids of Extracellular Vesicles

EVs can carry fragments of DNA and different forms of functional RNA molecules. First reports of mRNA presence in EVs were made with embryonic stem cell microvesicles in 2006 [33], followed by the first reports of mRNA and miRNA in small EVs, isolated from mast cells [34]. mRNA discovered in EVs are reported to be of a smaller base length compared to cellular mRNA – mRNA in EVs are generally <700 bps in length, while cellular mRNA can go up to 12,000 bps. The smaller size of mRNA can be due to space limitations, especially in small EVs. Both fragments of [73], and complete mRNAs [74] has been detected in EVs. However, instead of mRNAs, the majority of EV RNAs are miRNAs.

A study reported that the mRNA fragments present in EVs are enriched in 3' untranslated regions (3' UTR) [73], which are reported to be regulatory regions of miRNA binding [75]. The 3'UTR regions brought to a cell by EVs can act as competitive inhibitors to cellular miRNA, allowing EV mediated post-transcriptional regulation [73]. The ability to carry functional mRNA and miRNA allows EVs to act as 'shuttles' between cells, allowing

horizontal gene transfer [34]. Depending on the source of EVs, different functions such as enhanced survival [76,77], differentiation [78], immune activation [79], or metastasis [80].

The presence of DNA fragments in EVs has not been investigated as much as other nucleic acids. This phenomenon has been best studied as a diagnostic tool for different cases of cancer. Double-stranded DNA fragments were discovered in the EVs isolated from the serum of pancreatic cancer patients [81]. Another study reported that tumour microvesicles carried DNA fragments with amplified *c-Myc* sequences [82]. A final study looked at different subpopulations of EVs and reported differentiating compositions of double-stranded DNA [83].

1.3.3. Lipids of Extracellular Vesicles

While not as well studied as the proteins or nucleic acids, lipids of EVs are a crucial part of their function. The presence of a lipid bilayer is a mandatory part of the definition of an extracellular vesicle [1]. The lipid bilayer of EVs differentiates them from other biological macromolecules, allowing unique interaction pathways such as membrane fusion, which can attach functional transmembrane proteins to recipient cells [84], and disrupting this membrane can prevent the biological activity of EVs [85].

Like proteins, some lipids are enriched in EVs compared to the cells that secrete them. Specifically, the composition of EV lipids is akin to the composition of the plasma membrane compared to the other components of the cell. Studies show that EVs are enriched in cholesterol (CHOL), sphingomyelin (SM), glycosphingolipids, phosphatidylcholine (PC), phosphatidylinositol (PI), and phosphatidylserine (PS), with CHOL and SM being the most enriched lipids of EVs [86,87]. The composition of lipids of a sample can be used as an indicator when claiming whether or not isolation contains EVs [86]. For instance, the cholesterol-to-phospholipid ratio is higher when compared to intracellular compartments such as the endoplasmic reticulum or Golgi [88]. Moreover, different cells secrete EVs with varying concentrations of different lipids [87], and different EV subtypes can have different EV compositions, allowing lipid-based subcategorization of EVs [84,89,90]. However, while specific lipids can be used for subcategorization, concentrations of the primary lipid components such as CHOL or SM remains mostly similar between large and small EVs [89].

Enrichment of cholesterol also suggests an enrichment of lipid-raft domains on the membrane of EVs. Lipid rafts are stable sections of the plasma membrane that have been suggested as an element of membrane trafficking, recycling of membrane proteins, and signal transduction. They consist of regions enriched in CHOL and SM. The structure of CHOL allows densely packing the acyl-chains of lipids, which creates rigid domains of the membrane [91].

Studies report that EVS is enriched in lipids associated with lipid-raft domains, such as CHOL, SM and glycosphingolipids, as well as proteins associated with raft domains, such as Lyn, flotillin-1 and stomatin, as well as the glycolipid ganglioside G_{M1}. A study suggests, by slowing down the recycling rate of proteins, lipid rafts could influence cargo selection in EVs, as faster proteins are more likely to leave the confines of an MVB before budding [92]. Similarly, lipid-raft domains are associated with ubiquitination proteins such as Cbl and Nedd4 [93] – and the role of ubiquitination in the cargo selection of EVs is a major hypothesis in the EV field through the ESCRT mechanism [94].

Another function of extracellular vesicle lipids is their role in the biogenesis of exosomes – EVs created through the ‘blebbing’ of the plasma membrane. The lipid composition of cellular membranes shows asymmetry between the cytosolic and non-cytosolic (luminal) faces. During blebbing, cells break down sphingomyelin, which normally binds to membrane CHOL. An efflux of CHOL out of the membrane increases membrane fluidity [95]. Coupled with the generation of ceramide, which has a cone-shaped structure, a spontaneous curvature can form on the membrane, leading to the formation of an EV [96, 97].

Lipids of EVs can also initiate biological responses to recipient cells. Lipids of grape exosome-like particles have been shown to induce proliferation and immunomodulation in stem $Lgr5^{+}$ stem cells [5]. Lipids of ginseng EV-like nanoparticles (NPs), particularly ceramide, has been associated with macrophage polarization and activation via the TLR4 receptor [4], while another study identified 188 lipid species, belonging to 15 different classes of lipids, associated with PKC and protein kinase D pathways, affecting cell cycle progression, survival, oncogenesis, apoptosis, and melanogenesis [98]. Finally, plant-derived lipids have been shown to increase EV secretion and reduce amyloid-β plaque formation in neural cells, suggesting that plant-derived EVs could carry similar lipids, and have therapeutic properties against neurodegenerative diseases [99].

1.3.4. Nomenclature

Cells can produce extracellular vesicles through a variety of pathways, with different pathways producing EVs with different physical and biochemical properties, with different functions. Classically, EVs were divided into the three categories of exosomes, microvesicles and apoplastic bodies [100]. Exosomes are smaller EVs, described as 30 to 100 nm in diameter, and originate from a multivesicular body (MVB) that is formed through initial endocytosis of the cell membrane. Microvesicles are larger vesicles (200-1000 nm), produced through the 'blebbing' of the membrane, where the cellular membrane buds out and pinches off, producing an EV. Apoptotic bodies are vesicles secreted during the apoptosis of cells [101].

Traditionally, the study of exosomes has been significantly more frequent than the study of other EVs, to the point of negligence in the field of EV researchers. Furthermore, the term 'exosome' was often misused, referring to a mixture of EVs instead of the purely late-endosomal vesicles the term describe. . For example, it is now known that EVs formed through membrane budding can be as small as 50nm's, challenging the previous notion of all small EVs being exosomes [102], such as the smaller, non-membranous protein-lipid complexes dubbed 'exomeres' [89,103].

Inconsistencies such as these, coupled with non-standardized methods of EV characterization and study has led to the publication of studies that did not include the necessary steps to ensure reliable results. To combat nomenclature and procedural inconsistencies, members of the International Society of Extracellular Vesicles published three papers, the latest of which, MISEV2018, lists the minimal set of experiments and studies necessary to publish a complete and accurate study on EVs [1,104,105].

As most of the current isolation protocols isolate a combination of different EV subtypes and markers that differentiate different subtypes of EVs has not yet been conclusively determined, the umbrella term 'extracellular vesicles' can be used to describe an EV isolate. ISEV also endorses the use of operational terms based on the properties of EVs used in a study, such as their sizes, densities, biochemical composition, or their cell-of-origin [1].

1.3.5. Biogenesis

Biogenesis of exosomes is a multi-step process involving the formation of endosomes, intraluminal vesicles (ILVs), and MVBs. Initially, the plasma membrane invaginates, forming an endosome within the cytoplasm. Endosomes that do not enter recycling, degradation or secretion pathways transform into late endosomes. Inward folding of the endosomal membranes forms ILVs. The folding action also encapsulates cytosolic elements such as proteins, nucleic acids or lipids into the ILVs. Late endosomes with multiple ILVs formed within are called MVBs, which either enter recycling pathways through lysosomes or fuse with the plasma membrane to release their ILVs as exosomes [106].

Mechanisms behind the intraluminal folding of the endosomal membranes that result in the formation of ILVs determines the cargo of the secreted vesicle. There are two families of proteins that have been associated with the biogenesis and cargo selection of secreted vesicles – ESCRT proteins and tetraspanins. The first category, ESCRT proteins, handle the sorting of ubiquitylated proteins into endosomal spaces. ESCRT proteins are well conserved and present in all eukaryotic cells. ESCRT consists of four distinct elements, ESCRT-0, -I, II, and –III. ESCRT –I and –II, in particular, has been associated with vesicle formation. These proteins are enriched on endosomal membranes, and initiates the infolding of the early endosomal membrane, forming the ILVs [107]. ESCRT-III completes this process by recruiting the Vps4/Vta1 complex to the site, which close the open-side of the infold, forming an ILV [108].

The second family, tetraspanins, are a family of transmembrane proteins. They appear to be present in all mammalians and plants, with 33 tetraspanins proteins identified in mammalians, 17 in *Arabidopsis thaliana* [109]. No homologues have been reported in yeasts or prokaryotes [110]. Tetraspanins take part in both the biogenesis and uptake of EVs. 13 mammalian tetraspanins have tyrosine-based sorting motifs, which mediates sorting of transmembrane proteins to endosomes and other intracellular vesicles [111], while additional motifs and clathrin coating are required for the uptake of vesicles in plants [109]. Tetraspanin protein CD63, which was considered as a differentiating biomarker for exosomes over other EVs, can interact with AP-2 complexes through an evolutionarily conserved motif of GYEVM located at its C-terminus, which links CD63 with clathrin-

mediated endocytosis pathways, as well as with AP-3, which traffics endosomes to lysosomes [112].

Another pathway of EV biogenesis is through the sphingolipids in the lipid-raft domains of the endosomal membrane. These sphingolipids can form ceramides, which recruit other raft domains, forming larger domains, which can induce domain-induced budding. Additionally, the cone shape of ceramides can create a negative curvature due to the area difference between membrane leaflets [113]

1.3.6. Interaction Pathways

After secretion, EVs can interact with cells in a large variety of pathways. In biological systems, EVs stay in the circulatory system for a short period. Studies on the subject report a range of clearance times, between 2 minutes [114] to 3 hours [115], to up to 48 hours [57], albeit the latter article notes the risks of unspecific staining due to the long half-life of the dye. Both the half-life and biodistribution of EVs are affected by the type of EV used. Mesenchymal stem cell EVs selectively accumulate at injury sites [58,116], while dendritic cell EVs accumulate at lymph nodes [57]. EVs administered intravenously accumulate more at the livers, subcutaneously at the GI track, and intraperitoneal, at the pancreas [57]. Regardless of the type or path of administration, EVs are sequestered by phagocytic cells, with a majority of them accumulating at clearance organs such as the liver, lungs, spleen, or kidneys.

EVs must adhere to cells in most of their interactions. Several membrane proteins enriched in EVs can handle this action. For example, dendritic cell EVs carry ICAM-1 [117], coupled with CD9 and CD81 on their membranes [118], which facilitate their binding to T-cells. Different cells express different EV proteins, such as CD169 for macrophages, and heparin sulphate proteoglycans for glioblastoma and embryonic kidney cells [119].

After adhesion, EVs can affect cells through surface-to-surface interactions, by fusing with the cellular membrane, or through the internalization of the whole EV. The best example of surface protein-mediated activation is the presence of antigen-presenting major histocompatibility proteins on EVs of antigen-presenting cells. These proteins can activate

immune cells as if the EVs are an antigen-presenting cells, albeit at a lesser effectiveness [31].

Fusion refers to the membrane of the vesicle becoming part of the plasma membrane of the recipient cell. This action can attach the surface proteins of the EV to the plasma membrane of the recipient cell, allowing interactions such as sharing antigens between antigen-presenting cells [67]. This uptake pathway is particularly advantageous for drug delivery. EV fusion avoids lysosomal pathways, allowing fragile cargo, or cargo that cannot diffuse through the lysosomal membranes, to be effectively delivered via EVs [120].

The final pathway of EV-cell interactions is through the complete internalization of the EV. This can happen through a variety of pathways, such as phagocytosis, macropinocytosis, or receptor and raft mediated endocytosis. Phagocytosis depends on the opsonin receptors on the plasma membrane, which activates an actin-mediated process that leads to the internalization of the EVs. Macropinocytosis employs actin filaments at a larger scale, forming plasma membrane projections that create large infolds of the plasma membrane, collecting extracellular fluids and EVs alike. Finally, receptor or raft mediated endocytosis occurs when specific surface elements of an EV interacts with the plasma membrane. These signals result in the endocytosis of EVs through one of two pathways. Clathrin-mediated endocytosis happens through the interactions of clathrin and adaptor protein 2 complexes on the cell membrane, which creates an infold similar to the ones in micropinocytosis mechanisms of uptake [119]. Caveolae mediated endocytosis happens through the formation of invaginations by the caveolin proteins [121].

EVs can also affect cells independent without contacting them. Signal molecules on EV surfaces can be released through proteolytic cleavage. Examples of these are the FasL, TNF, and TRAIL proteins [122], however, these molecules have a reduced effectiveness after their cleavage [123].

1.4. PLANT EXTRACELLULAR VESICLES

1.4.1. History of Plant Extracellular Vesicles Research

While mammalian EVs have the attention of the majority of studies in the field, the discovery of plant and fungi derived EVs were not far behind their mammalian counterparts (Figure 1.1). Furthermore, evidence towards secretion and structure of plant and fungal EVs, and hypotheses on the mechanisms associated with, presided the ones for mammalian EVs. During observations with a transmission electron microscope, Girbardt discovered the presence of spherical structures between the plasma membrane and the cell wall of fungal hyphae [124]. These structures were named 'lomasomes' (meaning border bodies) by scientists Moore and McAlear, who proposed that these vesicles took part in the cell wall metabolism through the deposition of materials by the means of fusion with the cell membrane – which we now know to be a correct hypothesis [125].

Hypotheses on the biogenesis of these vesicles followed these findings. Scientists Peyton and Bowen observed that the parasitic *Peronospora manshurica* secreted vesicles during its invasion of soybeans, and suggested that the parasitic vesicles were produced by elaborations of the cell membrane and supported the uptake pathway suggested by Moore and McAlear [126]. Marchant claimed that, based on the heterogeneity of lomasome structures, their secretion must be based on two different pathways - through membrane elaborations and through multivesicular bodies formed by the endoplasmic reticulum [127]. Marchant's suggestion lead to the umbrella term 'paramural bodies' (PMBs), with two distinct subclasses of these vesicles – lomasomes, which originated from endosomal sources, and plasmalemmasomes, which originated from the cell membrane itself[21]. These discussions were in parallel with discussions into the origin of mammalian EVs.

While the prevalence of PMBs in fungal hyphae, and the claims of Marchant on lomasomes only being present in fungi with chitosan in their cell wall [127], researchers soon discovered similar structures in different plants and algae. Lomasomes were identified in 'Khapli' wheat [128], during viral infection in sugar beets [129,130], in cotyledons during germination [131], carrot cell cultures [132], and during the differentiation of xylems of willow [133].

In spite of a growing number of observations of plant EVs, researchers were still unsure of their biological functions. Some researchers suggested that they carried and deposited material for growth and expansion, primarily the expansion of the cell wall [131,133] – however, this hypothesis would not explain the presence of EVs outside the cell walls [132]. Other researchers observed an accumulation of MVBs at fungal infection sites in plants, but failed to identify their function at these sites [134,135]. The latter claim has been further investigated through the years, and several molecular mechanisms and models have been suggested as a defence mechanism [136]. Several studies suggest that plants may secrete vesicles carrying antifungal siRNAs to combat fungal infections [14,137].

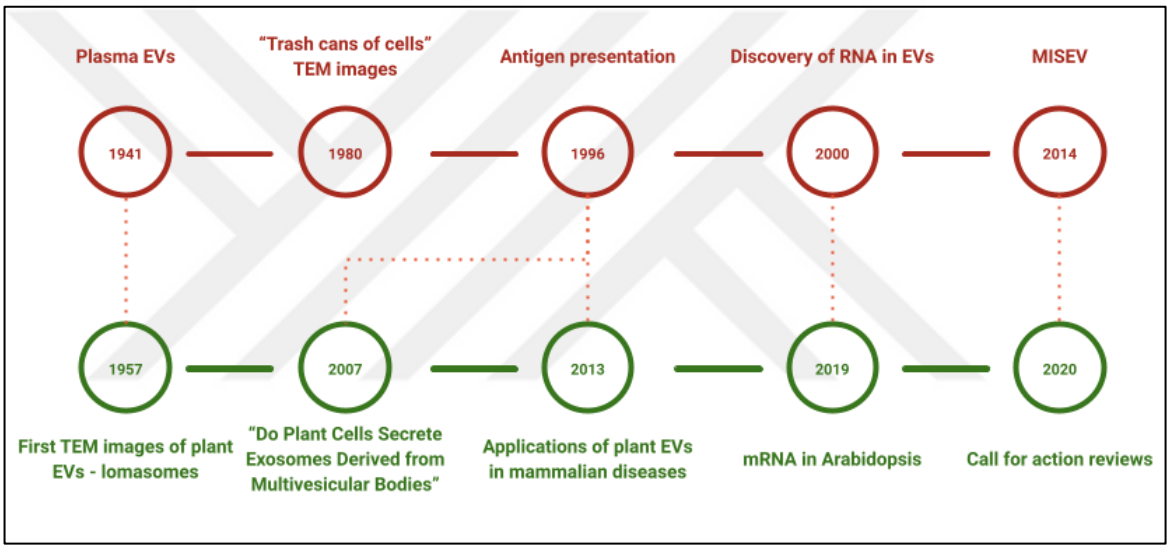

Figure 1.1. History of EV and plant EV research

1.4.2. Modern Plant Extracellular Vesicle Research

The discovery of functional mRNA and miRNA inside EVs attracted a large number of researchers interested in the study of EVs. It was during this revival that researchers started to experiment with plant-derived EVs.

Plants appear to be a promising subject for further research, either as a source of therapeutic EVs -where they are reported to be effective against diseases such as cancer,- or as a previously unstudied portion of the plant stress response. Plants produce the majority of the therapeutics used in medicine, meaning that plant EVs may be used as vehicles for the

administration of these therapeutics, or aid in their production and purification. Studies have already begun on discovering applications of plant EVs in the treatment of various diseases.

Cancer is one of the first fields in which the effects of plant EVs were studied. Raimondo et al. reported *Citrus limon* derived nanovesicles induced TRAIL-mediated apoptosis in human colorectal adenocarcinoma and human lung carcinoma cell lines [3]. Raimondo et al. further supported their findings with an *in vivo* uptake and tumour suppression study. Zhuang et al. demonstrated the ability of grapefruit derived nanoparticles as potential drug carriers, using them as vehicles to carry therapeutic microRNA past the blood-brain barrier, and treated mice with brain tumours [138]. Zhang et al. demonstrated that ginger-derived NPs could be used for immune suppression, and through it, colitis induced cancers [139]. Li et al. used modified ginger derived nanovesicles for the systemic delivery of siRNA, where they reported that the arrow-tail ligand they engineered onto ginger EVs could lead to increased uptake under *in vivo* conditions [140]. Lee et al. reported that *Dendropanax morbifera* derived nanoparticles were against melanoma, where vesicles extracted from both the leaves and stems of the plant had superior whitening effects than of the positive control [8]. Kim et al. used EVs isolated from the saps of four plants –including *D. morbifera*- on breast and skin cancer models [141] including a 3D microfluidic cancer metastasis model that mimics *in vivo* microenvironment on cancer-associated fibroblasts [142], and observed anti-tumour and anti-metastatic effects. Another study involving melanoma was published by Cao et al., where they used ginseng derived nanoparticles, and demonstrated the ability of these particles in macrophage polarization, where they showed that ginseng derived nanoparticles could influence the polarization tumour-associated macrophages towards the tumoricidal M1 phenotype [4]. A further study, by Stanly et al., targeted melanoma with micro and nanovesicles isolated from various *Citrus* species, where they observed differential metabolomes between the different species, and observed that *Citrus paradisi* derived vesicles induces cell cycle arrest and apoptosis while inhibiting cell growth, proliferation, migration, and the expression of oncogenes in melanoma [143]. Yang et al. used dialysis bags with 300 kDa membranes to isolate nanovesicles of lemon, and used these nanovesicles in the treatment of gastric cancer [144]. Finally, Zhang et al. reported that nanovesicles from *Asparagus cochinchinensis* inhibits the proliferation of hepatocellular carcinoma cells. Using PEG engineering, they optimized the pharmacokinetic properties of the vesicles, increasing circulation times and anti-tumour effects.

Closely correlated with cancer research with samples such as ginger [139] or ginseng [4] EVs, studies involving the immune system are another field that plant EVs and plant NPs are found to be effective. Ju et al. employed grape-derived nanoparticles in the treatment of colitis in an *in vivo* model, where they observed that grape NPs induced suppressive stem cells through blocking a β-catenin–mediated signalling pathway [5]. Mu et al. isolated vesicles from three different plants, and observed that these vesicles are resistant to digestion by stomach fluids, were taken up by intestinal macrophages and stem cells and induced the expression of immunosuppressive cytokines such as IL-10 [6]. Song et al. showed that garlic-derived nanovesicles are taken up by liver cells through the protein CD98, and had anti-inflammatory properties [145]. Teng et al. reported that the miRNA content of ginger derived nanoparticles are preferentially phagocytosed by the *Lactobacillaceae* of the gut microenvironment, which could affect the overall state of the gut microbiota [146]. Another study by Teng et al. reported that ginger derived nanoparticles can be used in the treatment of SARS-CoV-2, through the suppression of inflammatory cytokines in the lungs [147]. Yamasaki et al. observed that onion derived nanoparticles can be used to suppress LPS-induced nitric oxide production in mouse macrophage cells, and this suppression was not reliant on the endocytosis of the vesicles, suggesting interactions between surface elements of cells and nanoparticles to be behind this observation [148].

Another field where plant EVs attract attention is drug delivery. Instead of studying the effects of plant EVs on their own, researchers take advantage of the non-toxic, easy-to-upscale nature of plant EVs to deliver therapeutic agents. You et al. encapsulated therapeutics in vesicles isolated from standard and red cabbages [149]. Zhuang et al. made good use of EVs ability to pass through the blood-brain barrier and their ability to deliver fragile cargo such as miRNA through lysosome avoidance and delivered therapeutic miRNA to brain tumours [9]. Garaeva et al. delivered bovine serum and HSP70 in their functional forms to human cells. Using labelled proteins, they successfully delivered the functional protein to different organs in an *in vivo* model [10].

Traditional applications of plants in wound healing also find parallels in plant EV research. Savcı et al. reported that grapefruit-derived extracellular vesicles can induce proliferation and angiogenesis while reducing the production of reactive oxygen species [150]. Şahin et al. reports that wheat-derived nanovesicles can induce proliferation and migration in endothelial cells, epithelial cells, and dermal fibroblasts [151].

Anti-aging research commonly makes good use of plant-derived products, and plant EV research is no exception. Cho et al. isolated EVs of ginseng cultures and ginseng-NPs from ginseng roots, and observed that treating human dermal fibroblasts or melanocytes with these EVs downregulated senescence-associated and melanogenesis-associated molecules [98]. Perut et al. used EV like nanoparticles isolated from strawberries and observed these vesicles can reduce oxidative stress in human mesenchymal stem cells [152].

The final field of application in plant EV research employs the anti-microbial properties of some plant species. Pinna et al. reports that nanovesicles of *Thymus capitatus* and *Citrus limon* variant *pompia* has anti-microbial properties against the fungus *S. mutans* and yeast *C. albicans* [153]. Sundaram et al. reports that ginger derived nanoparticles can inhibit the pathogenicity of *Porphyromonas gingivalis*, and report that that they can be used in the treatment of periodontitis in an *in vivo* mouse model [154].

While reports of therapeutic success with plant EVs is promising, a large majority of plant EV research does not adhere to the MISEV criteria. Most plant-EV research isolates EVs or EV-like NPs from the lysate of plant material, usually prepared by juicing or blending fruits or plants acquired from local markets. One of the fundamental parts of the MISEV definition of EVs is that cells used during the production of EVs must remain intact before collecting the EVs. This is due to the presence of intracellular vesicles, which may be released if the integrity of the cells is compromised. Intracellular vesicles outnumber EVs, and resemble EVs in many ways, making the contamination difficult to overcome. This problem is exacerbated by the harsh disruption methods employed in plant EV research. Furthermore, due to a lack of knowledge and refinement in plant EV isolation methods, phytochemicals released during the disruption processes can also contaminate the EV isolates. The presence of these contaminants makes it unclear the observed biological effects are truly caused by the plant EVs, or caused by the phytochemicals present in the isolate.

These issues are not unique to plant EV research. The increasing popularity of EV research brought with it a large variety of nomenclature, invented by researchers to reflect the particular origin or functions of the extracellular vesicles they are working with. More crucially, due to a lack of standardization or scrutiny in EV research, many reports on the effects of EVs did not consider the importance of production and isolation methods on the effects they observed, such as the presence of soluble contaminants capable of raising similar responses.

While findings in these papers should be approached with a grain of salt due to the possible presence of non-EV contaminants used in these studies, possible therapeutic applications of plant-EVs demonstrated with these studies must be revisited with modern plant-EV isolation techniques to validate their findings. Isolation protocols used in these earlier studies did not properly address the difficulties of isolating extracellular vesicles from plants and did not fulfil the requirements laid down in the MISEV papers. Pioneering papers in this field correctly addressed this issue by avoiding the terms ‘exosomes’ or ‘extracellular vesicles’, and instead used more general terms such as ‘exosome-like’ or ‘nanoparticles’. However, these terminologies still infer that the isolate consists only of particles, and does not carry any contaminants such as soluble proteins or phytochemicals, and some of the studies themselves lack the rigorous control groups, such as the inclusion of a particle-depleted form of the isolate, to control against these contaminants.

There are some studies of plant EVs that fulfil the requirements set in the MISEV2018 guidelines. These studies acquire plant EVs without the use of harsh disruption methods, using protoplast cultures, apoplastic washes, and gentle dislocation methods. The correct nomenclature for vesicles isolated through these methods is called plant EVs, while vesicles isolated through harsher methods should be called plant-derived nanoparticles (PDNPs). So far, there have been five such articles, isolating EVs from Arabidopsis [15,152], olive pollen grains [153], sunflower seeds [16], and ginseng [95]. The first four of these studies were made with the apoplastic wash technique, where explants are vacuum infiltrated with a buffer, which washes the apoplastic space and recovers EVs from it. The study by Cao et al. published in 2021, however, employs plant cell suspension cultures as its source of EVs [95].

Plant cell suspension culture is the *in vitro* culture of a tissue culture derived from a particular explant of a target plant. Plant cells are grown in the suspension of a liquid culture media that contains optimized concentrations of hormones and nutrients tailored to the specific plant cell. Suspension cultures are generally used for the large scale production of plant metabolites [16] or recombinant proteins [17]. Plant cell suspension cultures are well established, but often neglected platforms of large scale production [18]. As a stable, liquid culture of plant cells, conditioned media collected from a plant cell suspension culture may be used to isolate plant EVs with ease, at high efficiency and purity. Suspension culture also allows the study of highly specific subgroups of plant cells, where EVs that represent the

cells of a single type of tissue can be isolated, compared to a mixture of EVs from different plant tissues.

In this study, we have developed the use of plant cell suspension cultures as a source of plant EVs. We isolated and characterized the EVs of *N. tabacum*, *V. vinifera* and *S. rebaudiana* using workflow. Furthermore, we conducted the first proteomics study of *N. tabacum* EVs, and the first proteomics study of plant cell suspension cell culture-derived EVs. Coupling our proteomic data with the previously published plant EV and PDNP proteomic data, we conducted a meta-study of plant EV proteomes to find protein enrichment patterns that can be used during the characterization of plant EVs. Finally, we created an orthologue-discovery assay against the top human EV proteins to identify potential plant EV biomarkers. We discovered 116 orthologous proteins, and 24 biomarker candidates, which were present in at least 5 of the plant EV proteomes studied. Our findings show that plant cell suspensions are an ideal source for producing plant EVs for research and therapeutic applications, and the biomarkers we discovered have the potential of catapulting plant EV research.

2. METHODS

2.1. PLANT CELL SUSPENSION CULTURE

The cultivar variants used in this study were *Vitis vinifera* cv. Öküzgözü, *Nicotiana tabacum* cv. Xanthi and *Stevia rebaudiana* L. Cell suspension culture media composition used in this was optimized in a previous study [157]. Suspension culture media consisted of Murashige and Skoog basal media supplemented with vitamins (4.405 g/L), sucrose (30 g/L), casein hydrolysate (250 mg/L), 1-Naphthaleneacetic (2.5 mg/L), and 6-Benzylaminopurine (0.2 mg/L).

2.2. ISOLATION OF PLANT EXTRACELLULAR VESICLES

Plant extracellular vesicles were isolated from suspension culture media using a modified version of the sucrose-cushion EV isolation method. Collected media were centrifuged at 300 x g for 10 min at 4^{o}C and 2,000 x g for 10 minutes at 4^{o}C to remove large contaminants and cellular debris. The resulting supernatant was then centrifuged at 10,000 x g for 20 minutes at 4^{o}C to collect lEVs. 10 ml of supernatant was transferred to a 13.2 ml ultracentrifugation tube, and 1.5 ml of 1M sucrose solution in PBS was gently layered to the bottom of the tube with the use of a glass Pasteur pipette. Samples were centrifuged at 100,000 x g for 2 hours at 4^{o}C using an SW 41Ti Beckman Rotor (331362). Top phases were discarded, and 1 ml were collected from each bottom phase to ensure that the samples were not contaminated from the top phases. EV containing fractions were then washed away from sucrose using 50 kDa ultrafiltration columns (Amicon, UFC905024). By repeating the washing process thrice, an 80,000 fold reduction of sucrose was achieved. EVs were stored at -20°C before use.

2.3. NANOPARTICLE TRACKING ANALYSIS (NTA)

Concentration and size distribution of plant vesicles were measured using Nanosight NS300 NTA (Malvern) device. Briefly, 20 µl of EVs were stained with 5 µl of 100 µM CellTracker Green CMFDA (C7025) dye for 30 minutes at 37°C. Samples were then passed through

exosome spin columns (Invitrogen 4484449) to remove unbound dye particles. Stained samples and their controls were then measured with the 532 nm laser module and the O-ring top plate. Samples and controls were diluted with particulate-free distilled water to have 20-200 particles in frame. Controls were used to test against the chances of lipophilic dye aggregates appearing as particles - if the control samples had over 5 particles, the samples would be rejected. 15 captures of 30 seconds each were captured for each sample, with additional liquid being introduced after 3 captures. Captures were analysed with appropriate threshold values using NTA software version 3.4. Measurements were individually analysed and tracks created by sudden flares and other complications were excluded.

2.4. FATTY ACID METHYL ESTER (FAME) ANALYSIS

Identification of fatty acid profiles of plant cell suspension culture derived EVs were made using. Samples were analysed with Agilent Tech GC-Midi 6890 N. Samples were prepared according to the manufacturer's instructions. Briefly, 1 ml of Reagent 1 (15 percent sodium hydroxide, 50 percent methanol), was added to EV samples and vortexed. Samples were heated at 100°C water bath for 5 minutes. Eppendorf's containing the samples were vortexed again, and placed in the water bath for an additional 25 minutes. After the incubation, samples were cooled in a 4°C water bath for 1 minute, and were then combined with 2 ml of Reagent 2 (10 percent hydrochloric acid, 45 percent methanol). Tubes were vortexed, and heated in an 80°C water bath for 10 minutes, and cooled for 1 minute. 1.25 ml of Reagent 3 (50 percent hexane, 50 percent methyl-ester-butyl ether). Samples were centrifuged at 3,000 rpm for 10 minutes, forming two distinct phases. Upper phases were collected into clean. Identification of fatty acids was confirmed manually in the Sherlock Microbial Identification System.

2.5. PROTEOMICS ANALYSIS

For proteomics analysis, sEVs were isolated from 200 ml of *N. tabacum* suspension culture media and concentrated to a volume of 200 µl using 50 kDa ultrafiltration columns (Amicon, UFC905024). The prepared concentrate was diluted with SDS-PAGE sample buffer. Protein concentrations were determined using Bradford assay (Biorad) with Nanodrop 1000

Spectrophotometer (Thermo Fisher). Proteins were separated using a 12 percent SDS-PAGE and Mini Protean Tetra System (Biorad). Proteins were precipitated and concentrated using ReadyPrep 2-DE Cleanup Kit (Biorad) according to the manufacturer's instructions. Prepared gels were fixed overnight in a fixation solution consisting of 40 percent methanol and 10 percent acetic acid, and colloidal Coomassie Brilliant Blue G-250 in distilled water. For in-gel digestion, spots were excised from the SDS gel and subjected to an in-gel tryptic digestion kit (Thermo Fisher) according to the manufacturer's instructions. Mass spectroscopic analysis of the digested peptides was made by nLC-MS/MS using an Ultimate 3000 RSLC nanosystem (Dionex, Thermo Fisher) coupled with a Q Exactive mass spectrophotometer (Thermo Fisher). Digested peptides were pre-concentrated and desalted on a trap column, and were then separated using high-performance liquid chromatography (HPLC) with an Acclaim PepMap RSLC C18 analytical column (75 μm x 15 cm x 2 μm, 100 Å diameter, Thermo Fisher Scientific). Samples were separated under the following sequence – 6-20 percent B for 45 minutes, 20-40 percent B for 30 minutes, 40-90 percent B for 15 minutes, 90 percent B for 30 minutes, 90-6 percent B for 5 minutes, and 6 percent B for 10 minutes, with a flow rate of 300 nL/min. Full scan MS spectra were obtained with a resolution of 70,000, scan range of 40-2000 m/z, spray voltage of 2.3 kV, target automatic gain control of 'AGC' $3x10^6$, maximum injection time of 60 ms. The top ten precursor ions were selected by data-dependent acquisition for MS/MS analysis. Protein identification was made using Proteome Discoverer 2.2 (Thermo Fisher) software with the following parameters: peptide mass tolerance of 10 ppm, MS/MS mass tolerance of 0.2 Da, mass accuracy of 2 ppm, tolerant miscarriage of 1, minimum peptide length of 6 amino acids, fixed changes cysteine carbamidomethylation, unstable changes methionine oxidation, and asparagine deamination. Final results were queried in the Uniprot/Swissprot database for protein identification.

2.6. BIOINFORMATICS ANALYSIS

Uniprot accession numbers were used for the functional annotation of the proteins. Gene ontology (GO) enrichment analysis of the *N. tabacum* sEV genome was made using Protein Analysis Through Evolutionary Relationships [155] (PANTHER) and Kyoto Encyclopedia of Genes and Genomes [156] (KEGG). Subcellular locations of the identified proteins were predicted using the CELLO2GO software [157].

For orthology analyses, proteomic data of human EV proteome was obtained from Vesiclepedia [158]. Proteins with <123 occurrences in Vesiclepedia, which make up the top 301 proteins of human EVs were used during orthology analysis. Proteomes of *Arabidopsis thaliana* [15], *Citrus limon* [3], grape [5], grapefruit [159], sunflower seeds [19] and watermelon [160] EVs were obtained from various articles. Protein sequences for *Arabidopsis* were obtained from the Arabidopsis Initiative, *Citrus limon*, grape and grapefruit from Uniprot database, for sunflower from HanXRQ database [161], and for watermelon from CuGenBit database [162]. Plant EV proteomes were aligned against the top human EV proteins using Basic Local Alignment Search Tool For Proteins (BLASTP) [163]. E-value was selected as 10^{-3}, and proteins with a query coverage of at least 70 percent with at least 50 percent identity within the region were considered orthologous proteins.

2.7. STATISTICAL ANALYSIS

Statistical analyses were performed on GraphPad Prism 9 unless otherwise stated. For physical characterization experiments, at least three biological replicates were used for each result. Replicate measurements were taken from distinct samples, except in the case of technical replicates. For bioinformatics analysis, data filtration and statistics of the data were handled using Microsoft Excel.

3. RESULTS

3.1. PHYSICAL CHARACTERIZATION OF PLANT CELL SUSPENSION CULTURE DERIVED EVS

To study plant EVs, sEVs and lEVs were isolated from the conditioned media of plant cell suspension cultures of *Nicotiana tabacum, Vitis vinifera,* and *Stevia rebaudiana*. lEVs were isolated by differential centrifugation, and sEVs were isolated from the resulting supernatants, which are further subjected to density cushion ultracentrifugation. The concentration of EVs were measured using NTA, and the size distributions of EVs were measured using dynamic light scattering (Zetasizer) and NTA.

The average diameter of the sEVs isolated were 146.4 nm, and did not significantly differ between different plant EVs. However, the average diameter of lEVs were significantly different from one another. Mean size of lEVs were 117.2 nm for *N. tabacum,* 363.3 nm for *V. vinifera,* and 241.3 nm for *S. rebaudiana* (Figure 3.1.).

Different plants yielded significantly different amounts of EVs from one another. The yield of EVs of *N. tabacum* was 3.14×10^{10}, of *V. vinifera* 5.56×10^{9}, and of *S. rebaudiana* 9.95×10^{9} particles per ml. Only the yield of *N. tabacum* lEVs were significantly higher than lEVs of other plants, with 6.62×10^{13}, 3.81 10^{9} for *V. vinifera*, and 3.93×10^{9} for *S. rebaudiana* (Figure 3.2.).

The yield of EVs increased significantly with the use of a bioreactor. *N. tabacum* cells in the bioreactor produced 100 times more EVs compared to the standard plant cell suspension culture setup (Figure 3.2.).

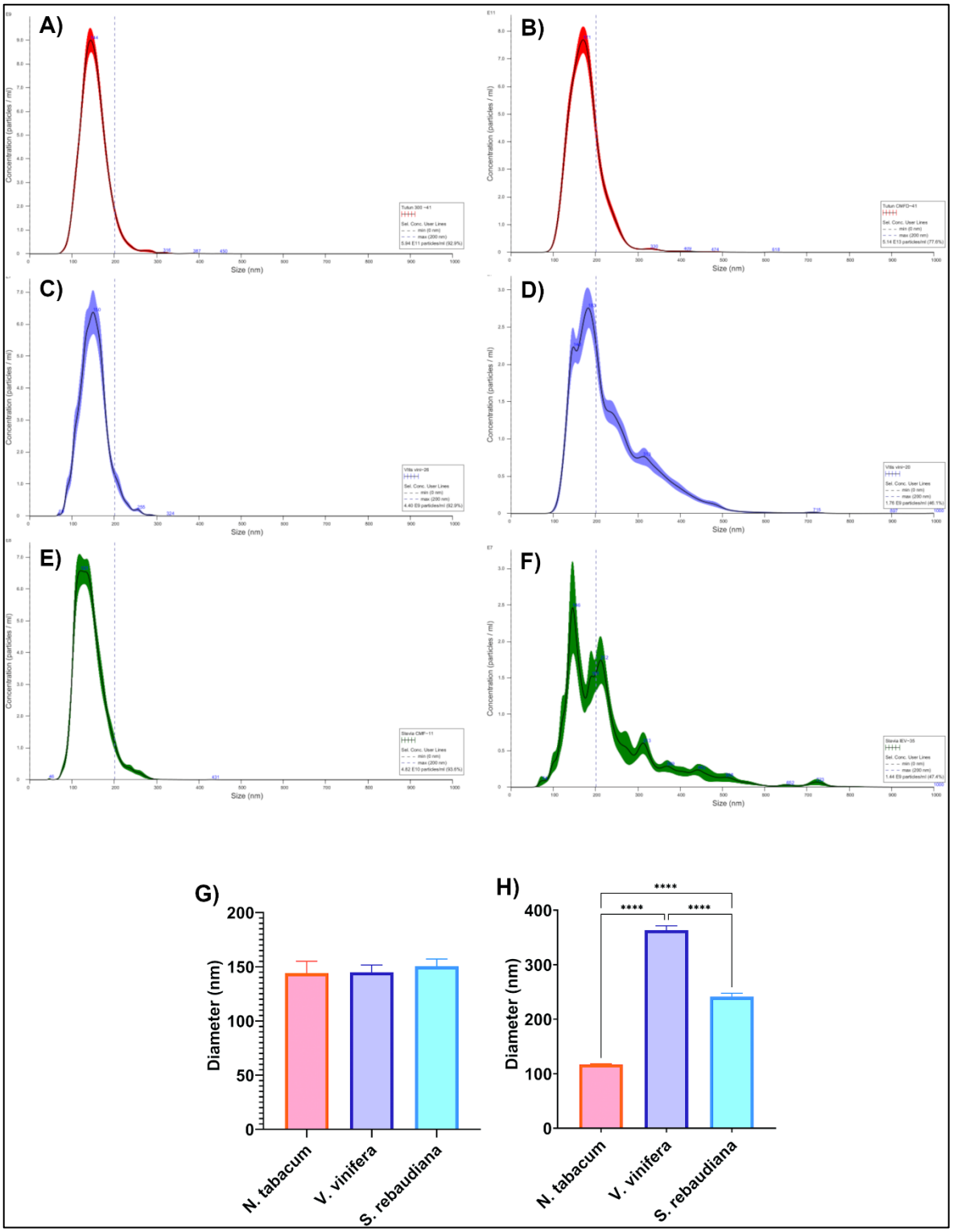

Figure 3.1. Physical characterization of plant cell suspension culture derived EVs. A) *N. tabacum* sEVs, B) *N. tabacum* lEVs, C) *V. vinifera* sEVs, D) *V. vinifera lEVs*, E) *S. ebaudiana* sEVs, F) *S. rebaudiana* lEVs, G) Mean sEV sizes, H) Mean lEV sizes

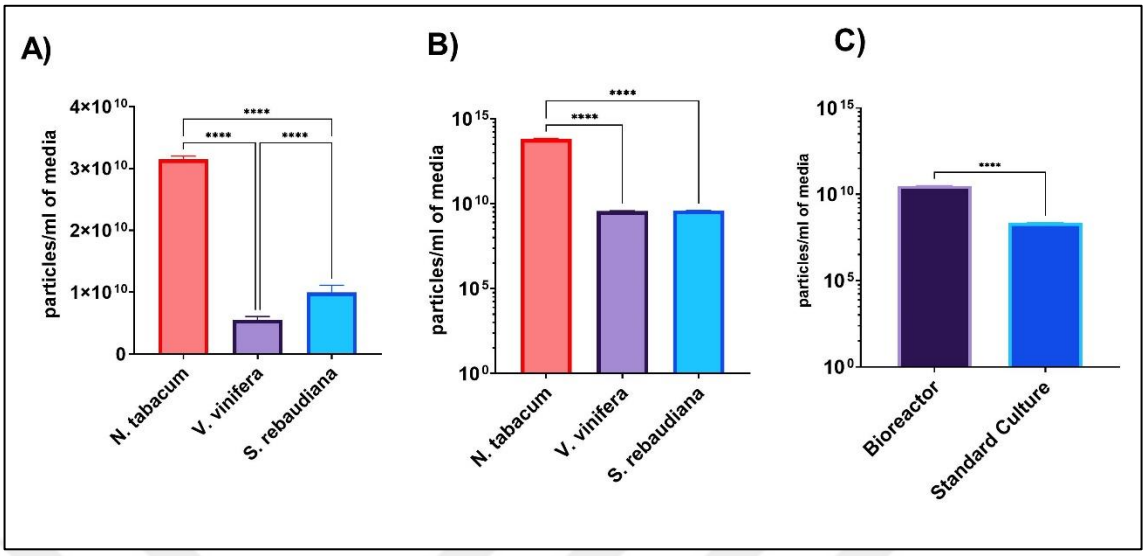

Figure 3.2. The yield of different EVs. A) sEV yields, B) lEV yields, C) Difference EV yields between the bioreactor and standard culture of *N. tabacum*

3.2. PROTEOMIC PROFILING OF PLANT EVS

Next, proteomic analysis of *N. tabacum* EVs was performed. *N. tabacum* was selected due to its status as a model organism. Approximately 3.2e12 *N. tabacum* sEVs were sent for proteomics analysis. A total of 135 proteins were identified, a full list of which is available in Appendix A.1. eight proteins that were previously identified as potential plant EV biomarkers were detected in *N. tabacum* sEVs (Table 3.1.) [11,19].

Table 3.1. Plant biomarker proteins detected in *N. tabacum* sEVs

Protein Name	Biological Function	Accession
Blue copper protein	Electron transfer activity	A0A1S3ZME8
Glucan endo-1,3-beta-glucosidase	Carbohydrate metabolism	A0A1S4D1I4
Glycerophosphodiester phosphodiesterase	Lipid metabolism	Q2WGN6
Homocysteine methyltransferase	Methylation	A0A1S3XCV6
Tubulin	Cytoskeleton	Q8VXC9
Heat shock protein 70	Stress response	Q67BD2
Lipid transfer proteins	Lipid transport	A0A1S4DJK5
Malate dehydrogenase	Carbohydrate metabolism	A0A1S3YXG6

Gene ontology analysis revealed that the enrichment profile of proteins in *N. tabacum* sEVs fits the profiles of mammalian EVs. *N. tabacum* sEVs were enriched in proteins related to the plasma membrane, cell wall and extracellular region, while depleted in protein related to the nucleus, organelles, and intracellular anatomical structures. Most enriched GO terms were ‘anchored component of plasma membrane’, ‘anchored component of membrane’, and ‘intrinsic component of plasma membrane’, with 42.0, 39.2 and 27.9 fold enrichment compared to reference background. Categories of ‘cell periphery’, ‘plasma membrane’ and ‘anchored component of membrane’ were linked with most proteins, accounting for 32.7 percent, 26.5 percent and 24.8 percent of the identified proteome respectively. 46.9 percent of the identified proteins could not be classified under any of the categories of the ‘Cellular compartments’ classification (Figure 3.3.).

N. tabacum sEVs were highly enriched in proteins associated with various metabolic processes, with 54.9 percent of the identified proteins taking part in various metabolic pathways. Over 100 times enrichment was observed in proteins related to D-xylose and arabinan catabolism. Proteins associated with the GO term ‘response to wounding’ were enriched 86.3 times, while proteins associated with glycerol, alditol, methionine, xylan metabolism, and cell wall polysaccharide catabolism were enriched more than 50 times. Molecular function analysis revealed that *N. tabacum* sEV proteome is enriched in proteins associated with catalytic activity and hydrolase activity, making up 63.7 percent and 33.6 percent of the total identified proteins respectively (Figure 3.3.).

KEGG BRITE results reveal that sEVs are enriched in proteins that fall under the categories of KEGG orthology and enzymes, with 34.2 percent and 23.7 percent of the queried proteins falling under the said categories respectively. Enzymes such as glycerophosphodiester phosphodiesterase, malate dehydrogenase, and peroxidase were detected in the sEVs. Notable proteins annotated were HSP70, Tubulin alpha, and 14-3-3 protein, which all fall under the protein family for exosomes in BRITE. Furthermore, KEGG pathway search matches 6 proteins to ‘metabolic pathways’ and 4 proteins to ‘biosynthesis of secondary metabolites’.

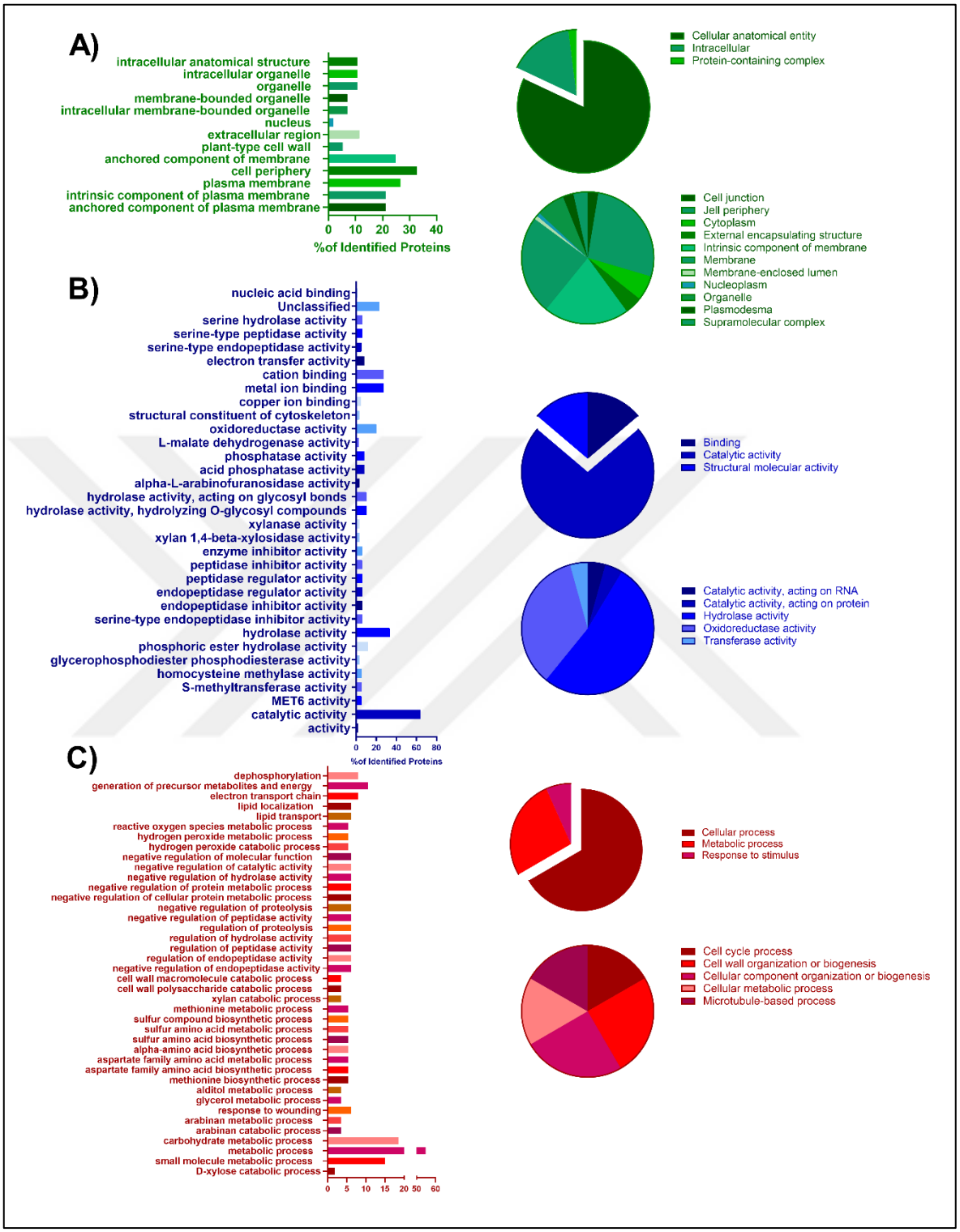

Figure 3.3. GO enrichment profiles of *N. tabacum* sEV proteome. A) Cellular compartment GO terms, B) Molecular function GO terms, C) Biological process GO terms

3.3. ORTHOLOGY

In order to discover potential plant EV markers, the proteome of *N. tabacum* sEVs and previously published plant EVs proteomes were compared against the proteins enriched in human EVs [158]. A total of 115 human EV proteins were found to be orthologous to one or more plant EV proteins. 24 of these proteins were matched in five out of the eight plants studied, and 62 proteins were matched in three or more plants.

There were 20 proteins in the *N. tabacum* proteome that matched with 4 human EV protein families – 14-3-3 proteins, Endoplasmic reticulum chaperone BiP, HSP 70, and Tubulin. There were human protein orthologs to four out of the eight plant EV biomarker proteins present in *N. tabacum* sEVs (HSP70, Tubulin, Glycerophosphodiester phosphodiesterase, lipid transfer proteins, Blue copper protein, glucan endo-1,3beta-glucosidase, and malate dehydrogenase) [11].

Orthologue discovery analysis between *N. tabacum* sEV proteome and other plant EV proteomes identified proteins such as adenosylhomocysteinase, 14-4-3 proteins, tubulun, and heat shock proteins as orthologues (Figure 3.4.).

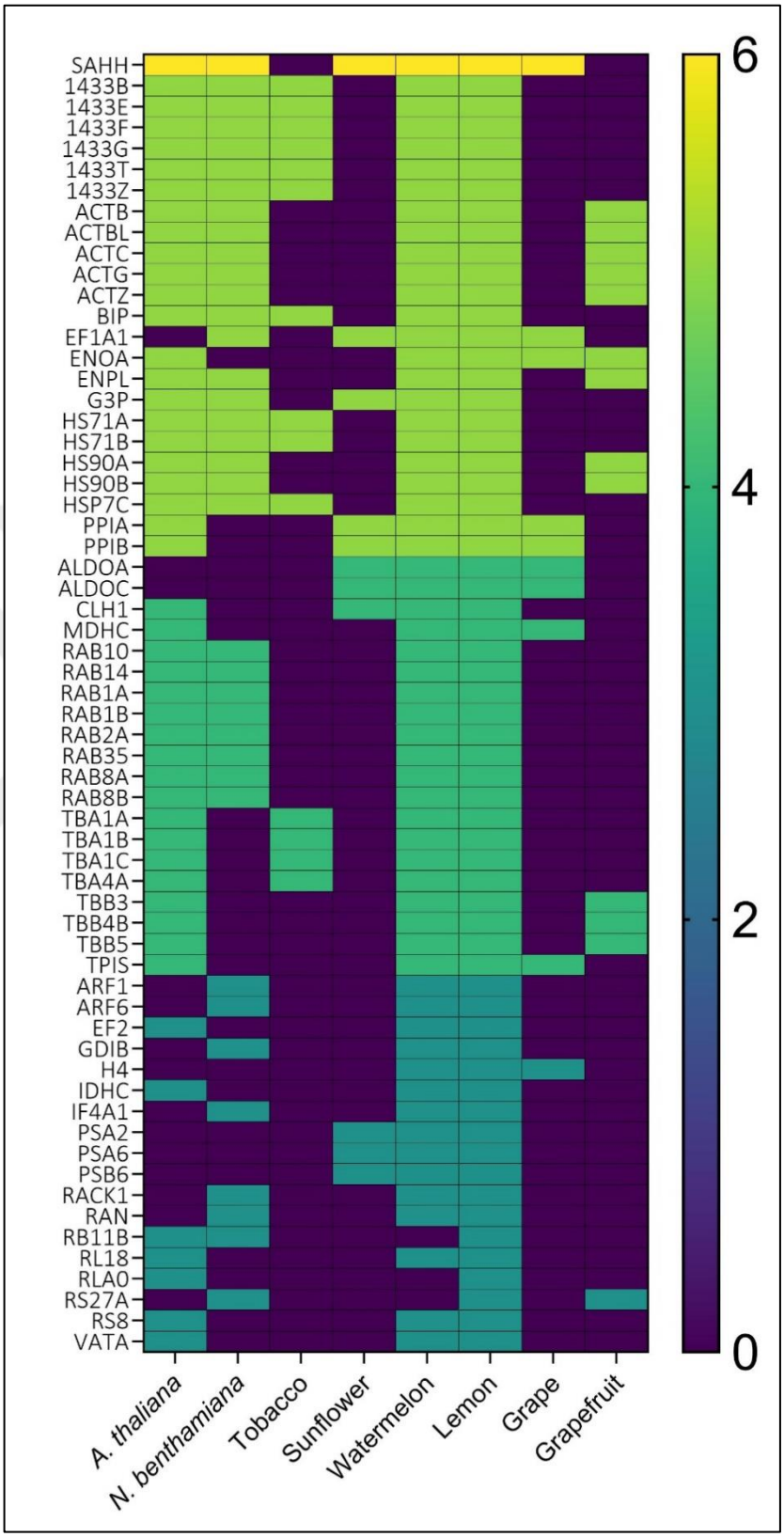

Figure 3.4 Human EV biomarker orthologues in different plant EVs. Colour indicates the number of plants that had an orthologous protein to the top human EV biomarker proteins listed to the left

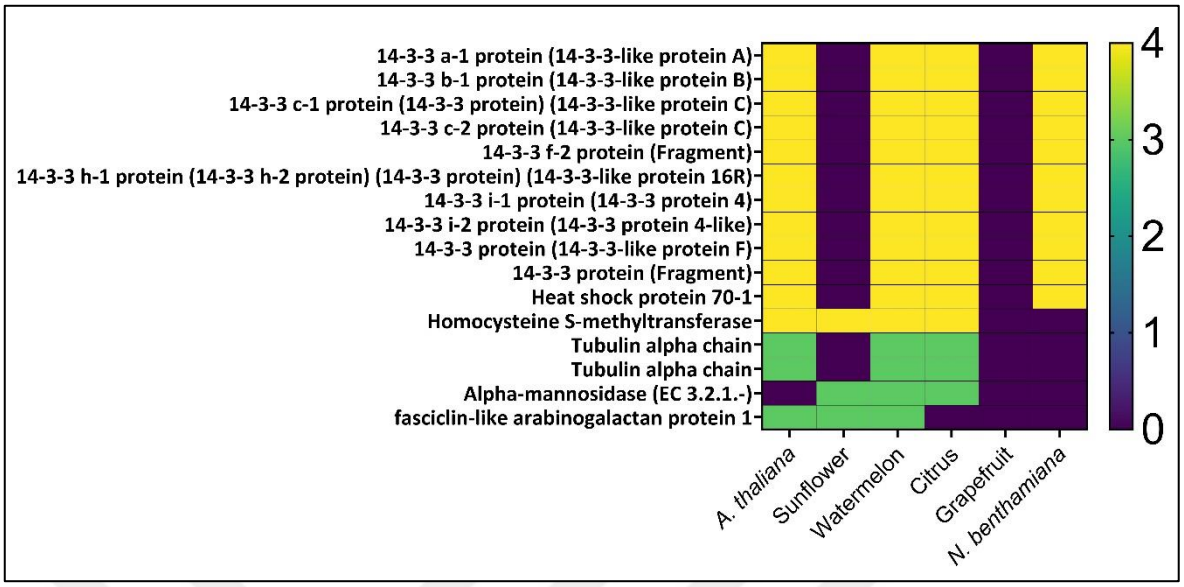

Figure 3.5 *N. tabacum* sEV protein orthologues in different plant EVs. Colour indicates the number of plants that had an orthologous protein to tobacco EV proteins listed to the left

3.4. SUBCELLULAR LOCALIZATION (CELLO) ANALYSIS

As a large percentage of the subcellular localization of *N. tabacum* sEV proteins, and most of the plant proteins included in this study were unannotated, CELLO [157] software was used to predict the subcellular locations to complement the cellular compartments. CELLO analyses were made for all eight plants and the top human EV proteins. A pattern of enrichment was observed in proteins predicted to locate into the cytoplasm, plasma membrane, or extracellular region, in that order. Human, Arabidopsis, grapefruit and *Nicotiana benthamiana* proteomes were also enriched in nuclear proteins, while sunflower and *N. tabacum* proteomes were more enriched in extracellular proteins than cytoplasmic or plasma membrane-associated proteins (Table B.1.)

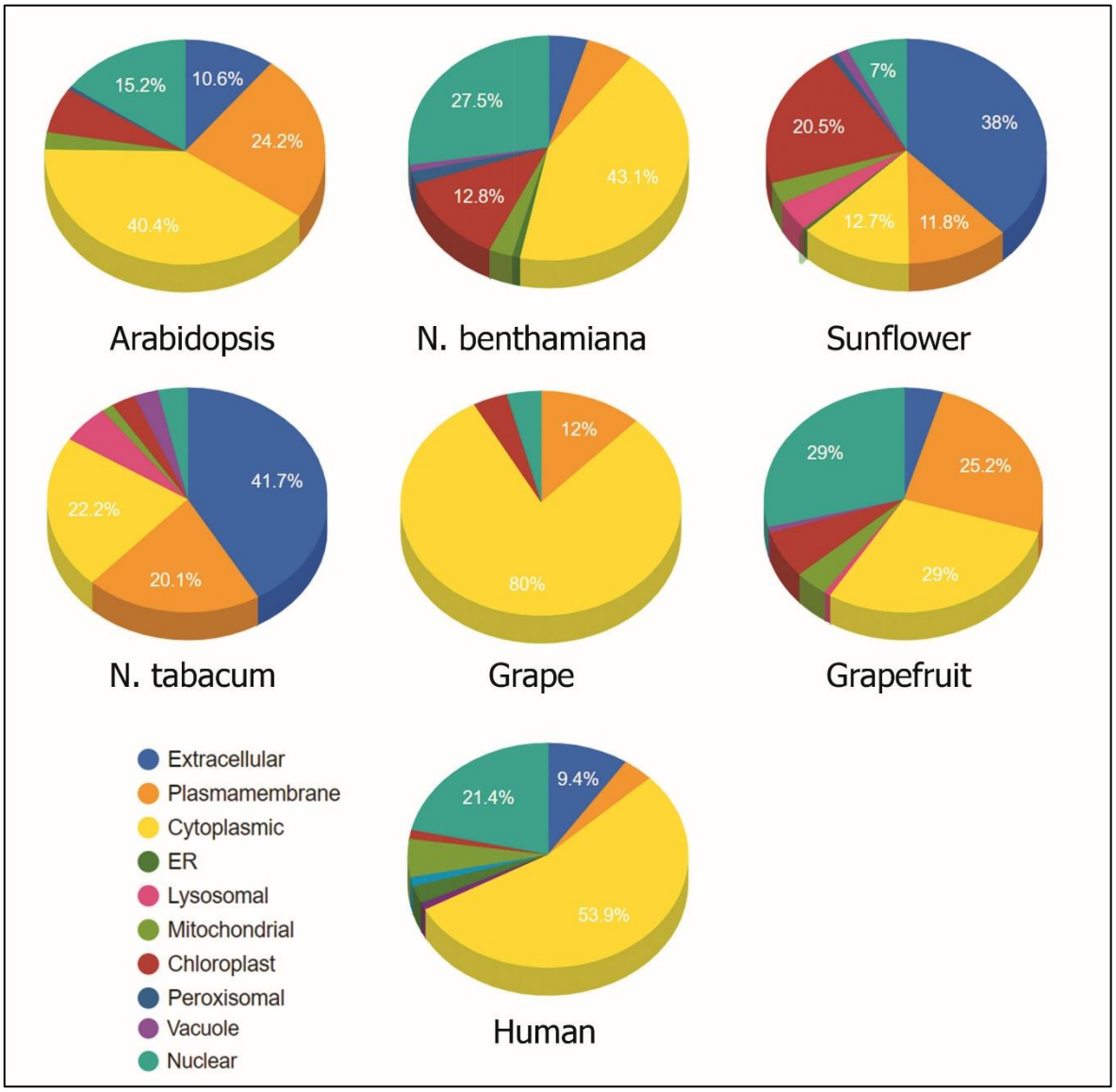

Figure 3.6. CELLO analysis of human and plant EVs

4. DISCUSSION

Despite the promising reports on the therapeutic applications of plant EVs, their study is severely lagging behind their mammalian counterparts. The harsh isolation methods used in these studies, which involve the homogenization of plant material, causes the isolate to be contaminated by a large variety of cellular material, intracellular vesicles, and soluble proteins or phytochemicals. The presence of such contaminants makes the isolate PDNPs, as the intracellular vesicle contamination makes them ineligible as EVs, and casts doubt to whether or not the results observed in these studies are solely due to plant EVs [1]. A lack of standardized isolation methods and biomarkers for the characterization of plant EVs exacerbates this issue.

In this dissertation, we present a complete workflow for the isolation and characterization of true plant EVs using plant cell suspension cultures. These EVs were pure of any intracellular vesicles or cellular debris, as they were isolated from a liquid culture media instead of tissue lysates. We have successfully isolated sEVs and lEVs of *N. tabacum*, *S. rebaudiana* and *V. vinifera.* Isolated EVs had physical characteristics similar to mammalian EVs (Figure 3.1.). Furthermore, we present the first proteomics data of *N. tabacum* sEVs, and the first proteomics data of plant cell culture EVs, which included several plant EV biomarker candidates (Table A.1.) [11], and had a similar GO enrichment pattern to mammalian EVs (Figure 3.3.). Finally, in order to discover potential plant EV biomarkers, we performed an orthologue discovery meta-study using *N. tabacum* sEV proteome and all seven previously published plant EV proteomes [3,5,15,16,152,162,163]. Comparing these proteomes against proteins enriched in human EVs [161], we identified 24 potential plant EV biomarkers, such as 14-4-3 protein family, actin, adenosylhomocysteinase (*SAHH)*, enolase, HSP70 or HSP90 (Figure 3.4.).

Proteomic analysis of *N. tabacum* sEVs identified 135 proteins. GO enrichment analysis of the *N. tabacum* sEV proteome revealed enrichment patterns that were consistent with the ones for mammalian EVs. As around 46 percent of *N. tabacum* sEV proteins were uncategorized under the cellular compartments GO annotation, we also performed CELLO analysis to predict the subcellular locations of the *N. tabacum* sEV proteome [160]. The findings of the CELLO analysis were consistent with the GO enrichment results (Figure 3.4.1.). Fatty acid profiling of *N. tabacum* small and large EVs were also made. However,

due to the small number of identified fatty acid species and a lack of fatty acid profiles for different EV subtypes, no conclusive findings were made (Table B.1.) (Figure B.2.).

Cellular sublocation of EV proteins for all plants EV proteomes and the human EV proteome are enriched towards the extracellular region, plasmamembrane, and cytoplasmic proteins, while depleted in proteins from membrane-bound organelles such as mitochondria or nucleus, and molecular functions such as binding and catalytic activity are also enriched in both instances. There was a non-insignificant presence of nuclear proteins in arabidopsis, grapefruit, human, and *N. benthamiana* EV proteomes, which suggests that these isolations may be. Considering these findings, we propose that plant EVs can be biochemically characterized using CELLO analysis. Patterns similar to those of plant EVs would mean the isolate is pure, while the percentage of proteins with nuclear or mitochondrial subcellular localization motifs would suggest the degree of impurity of a sample (Figure 3.6.). The enrichment of metabolic and catalytic proteins hints at the biological functions of *N. tabacum* EVs. These EVs could be a way for the plant to transfer catalytic enzymes to storage sites to aid in the breakdown of stored biological material.

Eight of the *N. tabacum* sEV proteins that were considered as plant EV biomarker candidates in a previous study [11]. *N. tabacum* sEV proteome include HSP-70, which was reported to be present in all of the studied plant proteomes, homocysteine methyltransferase in olive pollen grains and Arabidopsis leaves, while the remaining five proteins were reported in sunflower seeds. Interestingly, the *N. tabacum* sEVs had no proteins in common with the reported proteome of *N. benthamiana* sEVs, however, this is likely due to the small size of the *N. benthamiana* proteome (44 proteins) [152].

Bioinformatics analysis of the seven other published plant EV proteomes identified numerous orthologous proteins between plant and human EVs. For the 295 human EV proteins queried, 39.3 percent (116) of the proteins matched with at least one plant EV protein and 8.13 percent (24) of the proteins matched with plant EV proteins in at least five of the eight plants studied. Orthologues were found for proteins that were put forth as plant EV biomarkers in previous studies, such as adenosylhomocysteinase, actin, enolase and HSP70 (Table 3.1.). These proteins were also present in at least five plants, excluding tubulin which was present in four plants.

The parallels between plant and human EV biomarkers suggests to the evolutionary conservation of the EV secretion mechanisms. It can be suggested that proteins present in human EVs will likely have homologues in their plant counterparts. Crucially, the high degree of homology explains how plant EVs and PDNPs could have therapeutic effects in human cells. There is a high likelihood that the highly conserved proteins of plant EVs can enter biological processes in place of their human orthologues. This interaction could be present in other inter-kingdom interactions, such as with EVs and other membrane vesicles of pathogenic eukaryotes or prokaryotes [167].

Comparing the proteome of *N. tabacum* sEVs with the proteomes of other plant EVs revealed a high degree of homology between different plants. Proteins that showed homology to human EV proteins, such as 14-4-3 proteins, actin, adenosylhomocysteinase and enolase were also present as orthologues in this analysis. This parallel supports the evolutionary conservation of EV secretion in different forms of life (Figure 3.5.)

To our knowledge, there are only five other examples of true plant EVs isolated in the literature. Four of these studies employed the apoplastic wash method, where rosettes of the plant are vacuum infiltrated with a buffer. Collecting this fluid with syringes and low-speed centrifugation collects vesicles present at the apoplastic spaces of the rosettes. Developed by Regente et al. to isolate EVs from sunflower seeds [19], this method has been used to isolate EVs from *Arabidopsis* [15,164], *Nicotiana benthamiana* [164] leaves, and olive pollen grains [165]. The apoplastic wash method is time consuming and unsuitable for the large-scale production of plant EVs. EVs cannot be continuously produced in this manner, and the EVs of the apoplastic wash may not accurately represent the whole EV secretome, as these EVs are collected after the removal of the leaf from the plant. The fifth study, published by Cho et al. in 2021, used a similar suspension culture method to this study, isolating EVs of ginseng from a ginseng suspension culture [98].

While the size distribution of the sEVs isolated from all three plant cell suspension cultures were roughly identical, the size distribution of their lEVs were significantly different from one another. The smaller median size of *N. tabacum* lEVs is likely due to a large number of sEVs present in those samples, which also inflated the numbers of lEVs. We also looked at the efficiency of different methods in producing EVs. Using a bioreactor of *N. tabacum* cells produced more than 100 times sEVs per ml of media.

5. CONCLUSIONS

This dissertation is the first proteomic characterization of plant cell culture-derived EVs. In conjunction with the set of plant EV biomarkers discovered through the orthologue analysis of plant EV proteomes, we hope to present a clear workflow from isolation to characterization for plant EV research, which avoids the shortcomings of older research and allows for a unified, methodical approach to the study of plant EVs. Finally, we hope to draw attention on the high degree of homology between the proteins of plant and human EVs. Orthologue analyses could mark out potential plant proteins with the ability to affect particular biological processes in humans, allowing an informed approach in the discovery of therapeutic affects by plant EVs, or other non-mammalian EVs studied in a similar manner.

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APPENDIX A: TOTAL PROTEOME OF *N. TABACUM* VESICLES

Table A.1. List of identified proteins in *N. tabacum* sEVs

Uniprot Accession	**Protein name**	**Gene names**	**Length**
Q2WGN6	Glycerophosphodiester phosphodiesterase (EC 3.1.4.46)	NtGPDL LOC107795798	752
A0A1S4A171	fasciclin-like arabinogalactan protein 7	LOC107792706	264
A0A1S3ZNE8	Alpha-mannosidase (EC 3.2.1.-)	LOC107788691	1029
A0A1S4B2V1	Peroxidase (EC 1.11.1.7)	LOC107803841	330
A0A1S4C8B7	fasciclin-like arabinogalactan protein 1	LOC107816302	416
A0A1S3ZK57	proteinase inhibitor I-A	LOC107787825	107
A0A1S3XCV6	5-methyltetrahydropteroyltriglutamate--homocysteine S-methyltransferase (EC 2.1.1.14)	LOC107763602	765
A0A0R4WSF0	Proteinase inhibitor (trypsin inhibitor 1-like)	PI-2 LOC107814132	95
A0A1S4DJK5	non-specific lipid-transfer protein-like protein At5g64080	LOC107830512	186
A0A1S3YMI7	somatic embryogenesis receptor kinase 2-like	LOC107777855	197
A0A1S4AZR2	subtilisin-like protease SBT1.9	LOC107802951	763
A0A1S4D1I4	glucan endo-1,3-beta-glucosidase 14-like	LOC107824944	396
A0A1S3XGA2	beta-xylosidase/alpha-L-arabinofuranosidase 2-like	LOC107764751	784
A0A1S4APQ9	Beta-galactosidase (EC 3.2.1.23)	LOC107800119	824
A0A1S3ZB71	Laccase (EC 1.10.3.2) (Benzenediol:oxygen oxidoreductase) (Diphenol oxidase) (Urishiol oxidase)	LOC107784916	574
Q67BD2	Heat shock protein 70-1	HSP70-1	653

A0A1S3XY66	monocopper oxidase-like protein SKS1	LOC107770130	589
A0A1S4C9Y6	early nodulin-like protein 3	LOC107816699	181
A0A077DBL0	Xyloglucan-specific fungal endoglucanase inhibitor protein	x	437
A0A1S3YB08	early nodulin-like protein 2	LOC107774426	414
A0A1S3YXG6	Malate dehydrogenase (EC 1.1.1.37)	LOC107780752	341
A9XG40	Subtilisin-like protease	SBT1.1A	768
A0A1S4DJL6	beta-xylosidase/alpha-L-arabinofuranosidase 2-like	LOC107830518	778
A0A1S4CYF0	fasciclin-like arabinogalactan protein 17	LOC107823842	449
A0A1S3YVC7	uncharacterized protein LOC107780235	LOC107780235	147
F2ZAM0	Non-specific lipid-transfer protein	NtLTP3 LOC107814800	114
A0A1S3YN86	Glycerophosphodiester phosphodiesterase (EC 3.1.4.46)	LOC107778062	752
A0A1S4AYF3	Purple acid phosphatase (EC 3.1.3.2)	LOC107802681	611
A0A1S4DG28	trypsin inhibitor 1-like	LOC107829494	95
Q8VXC9	Tubulin alpha chain	tubA3 LOC107826406 LOC107827984	451
A0A1S3Z898	fasciclin-like arabinogalactan protein 9	LOC107784037	251
Q5KTN5	14-3-3 c-1 protein (14-3-3 protein) (14-3-3-like protein C)	14-3-3 c-1 LOC107777576 Nt14-3-3omega2	260
A0A1S4BVV4	early nodulin-like protein 1	LOC107812424	181
A0A1S3X916	basic 7S globulin-like	LOC107762538	438
A0A1S3ZME8	blue copper protein-like	LOC107788304	351
A0A1S4D534	xylogen-like protein 11	LOC107826061	189

A0A1S3X9W2	Peroxidase (EC 1.11.1.7)	LOC107762727	360
A0A1S4AAU3	glutamyl-tRNA(Gln) amidotransferase subunit A-like isoform X1	LOC107795571	629
A0A1S3Y0U6	lysM domain-containing GPI-anchored protein 1-like	LOC107770883	417
A0A1S3X7U9	beta-fructofuranosidase, insoluble isoenzyme CWINV1-like	LOC107762144	573
A0A1S3X6V0	Xylose isomerase (EC 5.3.1.5)	LOC107761872	481
A0A1S4C704	uncharacterized protein LOC107815754	LOC107815754	1066
A0A1S4BC16	fasciclin-like arabinogalactan protein 1	LOC107806676	415
A0A1S3X1G3	DNA-directed RNA polymerase subunit beta (EC 2.7.7.6)	LOC107760305	1243
A0A1S4ACF7	cytochrome b561 and DOMON domain-containing protein At3g25290-like	LOC107796023	242
A0A1S3XYP5	protein YLS9-like	LOC107770277	236
A0A1S4C159	Alpha-mannosidase (EC 3.2.1.-)	LOC107814068	931
A0A1S4B7K9	monocopper oxidase-like protein SKU5	LOC107805343	592
A0A1S4AMD8	fasciclin-like arabinogalactan protein 8	LOC107799234	422
A0A1S4CA17	beta-xylosidase/alpha-L-arabinofuranosidase 2-like	LOC107816752	779
A0A1S4BMJ4	early nodulin-like protein 2	LOC107809888	401
A0A1S4BVI1	Non-specific lipid-transfer protein	LOC107812329	114
A0A1S4A764	proteinase inhibitor I-B	LOC107794480	126
A0A1S3WZJ1	Purple acid phosphatase (EC 3.1.3.2)	LOC107759506	614
A0A1S3ZFA1	G-type lectin S-receptor-like serine/threonine-protein kinase RLK1	LOC107786027	435
A0A1S4CSI9	Carboxypeptidase (EC 3.4.16.-)	LOC107822067	499
A0A1S4B3Q6	monocopper oxidase-like protein SKS1	LOC107804105	585

A0A1S4CTD1	blue copper protein-like	LOC107822452	340
Q9FRU1	Tubulin alpha chain	BYtuba	450
A0A1S4AHL1	5-methyltetrahydropteroyltriglutamate--homocysteine S-methyltransferase (EC 2.1.1.14)	LOC107797732	765
A0A1S4BRD0	5-methyltetrahydropteroyltriglutamate--homocysteine S-methyltransferase (EC 2.1.1.14)	LOC107811094	765
A0A1S4D9K9	5-methyltetrahydropteroyltriglutamate--homocysteine S-methyltransferase (EC 2.1.1.14)	LOC107827442	765
Q6L7J8	Harpin inducing protein 1-like 9	hin9	229
Q8VXD1	Tubulin alpha chain	tubA1	450
Q75ZE5	14-3-3 a-1 protein (14-3-3-like protein A)	14-3-3 a-1 LOC107765116	255
Q75ZE3	14-3-3 c-2 protein (14-3-3-like protein C)	14-3-3 c-2 LOC107774394	260
A0A1S3XQ86	beta-xylosidase/alpha-L-arabinofuranosidase 2-like	LOC107767521	781
Q75XU9	14-3-3 f-2 protein (Fragment)	14-3-3 f-2	243
A0A1S4DQD9	non-specific lipid-transfer protein-like protein At2g13820	LOC107832326	186
Q9M663	Harpin inducing protein (Hin1)	hin1	229
Q5KTN6	14-3-3 b-1 protein (14-3-3-like protein B)	14-3-3 b-1 LOC107787833	255
A0A1S4C1T9	Peroxidase (EC 1.11.1.7)	LOC107814252	360
A0A1S3ZBM2	non-specific lipid-transfer protein-like protein At2g13820	LOC107785141	184
Q6L7J7	Harpin inducing protein 1-like 18	hin18	229
A0A1S3ZYM9	5-methyltetrahydropteroyltriglutamate--homocysteine S-methyltransferase (EC 2.1.1.14)	LOC107791827	765

Q549Z2	14-3-3 protein (14-3-3-like protein F)	Nt14-3-3omega3 LOC107801900	258
Q5DNZ8	Malate dehydrogenase (EC 1.1.1.37) (Fragment)	x	212
Q948K2	14-3-3 protein (Fragment)	D5	204
Q8H0I0	Beta-1,3-glucanase-like protein	B29	358
A0A1S4DH74	beta-fructofuranosidase, insoluble isoenzyme CWINV1-like	LOC107829669	569
Q8VXD0	Tubulin alpha chain	tubA2 LOC107781962 LOC107823267	451
A0A1S4A594	Peroxidase (EC 1.11.1.7)	LOC107793895	360
Q75ZD4	14-3-3 i-1 protein (14-3-3 protein 4)	14-3-3 i-1 LOC107813146	260
A0A1S4B2G1	Xylose isomerase (EC 5.3.1.5)	LOC107803724	481
P93353	Hin1 protein	hin1	221
Q948K4	14-3-3 b-2 protein (14-3-3 protein) (14-3-3-like protein B)	D31 14-3-3 b-2 LOC107828339	255
A0A1S4AJ21	fasciclin-like arabinogalactan protein 1	LOC107798150	306
A0A1S4CNK5	Malate dehydrogenase (EC 1.1.1.37)	LOC107820966	341
A0A1S4CDZ9	fasciclin-like arabinogalactan protein 1	LOC107817819	416
Q50LG4	Peroxidase (EC 1.11.1.7)	NtPOX2	360
A0A1S4CU51	Purple acid phosphatase (EC 3.1.3.2)	LOC107822622	611
Q75ZD6	14-3-3 h-1 protein (14-3-3 h-2 protein) (14-3-3 protein) (14-3-3-like protein 16R)	14-3-3 h-1 14-3-3 h-2 LOC107767929 LOC107817195 Nt14-3-3omega1	258
A0A1S4CAQ1	Purple acid phosphatase (EC 3.1.3.2)	LOC107816799	603

C7E4J6	Subtilisin-like protease preproenzyme	x	763
Q75ZD3	14-3-3 i-2 protein (14-3-3 protein 4-like)	14-3-3 i-2 LOC107786214	260
A0A170RBF6	Harpin inducing protein (Fragment)	Hin1	229
A0A1S4A7P5	14-3-3-like protein A	LOC107794654	255
Q50LG5	Peroxidase (EC 1.11.1.7)	NtPOX1 LOC107816453	360
Q75ZD8	14-3-3 f-1 protein (14-3-3-like protein F)	14-3-3 f-1 LOC107818314	258
A0A1S4AFG1	Tubulin alpha chain	LOC107797065	450
A0A1S3XSP4	5-methyltetrahydropteroyltriglutamate--homocysteine S-methyltransferase (EC 2.1.1.14)	LOC107768359	765
A0A1S3YZE6	protein YLS9-like	LOC107781320	236
A0A1S4AAP6	glutamyl-tRNA(Gln) amidotransferase subunit A-like isoform X2	LOC107795571	564
A0A1S3ZMU7	Tubulin alpha chain	LOC107788415 LOC107800766	450
A0A1S3YYW3	subtilisin-like protease SBT1.7	LOC107781223	773
A0A1S4AFH1	Glycerophosphodiester phosphodiesterase (EC 3.1.4.46)	LOC107797074	741
A0A1S4A2P8	subtilisin-like protease SBT1.9	LOC107793176	764
Q9FXL4	Glucan endo-1,3-beta-D-glucosidase (EC 3.2.1.39)	NtEIG-E76	467
A0A1S3XVY6	glutamyl-tRNA(Gln) amidotransferase subunit A-like isoform X2	LOC107769186	564
A9XG41	Subtilisin-like protease (subtilisin-like protease SBT1.7)	SBT1.1B LOC107789407	768
A0A1S3XKZ9	fasciclin-like arabinogalactan protein 17	LOC107766400	449
A0A1S3Y0G0	early nodulin-like protein 1	LOC107770784	180

A0A1S4AXK4	Purple acid phosphatase (EC 3.1.3.2)	LOC107802405	430
A0A1S4BGH4	monocopper oxidase-like protein SKU5	LOC107807992	592
A0A1S4B2G2	Glucan endo-1,3-beta-D-glucosidase (EC 3.2.1.39)	LOC107803828	467
A0A0A8JBT3	Alpha-L-Arabinofuranosidase/beta-D-Xylopyrianosidase (Fragment)	x	368
A0A1S4D9B7	Glycerophosphodiester phosphodiesterase (EC 3.1.4.46)	LOC107827414	461
A0A1S4DNN1	Purple acid phosphatase (EC 3.1.3.2)	LOC107831746	632
A0A1S3YNE4	Purple acid phosphatase (EC 3.1.3.2)	LOC107777959	632
A0A1S4AY08	Purple acid phosphatase (EC 3.1.3.2)	LOC107802405	391
A0A1S3Y1L6	cytochrome b561 and DOMON domain-containing protein At3g25290-like	LOC107771081	242
A0A1S4AUU3	early nodulin-like protein 3	LOC107801608	192
A0A1S4BB05	fasciclin-like arabinogalactan protein 8	LOC107806416	251
A0A1S3YJI6	trypsin inhibitor 1-like	LOC107776717	94
A0A1S3Y1D2	subtilisin-like protease SBT1.7	LOC107770994	773
A0A1S4ADG8	early nodulin-like protein 1	LOC107796455	181
A0A1S3XVH0	glutamyl-tRNA(Gln) amidotransferase subunit A-like isoform X1	LOC107769186	629
A0A1S4DIR5	Purple acid phosphatase (EC 3.1.3.2)	LOC107830287	611
A0A1S4DNL6	Purple acid phosphatase (EC 3.1.3.2)	LOC107831744	632
A0A1S4AXJ9	Purple acid phosphatase (EC 3.1.3.2)	LOC107802405	482
A0A1S4DNM3	Purple acid phosphatase (EC 3.1.3.2)	LOC107831744	568
A0A1S4B9H0	lysM domain-containing GPI-anchored protein 1-like	LOC107805926	417
A0A1S4CLZ3	Glucan endo-1,3-beta-D-glucosidase (EC 3.2.1.39)	LOC107820469	335

A0A1S3YUU4	subtilisin-like protease SBT1.9	LOC107780049	764
A0A1S4DGB9	trypsin inhibitor 1	LOC107829474	94

APPENDIX B: LIPIDOMIC PROFILE OF *N. TABACUM* EVS

Table B.1. List of fatty acids identified in *N. tabacum* sEVs

Common Name	IUPAC Name	%
Small EVs		
Palmitic Acid	(Z)-hexadec-8-enoic Acid 16:0	27.42
Civetic acid	cis-8-Heptadecenoic Acid 17:1 antesio w9c	27.58
Stearic Acid	Octadecanoic acid 18:0	44.6
Large EVs		
Myristic Acid	Tetradecanoic Acid 14:0	7.58
Palmitoleic Acid	(Z)-hexadec-9-enoic Acid 16:1 w7c/16:1 w6c	16.34
Palmitic Acid	Hexadecanoic Acid 16:0	39.7
Oleic Acid	(Z)-octadec-9-enoic Acid 18:1 w9c	7.51
cis-Vaccenic Acid	(Z)-octadec-11-enoic Acid 18:1 w7c	12.87
Stearic Acid	Octadecanoic acid 18:0	16

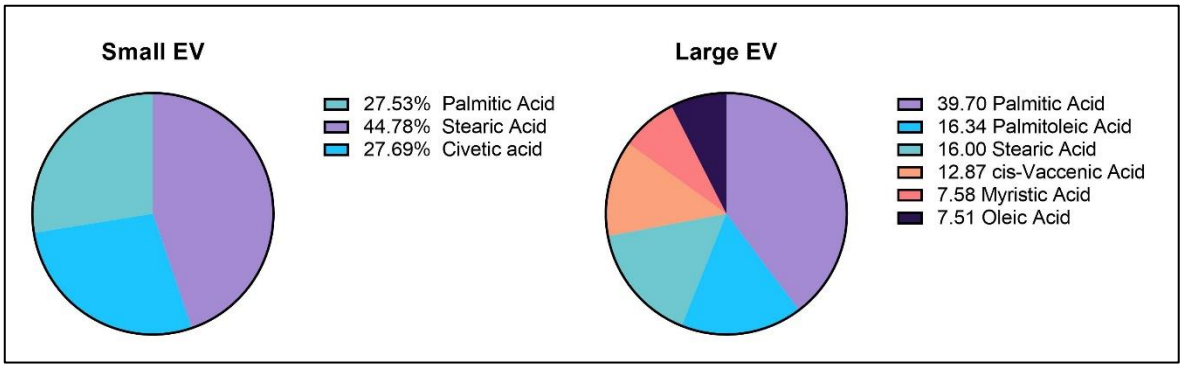

Figure B.2. Fatty acid profiles of *N. tabacum* EVs.