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UNIVERSITY OF STAVANGER

Dysfunction of Protein Homeostasis: Factors influencing the Formation of alpha - Synuclein (Lewy Bodies) in *Arabidopsis thaliana* plants

by

Jeffrey Bannor Frimpong

Declaration of Authorship

I, JEFFREY BANNOR FRIMPONG, declare that this thesis titled, 'Dysfunction of protein homeostasis: Factors influencing the formation of alpha - synuclein (lewy bodies) in *Arabidopsis* plants' and the work presented in it are my own. I confirm that:

- This work was done wholly or mainly while in candidature for a research degree at this University.

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"He who laughs last didn't get the joke"

Charles de Gaulle

UNIVERSITY OF STAVANGER

Abstract

Faculty of Science and Technology

Department of Biology, Chemistry and Environmental Engineering

Master Thesis in Biological Chemistry

by Jeffrey Bannor Frimpong

BACKGROUND:Protein homeostasis or proteostasis under the conscious control of a network of proteins including chaperones, transporters, ubiquitin-dependent proteasome control the proper expression, folding, translocation and clearance.This is necessary to keep an organism's health and and proteome in the functional state. Therefore, a derangement could possibly lead to aggregation or fibrilization of these proteins which is manifested in most of neuropathies. **OBJECTIVES**: Our objective was to determine the possible factors that could influence the accumulation of α-synuclein in *Arabidopsis thaliana* plant. METHODS: Three different *Arabidopsis thaliana* plant (Wild-type(WT) and 2 mutants - ISUSN5 and E46K12) were used for the study. Two sampling methods were used. In sampling method 1, 0.1 g of each of young and old leaves were harvested from the different plants after 4 months. Extracts were prepared from each of them to determine proteasome activity. In sampling method 2, the mutant plants, ISUSN5 and E46K12 were sectioned or divided into 4 quarter. Each quarter was harvested at an interval of 4 weeks within the 4 months period. Each quarter represented the different ages of the plants. Unlike the mutant plants, the whole plant was harvested at the first, second, third and fourth month. **RESULTS**: The average Relative Fluorescent Unit (RFU) measurement for young were and old leaves for the WT, ISUSN5 and E46K12 were 43568.29, 37182, 96420 respectively for the young leaves and 33982.41, 24145.43 and 159108.15 respectively for sampling method 1. In the second sampling method, the RFU measurements for the WT, ISUSN5 and E46K12 were 17981.05, 35052.42 and 18474.20 respectively. **CONCLUSION**: RFU measurements were higher in the young leaves than in the old leaves in the WT and ISUSN5 for sampling method 1. In sampling method 2, the average RFU measurement as a function of the proteasome for the first, second, third and fourth month were 4266.53, 10426.14, 26185.33 and 30146.20 respectively. The average RFU measurements obtained for the ISUSN5 mutant plants were 33650.30, 18455.63, 31502.64 and 29204.34 respectively. E46K12 mutant plant also had the following RFU measurements; 13266.84, 26824, 13600.38 and 20204.88 respectively. Therefore, sampling method 2 did provided some few evidence to support the hypohesis that ageing is a contributing factor in α - synuclein aggregation unlike the sampling method 1.

Keywords: Proteostasis, chaperones, proteome, $\alpha - synuclein$, Relative fluorescent unit, proteosome

Acknowledgements

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Abbreviations

PD	Parkinson's Disease
AD	Alzheimer's Disease
CNS	Central Nervous System
APP	Amyloid Precursor Protein
PS	Presenilin
PTEN	Phosphatase and Tension Homolog
PINK	PTEN - Induced Kinase
LRRK	Leucine - Rich Repeat Kinase
SOD	Superoxide Dismutase
DA	Dopamine
COMT	Cathechol -O- methyltransferase
DOPA	Dihydroxyphenylalanine
MAOB	Monoamine oxidase B
DRD	DOPA Responsive Dystonia
ROS	Reactive Oxygen Species
MPTP	1 - Methyl-4-phenyl -1,2,3,6 - tetrahydropyridine
MPP	1 - Methyl-4-phenylpyrinidium
NO	Nitric oxide
nNOS	Neuronal NO Synthase
C=C	Carbon-carbon double bond
HNE	4-HYdroxynonenal
ALS	Amylotrophic Lateral Sclerosis
DNA	Deoxyribonucleic acid
BBB	Blood Brain Barrier
AMC	7 - Amino -4- methylcoumarin (AMC)

ETC	Electron Transport Chain
BAD	Bcl-2- Associated Death Promoter
PKC	Protein Kinase C
ERK	Extracellular Signal-regulated Kinase
TH	TYrosine Hydrosylase
DAT	Dopamine Transporter
VMAT	Vesicular Monoamine Transporter
AR	Autosomal Recessive
UPS	Ubiquitin Proteasome System
CHIP	C-terminus of HSC70 - Interacting Protein
MHC	Major Histocompatibility Complex
HSP	Heat Shock Protein
sHP	small Heat shock Protein
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum - Associated Degradation
UPP	Ubiquitin Proteasome Pathway
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
UPR	Unfolding Protein Response
HECT	Homologous to -E6-AP carboxyl terminus
UBC	Ubiquitin Conjugating Enzyme
AAA	ATPases Associated with diverse cellular Activities
RUP	Regulated Ubiquitin Proteasome
UBA	Ubiquitin - like Domain
MS	Murashige and Skoog

Dedicated to my parents, Mr. and Mrs. Frimpong

Chapter 1

Introduction

1.1 Introduction

Cells' health and longevity of an organism is hugely dependent on the functionsl state of its proteome - the complete set of proteins in an organism [10]. The ability to control the amount, binding interaction, conformation and location of the constituent proteins within the proteome is known as protein homeostasis or protestasis. This is achieved by adjusting to the inherent biology of the cell usually through transcriptional and translational alterations [11]. Proper expression, folding, translocation and clearance is under the influence of effective homesostasis through a network made up of molecular chaperones, transporters,ubiquitin-dependent proteasome and autophagic activities [10]. Aggregation into highly organised and stable fibrillar or amyloid structures is a common feature possessed by most proteins. The demand for proteins to fold into specific three - dimensional (3D) conformational following translation in the ribosome is critical since regulation and control of necessary cellular task is under their control to keep cellular balance [12].

Parkinson's disease (PD) is a progressive movement disorder and the second neurological disorder following Alzheimer's disease (AD). In a monograph "An Essay on the shaking Palsy" in 1817 by James Parkinson, he explained the clinical features [13–15] using 6 subjects. Ageing is touted to be the major risk factor [16–18] for developing PD. Little knowledge has been exploited to comprehend the role of age in the pathogenesis of PD despite its certainty as a contributing factor[19]. The only explanation underlying this is the susceptibility of the dopaminergic neuron to toxic compounds owing to reduced or null normal cellular function and biochemical processes[20]. Neurological and clinical manifestation include severe motor symptoms and postural imbalance, slowness of movement, rigidity and uncontrollable tremor. PD affects at least 4 million people and

hence a debilitating neurological disorder[21]. Moreover, the average age of onset of PD is 55. The rate of incidence increases as one ages[14]. According to report, there is about 95% cases of sporadic PD, that is, there is no genetic linkage. The remaining 5% of PD cases owing to inheritance[14]. Clinical symptoms of PD get worse over time[14]. Before the introduction of Levodopa as a therapeutic intervention, mortality rates was thrice as much as normal individuals with the same matching age[14]. Prevalence rate reported worldwide ranges between 31- 347 per 100000[22]. Prevalence rate (1.7%) in China stands to be the lowest worldwide[23]. As a major neurotransmitter in the central nervous system (CNS), dopamine's neural dysfunction is touted to be a primary cause of PD[24]. Loss of nerve cells as a result of degeneration of dopaminergic neurons in the substantia nigra (a portion of the midbrain), locus ceruleus, nucleus basalis, hypothalamus, cerebral cortex, cranial nerve motor nuclei, central and peripheral divisions of the autonomic nervous system is typical pathological characteristic of PD[13, 25, 26]. The pathological feature of PD is the accumulation of aggregates or inclusions called alpha synuclein contained in Lewy bodies in the cytoplasm. Alpha–synuclein predominates the Lewy bodies[26]. Alzheimer's disease and dementia are also disorders associated with Lewy pathologies. Impaired mitochondria function, oxidative stress and excitotoxicity are thought to play a role in nigra degeneration, yet they remain not demystified[27].

TABLE 1.1: Proteins associated with some neurodegenerative diseases

Gene / protein	Role
APP	Yields Amyloid beta ($A\beta$), the main composition of senile plaques.
PSI and PS2	Forms part of γ-secretase. It cleaves APP giving rise to $A\beta$.
α - synuclein	A major paert of lewy bodies.
Parkin	Has ubiquitin E3 ligase activity.
DJ - 1	Shields the cell from death induced by oxidative stress.
PINK1	protective activity against cell death.
LRRK2	A kinase with an unknown function.
HTRA2	Breakdown proteins and also degrades inhibitors of apoptosis proteins.
SOD1	Detoxifies superoxide by converting it into hydrogen peroxide.
Huntingtin	Disease-associated mutations yields polyglutamine repeats.

Genetic polymorphisms associated with the metabolism of dopamine together with other compounds related in function, influence the susceptibility to PD[28]. Methylation by Catechol-O-methyltransferase (COMT) inactivates neurotransmitters and toxic catechol such as DOPA – immediate precursor of dopamine[28]. Dopamine metabolism is primarily regulated by an enzyme known as monoamine oxidase B (MAOB). A decline in COMT activity can lead to an elevated conversion of dopamine to neuromelanin and consequently result in the generation of cytotoxic radicals that contributes to the degeneration of neurons[28]. Degeneration of dopaminergic system in PD can be prevented if the activity of MAOB is inhibited[29].Smoking as a lifestyle activity has a beneficial

effect against PD since it reduces the activity of MAOB[30]. Based on the pharmaco-logical profiles, there are five dopamine receptors. This is further grouped into two: D-1 like which comprises DRD1 and DRD5 and D2- like which is made up of DRD2, DRD3 and DRD4)[28]. DRD2 and DRD4 are the two among the rest that control the signaling effect and regulate nigrostriatal neurons and motor activity[31]. An individual is predis-posed to PD as a result of genetic variation of these proteins that regulate dopaminergic neurotransmission[28].

Neuronal function is mitochondria-dependent taking into accounts, the biochemical, physiological and morphological features of the mitochondria. The mitochondria func-tions and respond quickly to cellular activities that require energy and hence normalizing fluctuations in bioenergetics. Intrinsic and extrinsic factors expose neurons to stress and hence require a more regulated system by the mitochondria. Therefore any compromise on mitochondrial function can lead to neuronal degeneration and dysfunction[3].

Chapter 2

Aim of the thesis

In this thesis, different strains of *Arabidopsis thaliana* plant was used. The aim of the thesis was to determine the factors that could promote of influence the accumulation of α-synuclein using *Arabidopsis thaliana* as the model plant

Arabidopsis thaliana plant was used for this study not only owing to the merits it has over other plant models which includes its small size, short generation time, small nuclear genome and large number of offsprings [32] but also permits the use of proteomics as a post-genomic tool [33]. In addition, the mitochondria and the chloroplast are the most predominant organelles that have attracted much studies at the sub-proteomic level [33]. It was thought that the expression of the various forms of the α-synuclein mutants could affect their rate of accumulation. The rate of accumulation could have a cytotoxic effect or affect downstream processes on other cells or tissues which might impair cellular function.

Confocal microscopy technique was used to observe the physical changes of the mitochondrial and the accumulutive effect of the labelled α-synuclein over-time. Lastly, the proteasome activity was measured to compare functional state of the different *Arabidopsis thaliana* plants at different plant stages.

Chapter 3

Background theory

3.1 ETIOLOGY AND PATHOGENESIS OF PARKINSON'S DISEASE

Attention has been drawn to environmental and genetic factor in the development of PD. The contribution to the initiation of PD could be a function of both or either factors[5, 27, 34], although there is still an existing debate. PD remains as a sporadic neurogenerative disease with unknown pathogenesis despite the times and years scientists have invested to unravel this mystery underlying its development and hence understanding the complication and relative selective degeneration of dopaminergic neurons. Cellular, molecular and organism studies has shown that exposure to the several toxins and agrochemicals may have an enormous effect on the pathogenesis of PD. Genetic contributions remain indispensable since some disease-related genes have been identified therefore making PD a multifactorial neurodegenerative disease[34]. Several monogenic hereditary forms of PD, although rare, appears to have early onset. Dominant and recessive trend of inheritance have been shown[5]. Intake of caffeine and Cigarette smoking appear to reduce the risk of PD and hence gives an important clue to the development of PD. The role of anti-inflammatories, exercise, antilipidaemics and calcium antagonists acting as antihypertensives in reducing PD remains ambiguous[20, 35, 36].

3.2 OXIDATIVE STRESS AND PD

Oxidative stress is said to occur when there is an imbalance between the generation and clearance of reactive oxygen species (ROS), that is, an unregulated production of ROS. These include nitric oxide,superoxide, hydrogen peroxide and hydroxyl radicals [2].An

elevatated ratio between high oxygen consumption and low antioxidant levels resulting in tissues regenerating at a lower pace in the brain, subject these tisses to the damaging effect of ROS[2].

Among the many factors that have been suggested to be involved in the pathogenesis of PD is oxidative stress that initiates from the glial cells which has been backed by postmortem studies and further investigations explaining the ability of oxidative stress and oxidizing toxins to cause the degeneration of nigral cells[37]. Oxidative stress indeed seems to be involved in the cascade of biochemical changes that bring about the death of dopaminergic neurons[37].

Heroin addicts, following the accidental use of 1-methyl-4-phenyl-1,2,3,6-tetrahydropypridine (MPTP)- synthetic analog of heroin, led to the discovery that, Parkinonism can be induced in humans and non-human primates by MTPT[38]. MTPT confers an irreversible and severe motor damage with features that are not different from those observed in PD[1]. MAOB oxidizes MPTP to MPP^+ (1-methyl-4-phenylpyridinium) in the central nervous system (CNS)[39]. Dopamine transfer has high affinity for MPP^+ and hence take them up into the dopamine neurons[40]. Following their translocation via active transport into the mitochindria, it inhibits the complex I by interfering with the michondrial respiration chain and consequently leading to the generation of superoxide anions[41]. Superoxide anions react with nitric oxide (NO) to yield another oxidant called peroxynitrite. Peroxinitrite has been implicated in several models of neurologic and neurotoxic diseases[42]. Supporting this observation is the protective action of neuronal NO synthase (nNOS) inhibitors that act against neurotoxicity against MPTP. Mice which were mutated to lack the nNOS gene were aslo not predisposed to the neurotoxic effect when compared to the wild-type[43–45].
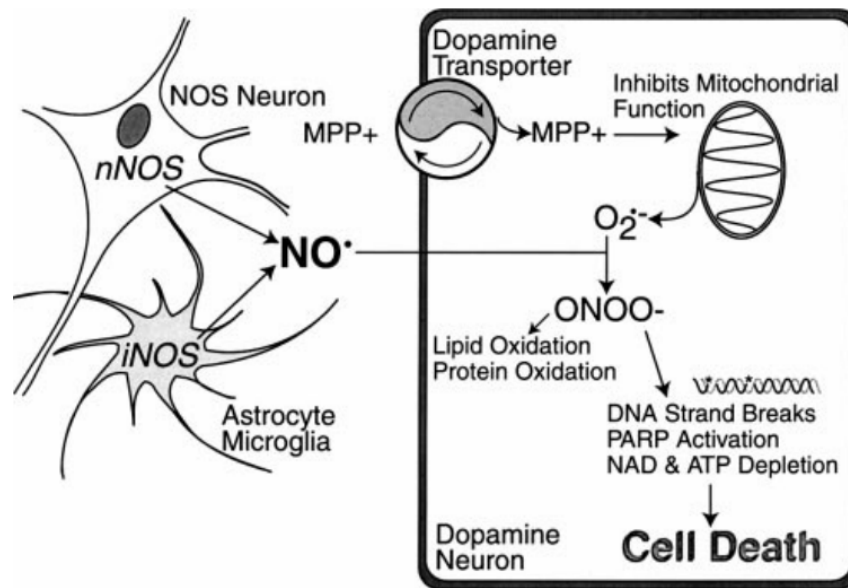
FIGURE 3.1: A model depicting cell-death of dopaminergic cells induced by MPTP. MPP^+ is transported via the high-affinity dopamine transporter (DAT) and gets concentrated in the mitochondrial of DA neurons thereby inhibiting Complex I. This leads to the generation of supeoxide anion and consequently reacts with nitric oxide (NO) to form peroxinitrite. The nitric oxide is generated by neuronal NO Synthase (NOS) and inducible NOS. The result is a damage of intracellular proteins and DNA, causing cell death. The effect of DNA damage is the activation of poly (ADP-ribose) polymerase (PARP). PARP depletes cells of high energy stores through a decline in NAD and ATP[1].

3.2.1 MANIFESTATION OF OXIDATIVE STRESS

3.2.1.1 Lipid peroxidation

The presence of double bonds in lipids enhances the oxidative changes by oxidants. Polyunsaturated fatty acids which include arachidonic and linoleic acids are mostly predisposed to this oxidative effect of ROS[2]. In lipid peroxidation, there is an abstraction of a proton from a C=C bond following an attack by ROS (usually radicals). This generates highly reactive lipid peroxy radicals capable of initiating chain reactions that attack other unsaturated fatty acids[2]. This often occurs within the side chain[46] and consequently results in breakdown product formation such as acrolein,malondialdehyde and 4-hydroxy-2,3-nonenal (HNE). An increased level of HNE and malondialdehyde have been implicated in PD and ALS[47, 48] and Azheimer's disease (AD)[49, 50] brain tissue.

3.2.1.2 Protein oxidation

The backbone and side chains of most proteins can be brought under the effect of oxidation by ROS with the side chain oxidation being most evident and hence investigated into when protein oxidation by ROS is suspected[46]. The oxidative action of these ROS produces several products which consequently react with the amino acid side chain to yield cabonyls.

3.2.1.3 DNA oxidation

The formation of DNA-protein cross-links, modification and release of purine and pyrimidine bases and break-of strands are some of the common evidence of ROS attack on nucleic acids[46]. A major class of DNA lesions induced by ROS is the oxidation of bases. Hydroxylated guanine is assayed and measured as 8-hydroxy-2-deoxyguanosine[46].

3.2.2 Metabolism of dopamine and oxidative stress

Neuromelanins present in the neurons within the substantiah nigra are known to accumulate with age. They function to accumulate metal ions, most especially iron[51]. As a neurotransmitter, dopamine also acts as a potent metal chelator and reductant[2]. It coordinates the cupric and ferric ions, thereby reducing their oxidation states and eventually trigger the generation of hydrogen peroxide. This provides the condition for Fenton reaction[2]. Synthetic melanins can be generated by incubating dopamine with curpric and ferric ions[52]. There has been a suggestion that supports the protective action of melanin against dopamine-induced redox associated toxicity[53, 54]. Depending on the metallic (iron) load - at low or high concentrations, neuromelanin can act as an antioxidant or a pro-oxidant respectively[55]. In addition to the antioxidant property, neuromelanins can also function as iron-storage molecules[2]. According to Double *et al.*, the presence of both high and low - affinity Fe^{3+} binding sites in neuromelanin obtained from human substantia nigra, has shown that the bound- iron has redox activity[56].

Evidence to show that α-synuclein plays a part in the activity of dopamine regulation is emerging[2]. The familiar form of PD resulting from A53T mutation of α-synuclein gene is known to disrupt vesicular storage of dopamine, leading to an age-related condition where there is an increasing concentration of dopamine in the cytoplasm and subsequently generate ROS after associating with iron[57, 58].

Also, according to studies, the aggregation of α-synuclein results from the direct association of α-synuclein with metal ions[59–61]. However, methionine, when oxidized prevent the aggregation of α-synuclein[62].
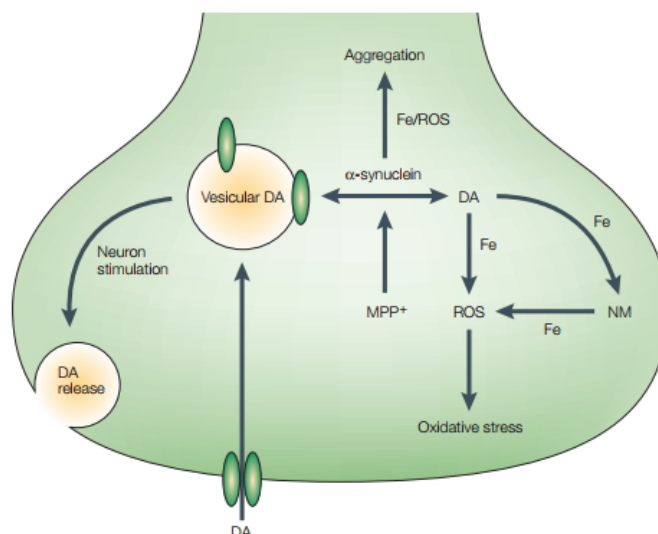
FIGURE 3.2: Regulation of dopamine (DA) activity and ROS generation. DA has a null redox activity when bound to vesicles and functons in neuronal signalling. It coordinates with with Fe when released into the cytoplasm to generate ROS and neuromelanin (NM). A transport of DA into the cytoplasm is favoured when dopamine release is not regulated by α-synuclein and hence results in the generation of more ROS and NM [2].

3.3   ENVIRONMENTAL AND GENETIC RISK FACTORS ASSOCIATED WITH PARKINSON'S DISEASE

A general potential influence on the incidence of PD ranges from rural settlement, industrialization, plant-derived toxins, well-water,viral and bacterial infections to a more specific , as occurrence is limited to carbon monoxide, carbon sulfide and organic solvents exposure[63]. Studies into pesticides as another potent agent that influence PD occurrence has been of interest recently. The only setback is the lack of specificity of which pesticide that might play a key role in PD incidence in individuals[64]. In rodents, rotenone and paraquat are the leading agro-chemicals that has been identified and known to cause nigra dopaminergic cell death in rodents[65, 66].
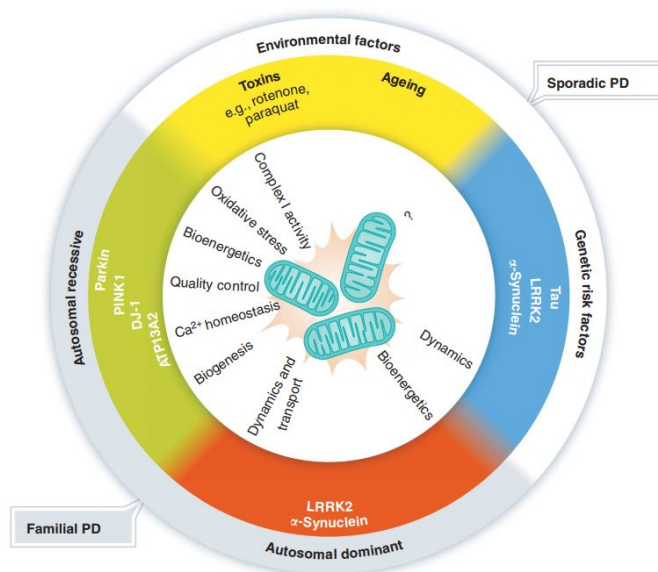
FIGURE 3.3: The cause of PD has been linked to both environmental and genetic factors including mutations. Either sporadic of familial forms of PD has a gross effect on the mitochondrial which is involved in many biochemical processes such as Complex I activity, quality control, dynamics and transport,etc. [3].

Rotenone is used to regulate uncontrollable fish population. It occurs naturally although highly toxic. Independent of transporters, Its lipophilicity makes it possible to cross membranes, blood brain barrier (BBB) and consequently accumulate in mitochondria[67]. Rotenone masks oxidative phosphorylation by blocking complex I in the electron-transport chain (ETC) which occurs in the mitochondria[68]. This inhibition consequently bring about loss of dopaminergic neurons in the substantia nigra and changes in behavior associated with PD in humans[69].
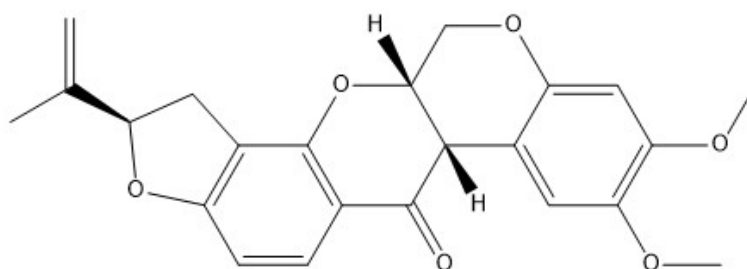
FIGURE 3.4: Structure of rotenone

Clinical and epidemiology studies have shown that herbicides and pesticides are potent risk factors for PD[70–73]. Paraquat (1,1' – dimethyl-4,4' -bi-pyridinium) is a herbicide that bears structural resemblance to MPP^+. Paraquat is known to induce oxidative stress[74]. MPP^+ is generated as active toxic product of MPTP metaolism[70, 72, 75, 76] and it is known to be toxic to neurons and hence purported as a risk factor for PD[77].

Much interest was given into investigating the neurotoxicity of MPTP by studying its mechanism of action in relation to the pathogenesis of PD[78, 79]. Parkinsonism induced by MPTP is not progressive, has no Lewy body formation and finally no changes in the affected area of the brains occurs as in normal Parkinsonism[20].

FIGURE 3.5: Structure of Paraquat (a) and MPTP (b)

Recent studies has focused on the identification of single genes (α-synuclein,DJ-1, PARKIN, PINK1, Ubiquitin C-terminal hydrolase isozyme L1 and nuclear-related factor 1) whose mutations could be responsible for the familiar forms of PD as this provides a better understanding into the molecular machinery underlying this progressive-neuropathological disease[34, 69, 80].

3.4 GENE IMPLICATIONS AND PARKINSON'S DISEASE

PARK1 is the first PD-gene that encodes alpha-synuclein. The synuclein family includes (*alpha*,*beta*, and *gamma* – synuclein)[81, 82]. In humans, α,β and γ have been identified in chromosomes 4q21, sq35[83] and 10q23[84] respectively. Although only α – synuclein is implicated in the diseased condition, yet has similar sequence as β and γ [5].

PARK2 or α-synnuclein gene

FIGURE 3.6: A multiple alignment sequence of α, β and γ - synuclein.
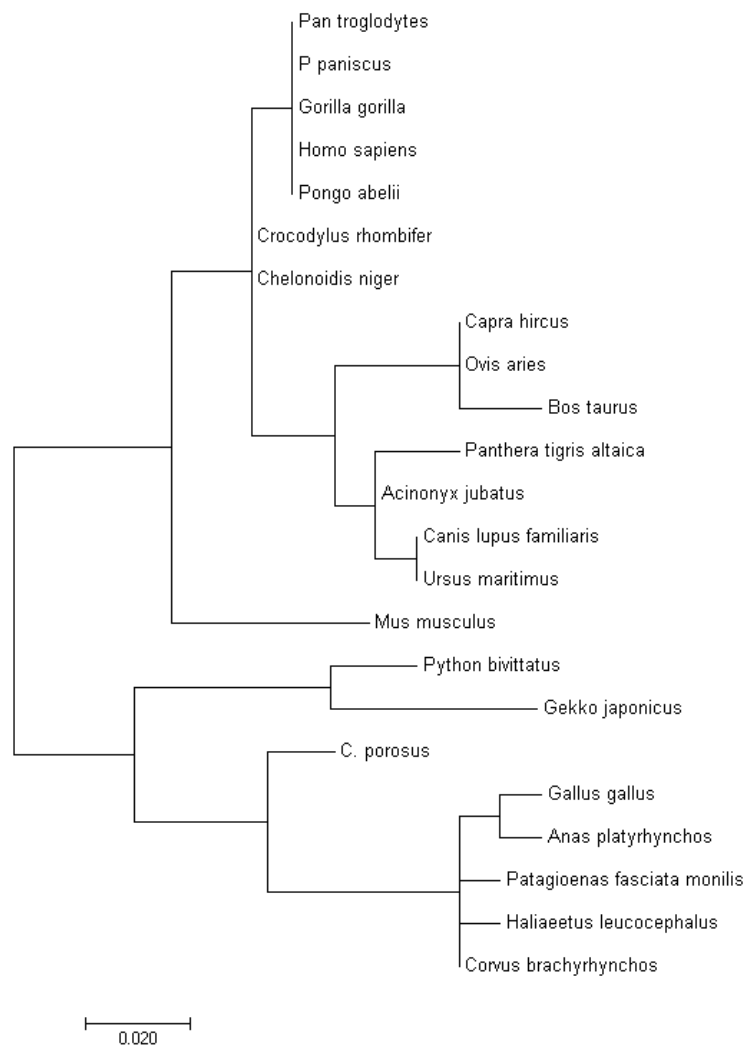
FIGURE 3.7: A phylogenetic tree of α - synuclein of some closely related organisms.

3.4.1 Stucture of alpha-synuclein in relation to mutation and function

Alpha synuclein is small pre-synaptic protein composed of 140 residues located widely in the brain within the hippocampus, olfactory bulb, thalamus, neocortex and cerebellum[82, 85]. The function of alpha-synuclein is undefined yet implicated in neurodegenerative associated diseases which includes PD and Alzheimer's disease. These are collectively known as synucleinopathies[86]. Its structure is defined by an N-terminal sequence which is fractioned into 11-mer repeats. From sequence 1-95 bears a consensus sequence of KTKGEV as shown in figure 3.6. Like apolipoproteins, with just a turn of 3, forms an alpha helix described as amphipathic[87]. Apolipoproteins play role in associating α-synuclein with lipid membranes[88, 89]. The N-terminal is known to contain the Nascent polypeptide-Associated Complex (NAC) domain which plays crucial role in α-synuclein aggregation and sensing lipid properties[90]. This domain lies between residue

60-95[87].In addition, synucleinopathies resulting from single residue mutation within the peptide occurs in the N-terminal. That is, A30P, E46K, H50Q, G51D, A53E, and A53T[91–94]. The high acidity and the unstructured nature of the C-terminus of alpha - synuclein[89] makes a target of most post-translational modifications[95]. The C-terminus interacts with proteins, polycation, ion and binding to polyamines. It also functions to protect alpha-synuclein from aggregation[96, 97]. There is 40% structural homology of the N-terminal of alpha synuclein to chaperone 14-3-3[98]. Its structural homology to 14-3-3, a chaperone protein following binding functions to block protein aggregation induced thermally[99, 100]. Therefore, any mutation associated with the PARK1 gene may facilitate the oligomerization or aggregation[101, 102]. In Lewy bodies(LB), chaperone 14-3-3 is involved in development of neurons and control of cell growth[103]. This prevents apoptosis by inhibiting BAD, a proapoptotic member of the Bcl–2 family. Like chapereone 14-3-3, alpha synuclein also binds to these proteins[98]. In PD brain, the formation of a 54-83kDa protein complex (selectively in the substantia nigra is due to the interaction between alpha synuclein and chaperone 14-3-3[104]). The interaction sequester chaperone 14-3-3 and consequently lead to a decline in the total amount of chaperone 14-3-3 available to combat apoptosis making the cells more predisposed to stress and other injury[104].

FIGURE 3.8: A multiple alignment sequence showing the first 140 amino acid sequence of chaperone 14-3-3 and α-synuclein.

Independently, alpha-synuclein acts as a chaperone to support cells to regulate the effect of stress[98]. However, dividing cells experience the toxic effect of overexpressed wild-type alpha- synuclein[98]. Toxicity effect is worse in overexpressed mutant alpha-synuclein owing to the inhibition of PKC and interaction of alpha -synuclein with proteins involved in signal transduction including ERK, BAD [98]. There is a divergent resultant effect of either alpha -synuclein binding or chaperone 14-3-3 binding. Unlike chaperone 14-3-3, the effect of alpha-synuclein has an inhibiting effect on tyrosine hydroxylase(TH)- the rate limiting enzyme in catecholamine synthesis[105].

3.4.2 Physiological roles of alpha-synuclein

Phospholipase D2 catalyzes the conversion of phosphatidylcholine to phosphatidic acid (PA) in the bovine brain. This triggers the production of secretory vesicles[106]. This enzyme is inhibited by alpha and beta – synuclein[105]. Alpha- synuclein is capable of binding to vesicles expressing high levels PA[107]. PA therefore regulates the trafficking

through vesicle budding or turnover[108]. There was an enhanced release of dopamine (DA) at nigrostriatal terminals following paired electric stimuli when alpha- synuclein were knocked out of mice. This observation suggests that alpha-synuclein represses dopaminergic neuro-release[109].

3.4.3 Synaptic activity of alpha-synuclein

Pre-synaptic localization and its association with curved membranes and proteins residing at the synapse highlights a more regulatory function of alpha-synuclein at the synapse including synaptic plasticity, vesicular trafficking, learning, DA metabolism and the release of neurotransmitters[87].
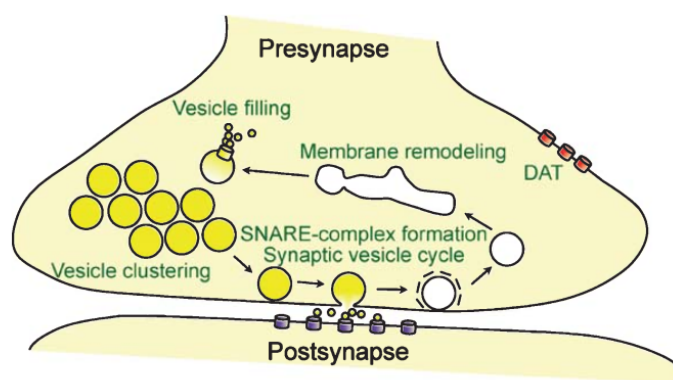
FIGURE 3.9: Synaptic roles of α - synuclein include membrane remodelling, modulation of the DAT - a dopamine transporter, vesicular monoamine transporter VMAT2, clustering of synaptic vesicles, etc [4].

3.4.4 Effect of alpha − synuclein on dopamine metabolism and dopaminergic neurons

Dopaminergic neurons remain the most noted neurons that are implicated in PD. Following its loss in the substantia nigra, there is lack or inadequate dopamine transmission and signaling[110–112]. The mechanism that explains the susceptibility of dopaminergic neurons following alpha -synuclein pathology is still unknown[87]. Interaction with serotonin transporter DAT has been the claim to explain that alpha-synuclein regulates the homeostasis of monoamines in synapses[113–115], although its mechanism of action is still controversial[116, 117]. Covalent modificstion either activates or renders tyrosine hydrosylase inactive through phosphorylation and dephosphorylation respectively[113, 114].

In understanding the role of α - synuclein and its dysfunction, the mechanism underlying the susceptibility of dopaminergic neurons to pathologies associated with α-synuclein is

still not comprehended. At the synapses, regulation of monoamines homeostasis through the interaction with the serotonin transporter is proposed to be function of α - synuclein [116]. Alpha-synuclein binds to and regulates the activity of DAT- the transporter of dopamine[113–115] although its mode of action remains demystified.

The expression and activity of tyrosine hydroxylase is inhibited by α - synuclein[118–122]. Alpha - synuclein achieves this by getting the phosphorylated state reduced, while the inactive dephosphorylated state becomes predominant through stabilization[119, 123–125]. A knockdown of α - synuclein is known to affect VMAT2- a vesicular dopamine transporter per vesicle by elevating the density of VMAT2 molecules.VMAT2 activity is impaired when α - synuclein is overexpressed, which eventually distorts dopamine homeostasis leading to an elevatd cytosolic dopamine concentration[126]. It is reported that, there is a decline in yield of dopamine uptake when α - synuclein is absent in the dorsal striatum [127]. This is followed by further decline in dopamine transporter,tyrosine hydrosylase, tyrosine hydrosylase-positive fibres in the striatum and more complicatedly, a decrease in the amount dopaminergic neurons in the substantia nigra[128–130]. Thus,dopaminergic neurons are dependent on α - synuclein function and impaired when there is loss of function α - synuclein. Neuronal function of α - synuclein is more profound when present in cells other than dopaminergic neurons[87].
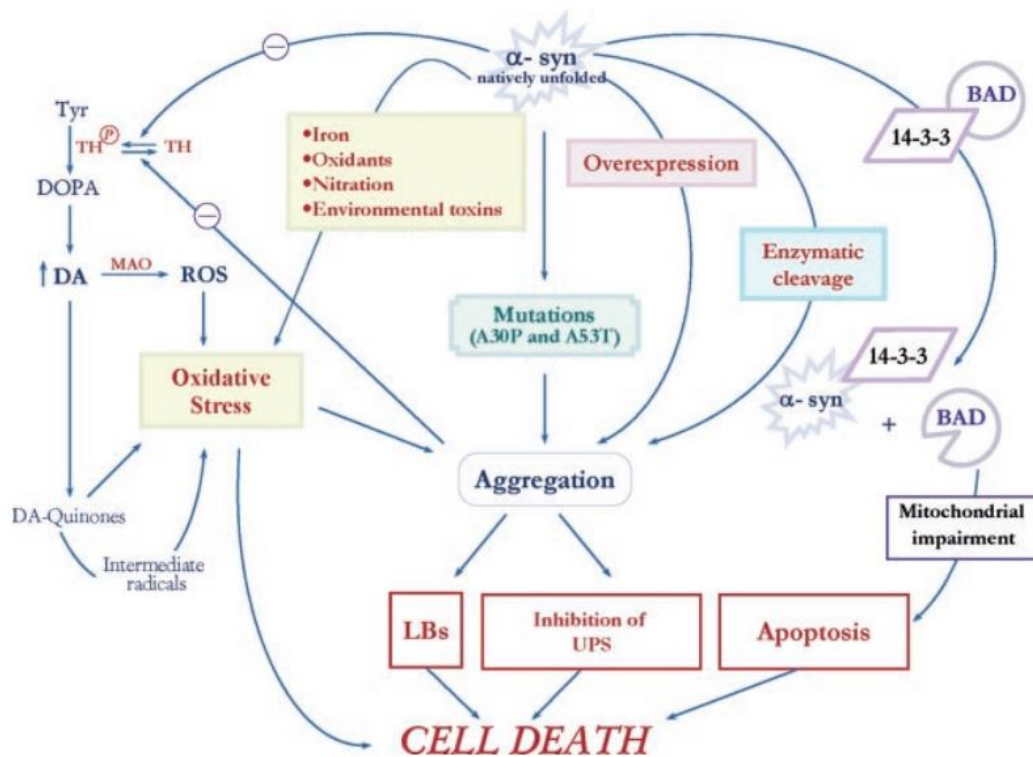
FIGURE 3.10: A hypothetical schematic representation of the leading pathways that result in the aggregation of α - synuclein. Loss of enzymatic activity and chaperone 14-3-3 lead to protein aggregation owing to unfloding events and consequently leading to the formation of Lewy bodies. Overexpression of α - synuclein also tend to clog the proteasom, that is when the rate of formation exceeds the rate of clearance leading to inhibition of the proteasome [5].

3.5 FUNCTIONS OF PARKIN

Subjecting *parkin* to different kinds of mutations including duplication or triplication of exons, deletion of single or multiple exons, point mutations, loss of a copy of a gene leads to an autosomal recessive (AR) form PD and hence the most common form of AR-PD[131–134]. *Parkin* protein constitute 465 amino acid residues. It is a RING-type, one of the two distinct forms of ubiquitin E3 ligase and functions to transfer ubiquitin directly from ubiquitin-activating enzyme (E2) to the substrate and hence E3 ubiquitin ligase activity [8, 134]. SH2 -like domain links the N-terminal to the C-terminal which are made up of ubiquitin-like domain and two RING finger domains respectively [131]. Parkin functions to render protein specificity prior to degradation in the ubiquitin proteasome system (UPS). Mono and polyubiquitination of Lysine-48 and Lysine-63 residues is carried out by parkin[132]. Receptor turn over under certain situations is achieved by parkin through its monoubiquitinating activity[135]. Protein

degradation and protein inclusions result from parkin-mediated Lysine-48 and Lysine-63 linkages respectively[136, 137]. The function and the type of ubiquitin modification is hugely dependent on the cellular context and machinery of ubiquitin that is used by parkin[132].

With its multifunction E3-ligase activity, parkin can perform several ubiquitin associations and enhance cellular role. In vitro studies suggest that parkin is primarily involved in mono-ubiquitination processes[137, 138]. Polyubiquitination is achieved when chaperone-dependent ubiquitin ligase, COOH terminus of heat shock protein 70-interacting protein (CHIP) is added [137, 138].

3.6 PROTEIN DEGRADATION

About more than 30 years ago, there was a demonstration that illustrated the ability of cells to breakdown abnormally folded proteins[139]. It was learnt that treatments that hampered the normal folding of proteins necessitated their hydrolysis and clearance[6]. In view of this findings, it was established that the structure of proteins does not determine only its catalytic features but also its stability in the cell[140]. However, the exact conformational changes in proteins that trigger the degradation machinery of the cell and consequent hydrolysis remain unclear[6].

Protein turn-over explains the continual removal of both intracellular and extracellular protein by breaking them down into their basic building block[4]. Protein degradation plays a crucial role in cellular function and survival. To prevent the toxic effect of aggregation of proteins following misfolding or damage, cells need to clear them[9]. Most neurodegenerative diseases are characterized by protein folding, deposition and aggregation and hence collectively called proteinopathies or protein formation disorders[141]. The multiple proteolytic system in mammals that undertake the continual degradation process ensure a high selectivity and thus prevent the unwanted degradation of the constituenyts of the cell[4]. There should be a overall balance in protein breakdown and synthesis. This is because a marginal increase or decrease in the two activities, uncontrolled can lead to a substantial loss of mass in the whole organism[4].

3.6.1 Importance of protein degradation

The entire protein pool is brought under strict regulation which ensures that a protein is synthesized, folded and localized[11, 142]. When the quality of cellular protein is compromised, homeostasis and function is impaired[143]. Generically, protein conformational disorders including pathologies such as myopathies, metabolic disorders,

neurodegenerative disorders and systemic disorders type of amyloidosis are as a consequences of alterations in different elements of the protein quality control system[144, 145]. Proteotoxicity describes the toxic effect of altered proteins in the cell. Complex cellular systems that function to conserve protein homeostasis are called proteostasis network[11] including chaperones and their regulators which are involved in de novo folding or refolding and the ubiquitin-proteasome and autophagy system which carries out irreversible degradation and clearance roles[7]. Within the cell are surveillance systems that detect altered proteins and execute their folding, elimination or repair. The elimination process involves the degradation by proteases. The type of protein, the location within the cell and the defective step in protein's quality control process define the magnitude of effect of poor quality control[143]. The accumulation of altered proteins is due the increased formation of toxic protein products, defunct surveillance system that monitors,detect and clear altered proteins. However, it is suggested that, with age, the major contributor to the formation and accumulation of high levels of abnormal or damaged proteins is reactive oxygen species(ROS) as proposed as the main element to the aging process as indicated by the free radical theory of aging[143, 146].

The degradation of cellular proteins is highly selective and regulated[147] so as to achieve proteostasis[4]. Proteins that are continually degraded are replaced by newly synthesized ones. proteins have different half-lives and hence the time rate at which the different proteins are degraded varies ranging from minutes to days[6]. The clearance of critical regulatory proteins including enzymes, inhibitors and transcripion factors is necessary for the control of metabolism and growth in cells. Protein degradation is irreversible , unlike other regulatory mechanisms[4].

Proteins adapt to new physiological conditions and changes in cell composition following their rapid degradation. The degradation of proteins in all cell types enables a quality control mechanism that specifically clears damaged or abnormal proteins as a consequence of missense or nonsense mutation, oxidation by reactive oxygen species (ROS), denaturation and biosynthetic errors[4].

For example, during starvation or in a catabolic-diseased state when caloric intake is low, the hydrolysis of proteins in the skeletal into their constituent amino acids becomes necessary so as to provide precursors essential for gluconeogenesis and thus yield energy from non-sugar sources[148]. In the immune system, protein clearance is necessary for the continual monitoring for abnormal proteins found within the extracellular and intracellularly[4]. Protein fragments or peptides that are usually produced during breakdown in the proteasome is taken up into the endoplasmic reticulum. These molecules are carried on cell surfaces adhered to major histocompactibilty complex (MHC)[149].Small, non-native peptides, located on the surfaces of cells, presented by MHC are continually

degraded and removed by circulating lymphocytes. In the same way, foreign bodies or antigens presented by MHC II molecules are taken up by antibodies and digested in the lysosomal-endosomal compartment[4].
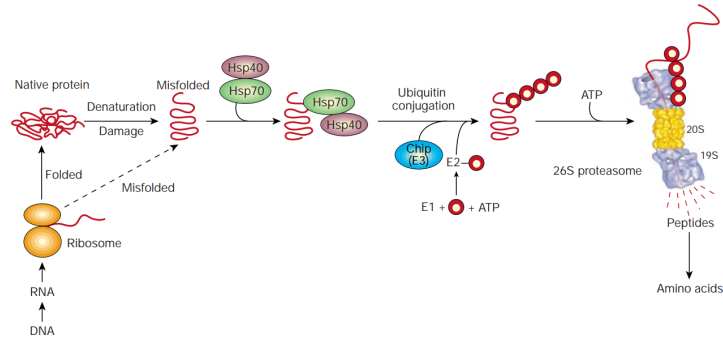
FIGURE 3.11: A diagram showing the life-phase of protein development from transcription through/involving an interplay of scaffolding proteins until it reaches the final degradative stage in the proteasome [6]

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3.7 MOLECULAR CHAPERONES AS A COMPONENT OF PROTEIN QUALITY CONTROL

Proteins are closely invloved in almost every biological process and hence the most versatile and complex macromolecule[7]. More than 10000 proteins are expressed and synthesized in the ribosome as chains of amino acids linked by peptide bonds. These chains need to fold into their native three-dimensional (3D) active state and hence to maintain the proteome integrity[150, 151]. The maintenance of the 3D conformation and flexibility of proteins is critical to their function. With a little margin, proteins are thermodynamically stable in their physiological state.

Chaperones also known as heat shock proteins are stress factors that associates, stabilizes or support other protein to achieve a stable native conformation. A typical feature of molecular chaperones is that they do not form part of the final structure[152, 153]. In response to high temperatures and other stress conditions, they are induced[7]. They are highly conserved molecules[143]. In eukaryotes, a fraction of 20-30 percent of the total proteins in mammals are inherently present in its folded-native state. Interaction with binding partners make them assume a folded conformation[154]. Otherwise, these metastable proteins such as alpha-synuclein and tau, by forming fibrillar aggreregates can give rise to parkinson's disease and dementia respectively[7]. Depending on the cellular location, molecular chaperones can be described as cytosolic or organelle-specific.

3.7.1 Cytosolic chaperones

Folding and unfolding events that are situated in the cytosol is modulated by cytosolic chaperones. To prevent clogging in the lumen, proteins that are synthesized in the polysomes or transported from other compartments in the cytosol are modulated by cytosolic chaperones[155–157]. Most the chaperones located in the cytosol are members of Heat-shock Protein (Hsp)40, Hsp60, Hsp90 family. They function co-operatively in their surveillance activity[143]. In a situation where the spontaneous folding of a de novo synthesized protein fails, Hsp70/Hsp40 may come into play accordingly and if this also fails in this regard, they are passed on to Hsp90/HOP stabilizing chaperone complex or Hsp60 chaperonin chamber[158]. sHSP and Hsp70 chaperones are the most readily deployed chaperones in response to cellular stress and thus being the most abundant among the chaperone family[143]. They bear 50% amino acid identity among other species and hence highly conserved. Cell resistance to heat shock is conferred by their overexpression and thus making whole organisms including flies tolerant[159, 160].

3.7.2 Organelle-specific chaperones

Folding events that are localised within organelles in the cell may also be essential and dedicated to maintain proteostasis [161–163]. Protein folding may be required for de novo synthesized proteins, like in the cytosol. Matured proteins that are already synthesized in the cytosol may also require folding after their transport across the mitochondrial membrane into the mitochondria through translocation complexes[143]. The endoplasmic reticulum (ER) is marked as a key organelle chaperones responsible for protein quality control and maintenance of protein homeostasis[143]. The high content of chaperones within the lumen of ER is due to their direct involvement in the synthesis of proteins. They are immediately brought into action once they sense that a protein is not folded and hence facilitate the folding process[143]. Like the cytosolic chaperones, the promiscuity of some ER chaperones enables them to act on a wide range of proteins by recognizing hydrophobic patches or oligomeric chains such as BiP and calnexin/-calreticulin respectivrely citehetz2009,scheper2009,todd2008. Some chaperones are also limited to specific substrate in function. A classical example is HSP47 which acts on collagen[143].

Unfolding protein response (UPR) describes the situation whereby the level of unfolding increases in the ER making the ER homeostasis get compromised. The presence of a complex network of proteins together with other factors in the ER upregulate ER chaperones synthesis. In effect of UPR, the amount of ER chaperones elevates and translation of proteins gets reduced to minimize ER clogging[164]. Like cytosolic proteins, organelle

proteins that escape folding are marked for degradation which occurs in the cytosol following retrotranslocation from the organelle's lumen. There is an intact relationship between the UPR and the proteosome, described as the ER-associated degradation (ERAD). Prior to protein degradation in the proteosome, proteins that skipped folding, they are tagged with ubiquitin[165, 166].

Clearance or proteolysis of the unfolded proteins in the ER is not only limited to the proteosome but also the lysosome. Unlike in the ER where there is a retrotranlocation of the destined unfolded proteins from the ER into the cytosol, the degradation of the proteins by the lysosome is achieved by engulfing the whole ER[167].

3.8 MAJOR CLASSES OF CHAPERONES

Based on their sizes, molecular chaperones can be classified as HSP90, HSP40, HSP70, HSP60 (chaperonins) and small heat shock proteins (sHP)characterized by a molecular weight of 12-43 kDa[168, 169].

3.8.1 HSP70

They are main the players in the folding process and maintenance of homeostasis. The toxic effect of protein aggregation reduces incredibly by increasing the amount of HSP70 in disease models[170]. The HSP70 reaction cycle is ATP-dependent. Regulation is under the control of HSP40 chaperones family and nucleotide-exchange factors[171, 172]. The linkage between the chaperone function, UPS and autophagy necessary for the degradation of misfolded proteins is coordinated by some of these factors[173]. Allosteric interaction mediates the binding and release by HSP70 chaperone. This event occurs by associating a conserved amino-terminal ATPase domain with a carboxy-terminal peptide binding domain.[171]. The carboxy-terminal peptide binding domain consists of an α -helical lid segment and a β sandwich subdomain[171].

The affinity state of the peptide in an ATP-dependent manner is regulated by the conformational change in the β sandwich domain and the α-helical lid[171]. In the presence of a bound ATP, the α-helical lid adopts an open conformation. The result is a high on rate and off rates for the peptide. Contrarily, when ADP is bound following the hydrolysis of ATP, leads to a lid closure and therby stabilizing peptide binding (low on rates and off rates for the peptide substrate. The hydrolysis of ATP to ADP is fascilitated by HSP40[7]. Direct interaction of HSP40 and the unfolded proteins can assemble HSP70 to the protein substrate[172, 174]. A nucleotide-exchange factor binds to the HSP70

ATPase domain and facilitates the exchange of ATP for ADP. This consequently results in lid opening an d substrate release. The fast-folding substrate bury their hydrophobic residues. Molecules that require a longer time to fold rebound to HSP70. This ensures aggregation[7].Rebinding to HSP70 may perhaps kinetic barriers in the folding process, thereby reducing folding time and bring about conformational remodelling[175].

After the HSP70 cycling process, proteins that skipped the accelerated folding event may be transferred into the chaperonin cage - a specialized environment for folding. A clear example includes actins and tubulins[156]. In dilute solution in vitro, these proteins are known to be confronted with high energetic barriers in the folding process and hence are not able to assume their native states spontaneously[7].
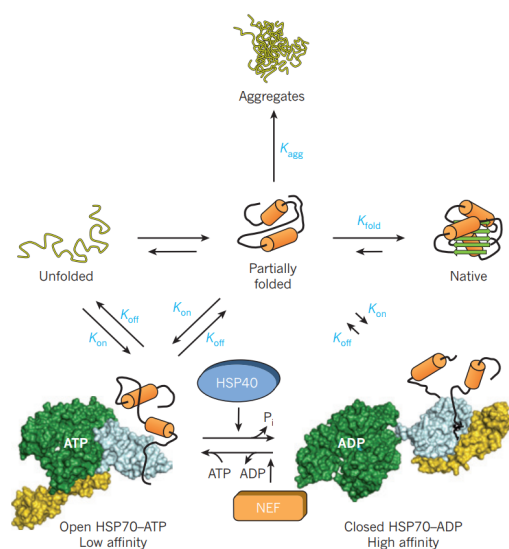
FIGURE 3.12: The different functional states of HSP70 depicting the role of ATP and co-chaperone, HSP 40 in folding nascent or partially folded proteins into their native state[7].

3.8.2 HSP90

In eukaryotes, telomere maintenance, apoptosis, mitotic signal transduction, cell-cycle progression, innate immunity and targeted-protein degradation are among the many signalling pathways are under the control of a proteostasis hub formed by HSP90[176]. Structural maturation and conformational regulation of many signal-transduction such as steroid receptors and kinases are influenced by HSP90 downstream of HSP70[176, 177]. However, the machinery by which HSP90 and its cofactors mediate conformational change is still not demystified[178].

3.8.3   HSP60 (Chaperonins)

They widely enclose substrate protein of molecular size of about 60 kDa. Chaperonins are grouped into two - group I and II. In eukaryotes and bacteria, group I chaperonins are also called also known as HSP60 and GroEL respectively [7]. The more complex group II chaperonins which include archael chaperonins(thermosomes) is made up of octameric or nonameric rings [179]. These rings are composed of one, two or three different subnunits. In bacteria, mitochondria and chloroplasts, there are 7-membered rings of group I chaperonins that function to co-operate with HSP10[7] Extensive studies on GroEl-GroES in *E.coli* has been carried out [153, 180]. More than 250 cytosolic proteins with molecular weight between 20 and 50 kDA are known to have interactions with GroEL [7]. However, structural and functional differences have been idenfified between the two groups of chaperonins. The formation of a temporal complex with HSP10 (co-chaperone) that enclosed the central cavity is the main closure mechanism in group I chaperonins as opposed to the presence of an extra helix found at the terminal of the helical domain that seals the central folding chamber in group II [181, 182].

The general idea to explain the intricate principle behind protein folding and encapsulating by GroEL and GroES has been defined. Here, three to four of the seven hydrophobic sites on an open ring of GroEL captures by binding to non-native polypeptide[183]. ATP and GroEs binds to GroEL ring and induces comformational changes. This subsequently results in both the sequestration of the binding sites and creating an expanded closed depression or cavity that trap the substrate polypeptide in the hydrophobic chamber for polypetide folding[184].

Separation, unfolding and confinement (within the GroES-GroEL cavity where there is acceleration of folding) are the significant features governing the interaction between non-native and GroES[185].

3.9   CELLULAR DEGRADATIVE PATHWAY

There are several protein degradative pathways in cells. The lysosome and the ubiquitin-proteosome are the major degradative pathways present in cells. However, protein degradation can also occur in the cytoplasm and nucleus[4].
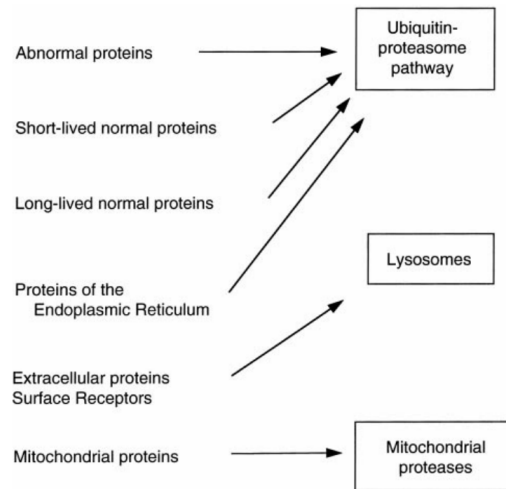
FIGURE 3.13: The various degradative pathways through which a substrate can be degraded within cells [4].

3.9.1 Lysosomal pathway

The process of endocytosis take up hormones or plasma proteins and phagocytosed bacteria and consequently degraded in the lysosomes. Within the lysosome are acid-optimal proteases that includes cathepsins B, H and D and other acid hydrolases[4]. An evolutionarily conserved process - autophagy, strictly regulates lysosomal pathways that are involved in the breakdown of cytoplasmic material and organelles[186, 187]. In response to stress conditions such as viral infection, unfolded protein response and amino acid starvation, autophagy is activated[188]. Macroautophagy (autophagy), microautophagy and chaperone-mediated autophagy are the known autophagic routes depending on the delivery route of the cytoplasmic material[188].

Macroautophagy is the most predominant route that sequesters the portion of the cytoplasm destined for degradation into its lytic compartment. This is achieved by wrapping inside a two-walled organelle called autophagosome[189]. The autophagosome subsequently fuses with the lysosome. The content within the autophagosoes are degraded and re-used[190].

3.9.2 Ubiquitin - Proteosome (UP) pathway

To degrade proteins, two distinctive processes are involved: protein signalling through covalent attachment of multiple ubiquitin to proteins and the degradation of the modified protein and consequent release and utilization of ubiquitin molecules[191]. Proteins that are committed as target for degradation often requires the attachment of

multi-ubiquitin residues. Ubiquitin moieties are linked through their Lys48 residues. The presence of escort factors also helps to deliver ubiquitin-tagged proteins into the proteasome[192, 193] The role played by protein ubiquitination is important in many cellular regulation processes in eukaryotes[147, 194]. Ubiquitin is a globular protein that is highly conserved and it is made up of 78 amino acid residues[8]. Some proteins whose levels are regulated or maintained constitutiely or in response to fluctuations in their environment and hence, the covalent conjugaton of ubiquitin (ubiquitination or ubiquitylation)to these proteins leading to degradation remain essential[8]. Ubiquitylation is therfore seen as targeting sunstrates for breakdown in the proteasome, a multi-subnit protease which is ATP dependent[8]. Ubiquitination is crucial in multitudinuous process that encompasses organelle biogenesis, apoptosis, cell cycle progression, cellular differentiation, protein transport, antigen processing, inflammation, DNA repair and stress responses[8]. Like phosphorylation, most substrates through their linkage via lysine residue associate with ubiquitin in a complex post-translational modification[195]. The activity of ubiquitination can be enhanced or inhibited by phosphorylation, either by modifying the destined substrate prior to ubiquitination or the enzymes involved in the process of ubiquitination[8].

3.10   ACTIVATION OF UBIQUITIN AND SUBSTRATE TAGGING

The multistep process of ubiquitylation involves three distinctive enzymes; Ubiquitin -activating enzyme (E1). E1 forms a thiol-ester linkage with the C-terminal glycine of ubiquitin. The activation process is ATP dependent[8]. Following activation, ubiquitin is transferred from E1 in a reaction catalysed by Ubiquitin carrier protein or E2 or ubiquitin-conjugating enzyme (UBC) to Uniquitin-protein ligase (E3) which is bound to the substrate. There are two distinct E3 families; Homologous to -E6-AP carboxyl terminus (HECT domain) which mediate the transfer of Ubi from E2 to E3 and finally to substrate and RING FINGER which mediate the direct transfer of Ubi to the substrate [9, 196, 197]. Following the conjugation of ubiquitin to the substrate, an isopeptide linkage is formed between the activated C-terminal of glycine of Ubi and an ϵ-amino group of a lysine residue of the substrate[191]. Conjugation between ubiquitin and the N-terminal residues of lysine or cysteine residues has been reported but although rare[198].

Seven lysine residues are present in ubiquitin and hence formation of polyubiquitin chains can be formed through different linkages[194]. To generate signals neccesary

for proteasome targeting, a chain consisting of four ubituitin residues in a Lys48 linkage is required[199]. Substrates that are marked with ubiquitin through Lys11, Lys63 and other lysine residue linkage can be presented for proteasome degradation[200]. Monoubiquitination prepares a protein for proteasome degradation, although in less cases, proteins marked with single ubiquitin is enough to serve as a target for proteasome-mediated degradation[201–203]. Membrane trafficking is other known function of both mono and polyubiquitination[204].
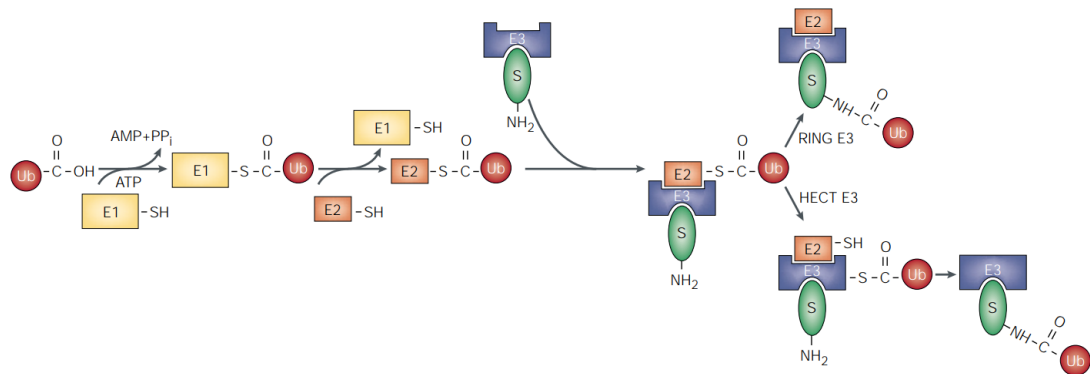
FIGURE 3.14: The delivery and activation pathway of ubiquitination [8].

3.11 THE DYNAMICS OF UBIQUITINATION

Ubiquitination is an important signal for proteasome degradation. However, ubiquitin, once conjugated to a protein does not always results in the degrdation of the protein. This is due to the play of ubiquitin dynamism[9]. Within the cells are numerous enzymes that dissociates the chain of ubiquitin from their substrates. Some of these ubiquitin enzymes are part or in association with the proteasome[205]. Rpn11, a 19S subunit, present in yeast, by hydrolyzing the isopeptide bond between the lysine in the substrate and the ubiquitin, gradually and progressively removes the ubiquitin tag[206, 207]. The removal of ubiquitin from the destined substrate occurs when a substrate is fully ready for degradation. The ubiquitin then escapes from the proteasome and hence, recycled[206, 207].

Inactivation of Rpn11 leads to protein degradation impairment[206, 207] suggesting that the folding of substrates is inhibited following polyubitination[208]. Associated with the proteasome are Ubp6 and Uch17 - de-ubiquitin enzymes that sequentially remove from the distal ends by trimming the ubiquitin residues[209, 210]. The action of these aforemention enzymes can be seen as a timer. The proteasome begins to degrade the ubiquitinated substrate after it binds whiles the ubiquitin consequently start to shrink until the

tag is finally removed from the substrate[9]. There is an escape of the substrate if degradation initiation fails. Longer ubiqutitin chains is aimed to decrease the susceptibility of ubiquitinated substrates to de-ubiquitin enzymes and thus ensure that the substate stay within the proteasome cavity for a relatively longer period of time[199, 210]. Another function of these de-ubiquitination enzymes is to prevent clogging in the proteasome. They remove ubiquitin from proteins that seem to retire in the proteasome, allowing them to escape and hence prevent blockage and hence allow other destined proteins to enter and be degraded within the proteasome[9].

Hul5, a ubiquitin ligase, is in association with the proteasome. By extending the ubiquitin residues on a substrate, Hul5 counteracts the process of de-ubiquitination[211, 212]. This consequently prolongs the time at which a substrate stay in the proteasome with a high propensity of being degraded[9]. This two opposing mechanisms on the substarte functions to select substrates for the degradation in the proteasome[9].

3.12 PROTEASOME

The Proteasome is described as a large cylindrical protein. Its overall dimension for length, maximum diameter and minimum diameter measures at 148 Å, 113 Åand 75 Årerspectively [213]. The proteasome comprises of at least 33 subunits with a molecular weigtht of 2.5 MDa[214, 215].

The 26S proteasome is found in all living cells and it functions to degrade regulatory proteins and degraded proteins. The 26S proteasome comprises a 20S core particle. The 20S proteasome forms the core and proteolytic chamber or unit[214] which is capped or flanked on either one side or both sides by a 19S regulatory particle[214, 215]. The lid and the base of the 19S regulatory particles are formed from at least 19 subunits. The 19S regulatory particle possesses ATPases and provides a barrier or gate to the degradative route. It also functions to recognise, unfold and transport substrate into the 20S proteolytic cavity[208, 215]. The 20S core particle is a cylindrical structure which is formed by four stacked heptameric rings[214]. The two outer and inner rings comprises α and β subunits respectively. The proteolytic active site can be located within the cental cavity - β subunit. The 20S present in bacteria are different proteases which bears structural resemblance to proteasome and yet perform equal function[9]. These bacteria proteases belonging to ATPases associated with diverse cellular proteins (AAA+ protein) and the proteasome have a distant relationship.

The proteasome plays significant roles in several biological processes that includes the clearance of misfolded or abnormal proteins, control of cell cycle and cellular immune

response[214]. These aforementioned cellular functions are closely linked to ubiquitin and ATP-dependent protein clearance or degradation pathway which involves the 20S proteasome.
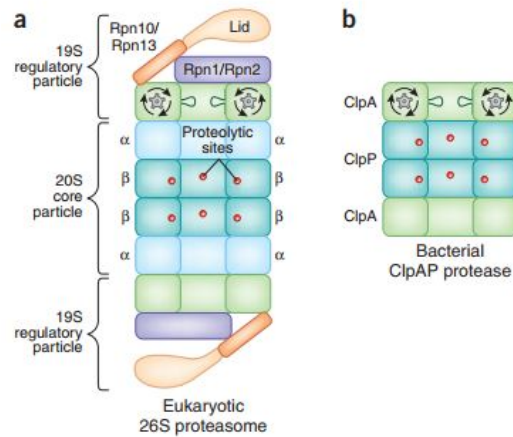
FIGURE 3.15: Structure of the proteasome showing the two main subunits, 19S and 20S which are invloved in regulatory activities and the proteolytic chamber (which is flanked by two 19S reglatory particle) respectively [9].

3.13 INITIATION OF DEGRADATION

Tagging proteins with ubiquitin is not enough to drive the destined protein into the proteasome for degradation[199, 216]. Coupled to the tagging, another degron, that is, an initiation site must be present on the protein[217, 218]. The initiation site is a portion of the protein where the proteasome associates with the substrate, followed by degradation. This degron - aportion of the protein crucial in the rate of degradation, can be situated either at the end of the protein or within. When located within the protein, it is flanked by folding domains on both sides of the protein[217, 219, 220]. Following the covalent attachment of ubiquitin, substrates are tethered to and fixed in the proteasome where there is an engagement by the translocation motor within proteasome[9]. The translocation motor can be located within the ATPase ring in 19S regulatory particle according to studies using a bacterial analogue of proteasome[221–224].The unstructured region of the substrate that is grabbed by loops is pulled into the central pore of the ATPase ring. This consequently results in unfolding and transport of the substrate into the proteasome[222, 224]. Hydrolysis of the substrate into peptides occurs. Proteolysis proceeds until the fragments become small enough that they can leave the proteasome by diffusion[225, 226]. According to studies, on average, amino acids of about 5-20 residues in length are further broken down into single or free amino acids in the cytosol by proteases[227].

However, an increasing number of destined substrates are not completely degraded according to recent studies[228]. A phenomenom, called regulated ubiquitin proteasome-dependent processing (RUP) results from partial elimination of the polypeptide chain, and the other part leaving the proteasome as a stable protein fragment. This stable species or fragment has a biological aactivity[228, 229]. RUP consequently activates inactive or dormant proteins including NFkB and NFkB-related transcription factors of mammnal and yeast respectively [230, 231], which are all ubiquitinated prior to their degradation in the proteasome[220].

3.14 DELIVERY OF SUBSTRATES TO THE PROTEA-SOME

The delivery of ubiquitinated substrate prior to degradation in the proteasome has two fate; first by asssociating directly with the 19S regualtory protein of the proteasome by interacting with regulatory subunits such as Rpn10, Rpn13 or Rpn5[9]. In other instance, adaptors bring ubiquitinated substrates to the proteasome. In this way, the adaptor binds to both the substrate-bound ubiquitin to present it for degradation[206, 232, 233]. A greater fraction of Rpn10 may function as an adapter owing to the fact that they are not in association with the proteasome[9]. Rad 23, Ddil and Dsk2 perform similar function as adaptors. They are characterized by the presence of ubiquitin-like domain (UBA)that bind multiubiquitin residues[234–236]. These aforementioned adaptors can bind to the proteasome and the ubiquitinated substrate at the same time whiles facilitating the degradation of the ubiquinated substrate by engaging the substrate to the proteasome at the initiation site. Indirectly, these adaptors can also hand off the substrate to the ubiquitin-binding proteasome subunit[208]. Differentiating between the proteins which directly get to the proteasome and which flows through the adaptor remains a debate, but the likelihood is that, ubiquititinated proteins may raech the proteasome directly[193].

Chapter 4

EXPERIMENTAL SETUP

4.1   Strains of *Arabidopsis thaliana seeds*

6 different strains of *Arabidopsis thaliana* were used for this thesis. The mutants had variant forms as shown in Table **??**.

4.2   Description of mutants

1. ISUSN: A transpeptide obtained from the gene, AtIscU1 guides the direct transport of α-synuclein into the mitochondria.

2. E46K: A mutant α-synuclein with a single amino acid residue substitution of glutamic acid (E) with lysine (K) at position 46.

3. A53T: A mutant α-synuclein with a single amino acid residue substitution of alanine (A) with threonibe (T) at position 53.

4. A30P: A mutant α-synuclein with a single amino acid residue substitution of alanine (A) with proline (P) at position 30.

5. Sterile SN: *Arabidopsis thaliana* plants modified to produce very few seeds.

4.3   STERILIZATION OF *ARABIDOPSIS THALIANA SEEDS*

1 mL of 10 % chlorine was added into Eppendorf tubes containing the seeds. The seeds were then vortexed shortly and incubated for 12 minutes. Following incubation the

seeds were centrifuged at 13.2×10^3 rpm for 2 minutes. The 10 % chlorine was removed by pipetting. The seeds were washed with distilled water to remove residual chlorine. The tubes containing the seeds were vortexed and centrifuged at 13.2×10^3 rpm for 2 minutes. After every washing, the supernatent was removed and vortexing, washing and centrifugation were repeated for 3 times and left to dry.

4.4 SOWING OF SEEDS AND TRANSFER OF SEEDLINGS.

Distilled water was added to the dried seeds and pipetted onto an a petri dish containing an MS agar. Following germination after 21 days, the seedlings were transplanted onto a soil. The plastic bed containing the transferred seedlings were placed under controlled growth conditions which with varying light and dark conditions (16 and 8 hours light and darkness, respectively) for 6 weeks.

4.5 CONFOCAL MICROSCOPY: SCREENING AND SELECTION.

4.5.1 Preparation of slides, scanning and selection

Six weeks after the seedlings have been transplanted, leave samples were obtained from each plant. Prior to confocal screening, wet glass slides were prepared for all the 15 (including variant) leave samples.The slides were viewed under white light. The samples were focused under white light. Scanning and adjustment was done with a X60 W objective lens. Alexa fluorophore was used for the laser screening. to observe α-synuclein expression both in the cytosol or mitochondria. An unbiased selection was done to select plants that exhibited high expression of α - synuclein in their respective cellular location.

4.6 SECTIONING OF SELECTED PLANTS

Unlike the wild-type (where the whole leave samples were harvested), each selected plant sample was sectioned into four(4) parts. Leave samples were collected from each quartet, wrapped in an aluminium foil and stored in liquid nitrogen at -74°C. This step was repeated every one (1) month for a total of 3 months. Subsequent quartet from each plant was collected and stored at -74°C.

4.7 SAMPLING OF PLANT SAMPLES.

Two sampling methods were developed and used:

1. Young and old leaves from the same plant were harvested.

2. The whole plant was divided into quartet where each quarter represented different developmental stages with respect to age. Harvesting were made at one (1) month interval. That is, the first quarter that was harvested becomes the youngest plant, followed by the second quarter until the fourth quarter which is considered to be the oldest plant sample. Each quarter was wrapped in an aluminium foil, labelled and stored shortly in liquid nitrogen and later stored at -78°C.

4.8 DETERMINATION OF PROTEASOME ACTIVITY USING CHEMICON 20S PROTEASOME ACTIVITY ASSAY, CAT NO. APT280.

4.8.1 Preparation of extract

4.8.2 Extract preparation for sampling method 1.

Triplicates of old and young leaves were obtained from the same plant for the different *Arabidopsis thaliana* plants. Both young and old leaves from the same plant were harvested, weighed, wrapped in an aluminium foil and stored in liquid nitrogen shortly proir to gringing using a mortar and pestle.The leaves were then transferred into a mortar. The grinded leave samples was transferred into an Eppendorf tube. 200 µL of 1X buffer was added, vortexed shortly and centrifuged at 14000 rpm for 5 minutes to obtain a clear supernatant and then stored on ice.

FIGURE 4.1: An image showing the sizes of both young(marked in the yellow ring), old leaf (marked in red ring) and a 2 cm grid (marked in sea-blue).

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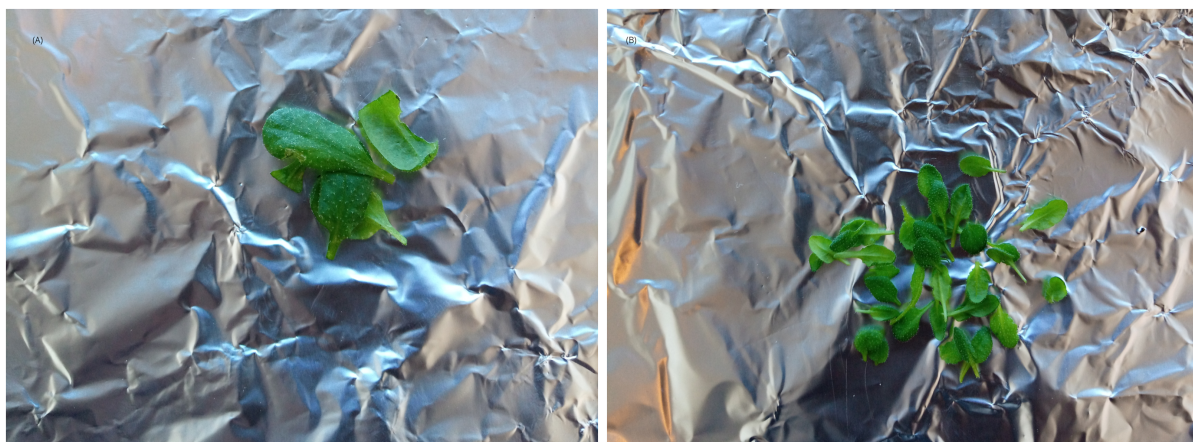
FIGURE 4.2: A picture showing 0.1 g of young and old leaves prior to extraction.

4.8.3   Extract preparation for sampling method 2.

Here, a quadruplicate of the whole wild-type (WT)plant was weighed unlike the mutants where each quarter was weighed to obtain different masses. Grinding procedure was the same as done in sampling method 1 but different known volume of 1X buffer was added respectively. The resulting mixture was vortexed shortly and centrifuged at 14000 rpm for 5 minutes to obtain a clear supernatant.

4.8.4 Preparation of assay mixture.

10 µL of the supernatant (proteasome sample) from each replicate of the different *Arabidopsis thaliana* plant was pipetted into 3 different labelled PCR tubes marked for each replicate. 4 different labelled PCR tubes marked for each replicate was made for sampling method 2. The test sample was prepared as shown in Table 4.1 below. The test sample was then incubated at 37°C for 2 hours. Fluorescence was read at 380/460 nm. The principle behind this test is that, a fluorophore, 7-Amino-4-methylcoumarin (AMC) is detected after cleavage from a labelled proteasome substrate (LLVVY-AMC). The AMC is then quantified as the relative fluorescent unit at380/460 nm using a fluorometer (Chemicon 20S Proteasome Activity Assay Kit, CAt. No. APT280).

TABLE 4.1: The different assay composition mixture using Chemicon Proteasome activity assay protocol

Sample	Assay mixture				
	10X Assay buffer	Proteasome sample	Distilled water	Proteasome substrate	Total volume
Buffer blank	10 μL	0 μL	90 μL	0 μL	100 μL
Substrate blank	10 μL	0 μL	80 μL	10 μL	100 μL
Test sample	10 μL	10 μL	70 μL	0 μL	100 μL

4.9 DETERMINATION OF TOTAL PROTEIN CONCENTRATION USING THE BCA METHOD BY THERMO SCIENTIFIC

4.9.1 Preparation of 1:2 serial dilution of BSA standard solution

A seven 25 μL serial dilutions were made from a 50 μL 2 μL/μg BSA stock solution.

4.9.2 Preparation of working reagent

Two solutions labelled A and B were provided by Thermo Scientific. 50 part of working reagent A was mixed with 1 part of reagent B. A green colloidal solution was obtained. The mixture was then dissolved evenly to obtain a clear green solution.

4.9.3 Preparation of assay mixture for total protein concentration

Four replicates of the plant extract were made for each Arabidopsis plant. 25 µL of both the extract and the BSA standard dilutions were pipetted into a PCR tube. 200 µL of the working reagent was added to each tube and mixture evenly. A purple colour developed. The tubes were on water bath at 37°C for 30 minutes. After 30 minutes, a higher colour intensity developed.The tubes were cooled down for 5 minutes. Absorbance was read at 562 nm to obtain theconcentration.

The principle underlying this test is that Cu^{+2} is reduced to Cu^+ by a protein in a basic solution. The Cu^+ is selectively detected by bicinchoninic acid (BCA). The result is a purple-coloured product which is formed by chelation of the Cu^{+1} by two molecules of BCA. This water-soluble complex has maximum absorbance at 562 nm.

Chapter 5

Results and Discussion

5.1 Plant selection

Results from the confocal microscopy screening showed that the following plants marked with asterics in the table below were selection for further investigation.

These were; E46K 12, ISUSN 5, A53T T3 - 1 and wild - type (WT). Out of the 10 replicates for each class of *Aradopsis thaliana*, those that showed more than 70% expression were deemed ideal.

TABLE 5.1: Summary of the degree of expression for the different Arabidopsis thaliana strains.

Sample ID	Cellular location of expression	Degree of expression (low\high)
ISUSN 1	Mitochondria	low
ISUSN 4	Mitochondria	low
E46K 12*	**cytosol**	**high**
E46K 11	cytosol	low
ISUSN 5*	**Mitochondria**	**high**
E46K 13	cytosol	high
A30P 6	cytosol	high
A30P 16	cytosol	high
A30P 1	cytosol	low
A53T T3-5	cytosol	high
A53T T3-4	cytosol	high
A53T T3-1*	**cytosol**	**high**
SN Sterile 2	cytosol	high
SN Sterile 1	cytosol	high
SN*	**cytosol**	**high**

5.2 Weekly observed features of a variant form of *Arabidopsis thaliana*, E4KK12 prior to screening.
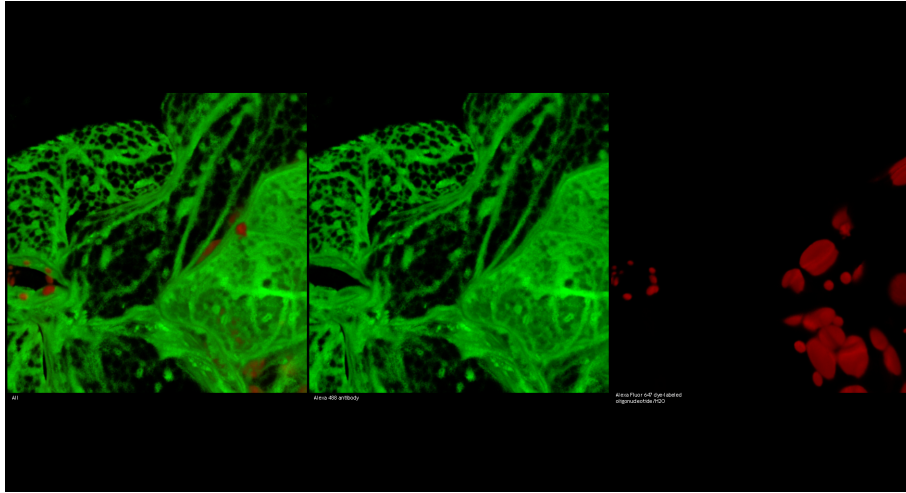
FIGURE 5.1: A picture of E46K12 plant viewed under confocal microscope. Cytoplasmic cellular content marked green and red are α - synuclein and chloroplast labelled with Alexa 488 antibody (green) and Alexa Fluor 647 dye-labelled oligonucleotide water (red) respectively.

After week 18, almost all the plants were physically visualized as developing flowers and shoots as shown in the image above. Also observed were thick and crowded leaves for each plant.

FIGURE 5.2: A picture of ISUSN 5 plant at 6 weeks (left), 19 weeks (right) and 21 weeks old (bottom).

5.3 Structural dynamism of the mitochondria.
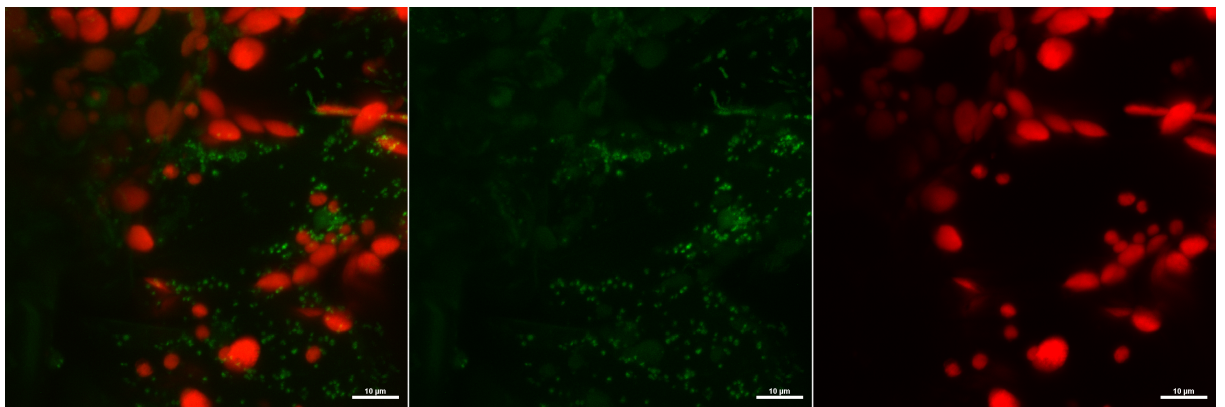
FIGURE 5.3: The mitochondria and chloroplast labelled with Alexa 488 antibody (green) and Alexa Fluor dye - labelled oligonucleotide water (red) after week 18 for the ISUSN5 *Arabidopsis thaliana* plant.
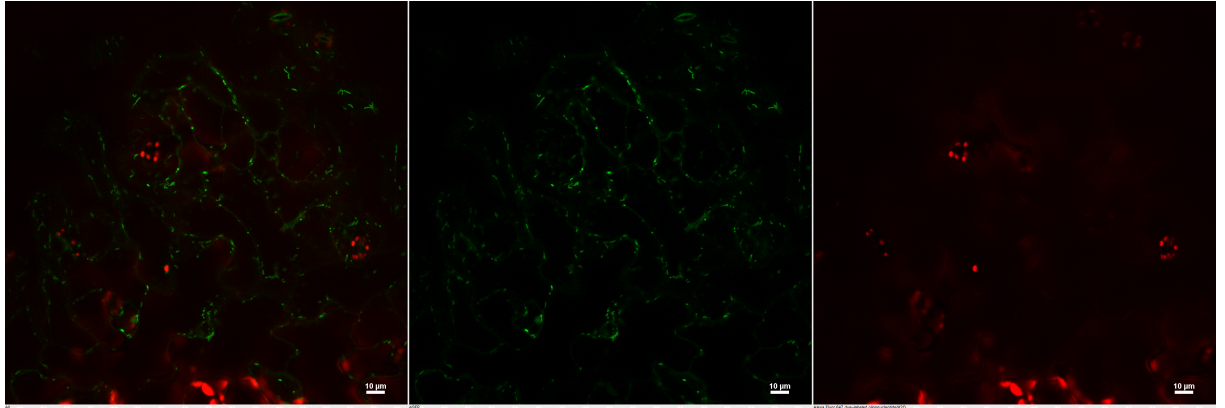
FIGURE 5.4: The mitochondria and chloroplast labelled with Alexa 488 antibody (green) and Alexa Fluor dye - labelled oligonucleotide water (red) after week 22 for the ISUSN5 *Arabidopsis thaliana* plant.
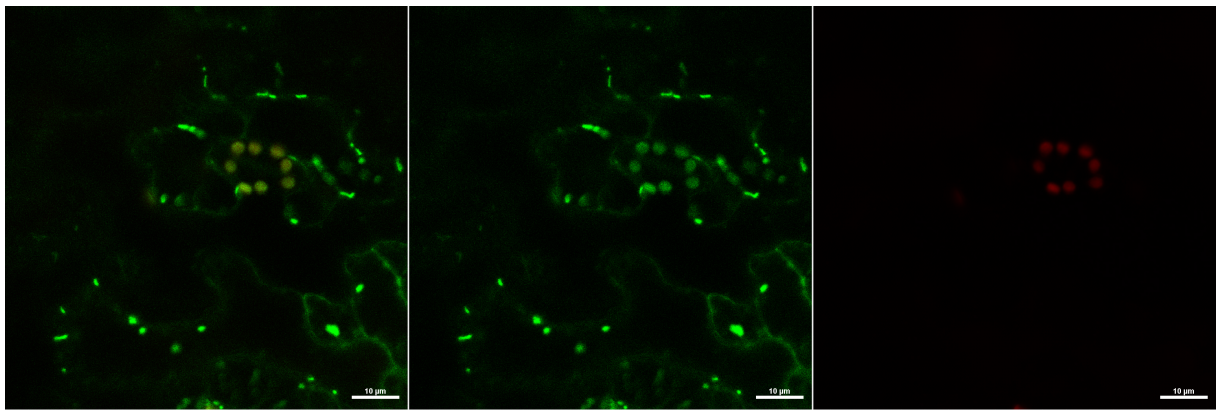
FIGURE 5.5: The mitochondria and chloroplast labelled with Alexa 488 antibody (green) and Alexa Fluor dye - labelled oligonucleotide water (red) after week 18 for the ISUSN5 *Arabidopsis thaliana* plant.

α - synuclein is known to be predominantly localized in the cytosol, although a fraction of the total ocmposition has been been suggested to be present in the membrane of the mitochondria in functional dopaminergic neurons [237]. .

The dynamism and different morphology assumed by the mitochondrial are hugely in response to the various physiologic function and partly owing to stress condition such oxidation and aggregation of intracellular proteins within the subcellular organelle [238]. In the figure above, it could be observed that the amount of α - synuclein concentration in the cytoplasm gradually decreases with age. This observation is manifestation by the aggregation of α - synuclein and hence the apperance of brighter spots.

5.3.1 Generation of standard curve.

5.3.1.1 Preparation of dilutions for a standard curve

A dilution series of 1:2 was made from a 125 μM AMC. The dilution concentration range was set between 0.04 μM - 6.25 μM.
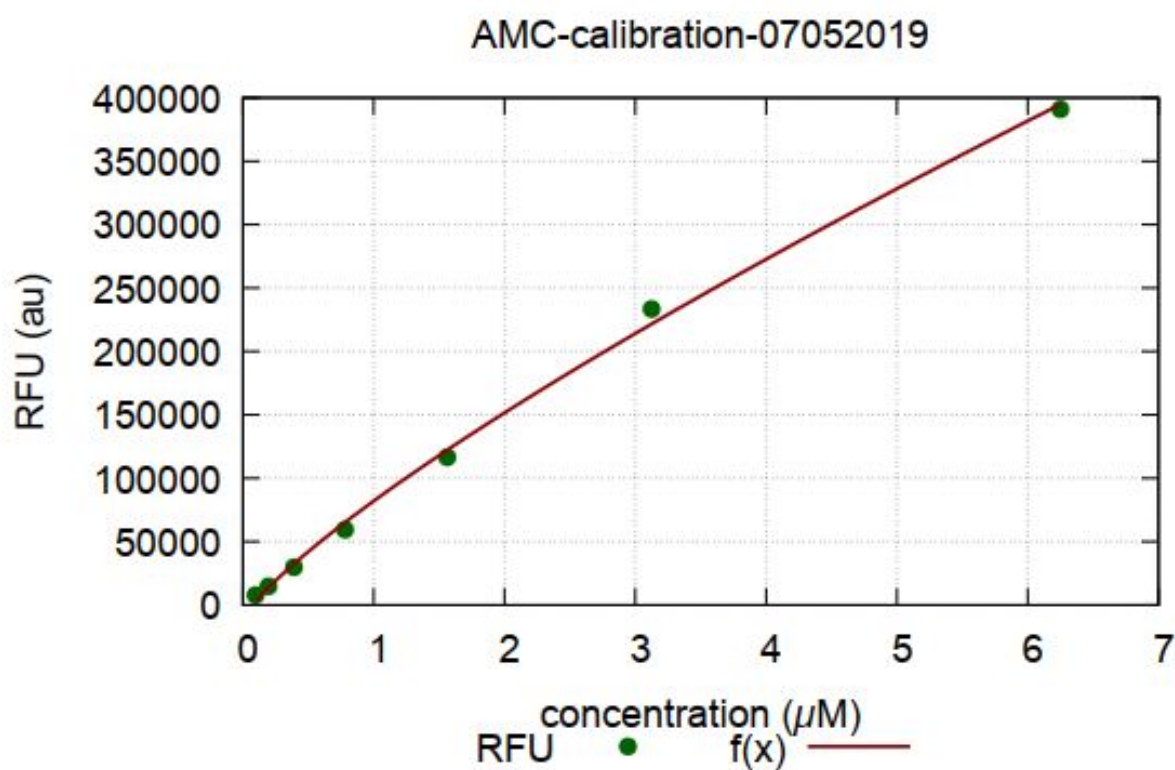
FIGURE 5.6: A calibration curve obtained for AMC. This function illustrates a direct relationship between the concentration of the fluorophore (AMC) and emission at 480nm. The higher the concentration, the higher Relative fluorescent unit (RFU).

TABLE 5.2: Fluorescent measurements obtained for both young and old leaves of the ISUSN 5 mutant Arabidopsis plant for sampling method 1 after week 28.

Sample ID / Assay component		Fluorescent measurement	
Replicate	Replicate number	Young leaves	Old leaves
2	1	44495.036	25630.341
	2	46008.709	30580.668
	3	50575.406	28239.481
	4	52139.844	32953.397
5	1	11634.188	17948.071
	2	42139.262	22851.964
	3	63835.613	24422.502
	4	35543.478	23105.330
8	1	14634.261	17450.917
	2	18553.665	22201.015
	3	30456.988	21086.994
	4	36171.013	23274.551
DMSO		657.849	
Distilled water		359.618	
10X buffer		306.360	

TABLE 5.3: Fluorescent measurements obtained for both young and old leaves of the Wild-Type (WT) Arabidopsis plant for sampling method 1 after week 28.

Sample ID / Assay component		Fluorescent measurement	
Replicate	Replicate number	Young leaves	Old leaves
1	1	39138.907	39997.568
	2	40465.135	35953.953
	3	41387.173	43553.392
	4	44623.7515	45338.496
2	1	40110.838	7547.107
	2	41982.490	9800.748
	3	38234.185	8865.939
	4	37630.586	8224.409
5	1	49084.300	37361.526
	2	48002.767	36652.918
	3	54665.635	37922.188
	4	47493.705	33982.408

TABLE 5.4: Fluorescent measurements obtained for both young and old leaves of the E46K12 mutant Arabidopsis plant for sampling method 1 after week 28

Sample ID / Assay component		Fluorescent measurement	
Replicate	Replicate number	Young leaves	Old leaves
2	1	90139.14	301239.32
	2	111341.93	318900.41
	3	96330.76	292147.18
	4	99933.56	291030.28
3	1	95890.25	59443.61
	2	93716.48	147102.73
	3	153856.73	79691.23
	4	100585.26	151683.26
4	1	71652.36	138552.04
	2	75491.72	68217.09
	3	86506.66	30661.68
	4	81601.87	30629.01

TABLE 5.5: A 1:16 dilution of extract to buffer to determine the total protein concentration and absorbance values for young and old leaves of E46K12 mutant *Arabidopsis thaliana* plant after week 28.

Proteasome sample		concentration of young	mass	concentration of old leaves	mass
		leaves	mg protein		
replicate	replicate	mg/mL	mg/mL	mg/mL	mg protein
	number		per g leaves	mL	per g leaves
			fresh wt.		fresh wt.
2	1	13.062	26.124	6.851	13.702
	2	13.110	26.022	6.611	13.222
	3	13.014	26.028	10.068	2.136
	4	13.879	27.758	9.636	19.272
3	1	10.606	21.212	8.227	16.554
	2	13.479	26.958	8.659	17.318
	3	12.598	25.196	8.867	17.734
	4	13.318	26.626	8.707	17.414
8	1	12.870	25.740	6.675	13.250
	2	13.062	26.124	7.827	15.654
	3	12.710	25.42	9.124	18.248
	4	13.094	26.188	8.803	17.606

TABLE 5.6: A 1:8 dilution of extract to buffer to determine the total protein concentration and absorbance values for young and old leaves of ISUSN5 mutant *Arabidopsis thaliana* plant after week 28.

Proteasome sample		concentration	mass,	concentration	mass,
replicate	replicate	of young leaves	mg protein	of old leaves	mg protein
	number	mg/mL	per g leaves	mg/mL	per g leaves
			fresh wt.		fresh wt.
2	1	6.211	12.422	2.561	5.122
	2	4.716	9.432	1.597	3.194
	3	6.140	12.280	3.200	6.400
	4	6.326	12.652	2.518	5.036
5	1	5.303	10.606	1.201	2.402
	2	5.096	10.192	1.580	3.160
	3	5.076	10.152	1.752	3.504
	4	5.515	11.030	1.653	3.306
8	1	3.948	7.896	2.119	4.238
	2	4.711	9.422	2.286	4.572
	3	4.800	9.600	2.245	4.490
	4	4.072	8.144	1.757	3.514

TABLE 5.7: A 1:16 dilution of extract to buffer to determine the total protein concentration and absorbance values for young and old leaves of Wild-type (WT) *Arabidopsis thaliana* plant extract after week 28.

Proteasome sample		concentration	mass	cncentration	mass,
Replicate	replicate	of young leaves,	mg protein	of old leaves	mg protein
	number	mg/mL	per g leaves	mg/mL	per g leaves
			fresh wt.		fresh wt.
1	1	13.447	26.894	7.011	14.022
	2	12.246	24.492	6.851	13.702
	3	11.574	23.148	8.036	16.072
2	1	11.494	22.988	2.417	4.834
	2	10.389	20.778	2.897	5.922
	3	10.546	21.092	2.961	5.922
5	1	13.463	26.926	6.131	12.262
	2	13.126	26.252	7.187	14.374
	3	13.446	26.892	7.764	15.528

TABLE 5.8: Summary of Total protein concentration obtained for young and old leaves in Sampling method 1 after week 28.

Plant ID	Young leaves, average [ ]± SD	Old leaves, average [ ]± SD	young leaves, average RFU	Old leaves, average RFU
Wild-type (WT)	12.192 ± 1.18	5.695 ± 2.14	43568.29	33982.41
ISUSN 5	5.160 ± 0.75	2.039 ± 0.53	37182.29	24145.43
E46K 12	12.899 ± 0.73	8.334 ± 1.09	96420.54	159108.15
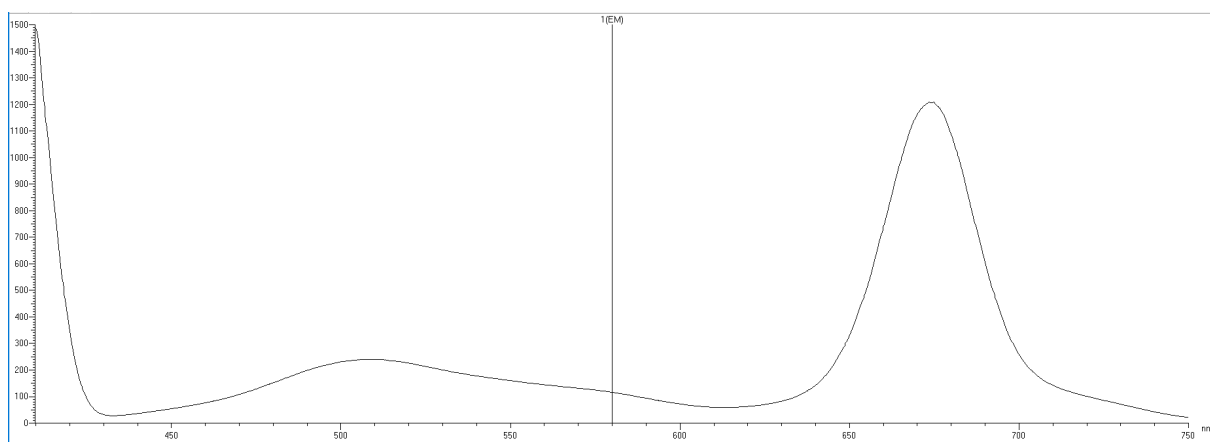
FIGURE 5.7: Excitation spectrum of extract measured at 400nm. This was obtained to account for any possiblity of Chlorophyll or other plant pigment interference.
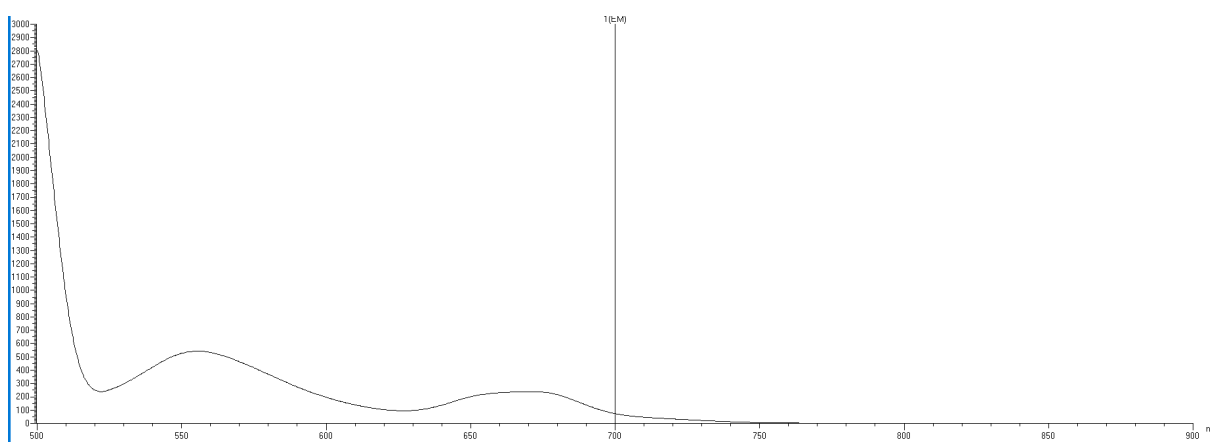
FIGURE 5.8: Excitation spectrum of extract measured at 490nm. This was obtained to account for any possiblity of Chlorophyll or other plant pigment interference

TABLE 5.9: Showing the different masses of the whole (for WT) and plant quartets with their respective volumes of added buffer.

Plant sample		weight (g)	volume of buffer (mL)
Wild-type (WT)	Replicate number		
	1	3.355	6.71
	2	3.221	6.44
	3	6.969	13.94
	4	5.375	10.75
ISUSN 5			
Plant number	Quarter order		
1	1	0.219	0.44
	2	1.075	2.15
	3	0.368	0.74
	4	0.128	0.26
2	1	0.526	1.05
	2	0.902	1.8
	3	0.241	0.48
	4	0.185	0.37
3	1	0.504	1.01
	2	1.307	2.61
	3	0.392	0.79
	4	0.288	0.58
4	1	0.867	0.18
	2	1.060	2.12
	3	0.926	1.85
	4	0.25	0.50
E46K 12			
Plant number	Quarter order		
1	1	0.487	0.97
	2	1.533	3.07
	3	1.133	2.27
	4	0.191	0.38
2	1	1.012	2.02
	2	1.586	3.17
	3	1.285	2.57
	4	0.242	0.48
3	1	0.588	1.18
	2	1.438	2.88
	3	1.155	2.30
	4	0.240	0.48
4	1	0.642	1.28
	2	1.130	2.26
	3	0.273	0.55
	4	0.163	0.33

TABLE 5.10: Fluorescent measurements obtained for the different quarters of E46K 12 mutant Arabidopsis plant for sampling method 2. The first, second, third and fourth quarter were 12, 16, 20 and 24 weeks old respectively. The emission wavelength was 460 nm.

Proteasome sample		Fluorescent measurement
Replicate	Quarter	
Plant 1	1st	5798.57
	2nd	5813.10
	3rd	28288.22
	4th	13165.45
Plant 2	1st	899.92
	2nd	4367.19
	3rd	32526.25
	4th	69506.44
Plant 3	1st	10171.86
	2nd	9233.98
	3rd	25314.36
	4th	9681.30
Plant 4	1st	11091.98
	2nd	9141.88
	3rd	24117.67
	4th	36468.97

TABLE 5.11: Fluorescent measurements obtained for the different quarters of ISUSN 5 mutant Arabidopsis plant for sampling method 2. The first, second, third and fourth quarter were 12, 16, 20 and 24 weeks old respectively. The emission wavelength was 460 nm.

Proteasome sample		Fluorescent measurement
		at an emission wavelength of 460 nm
Replicate	Quarter	
Plant 1	1st	29327.17
	2nd	17763.22
	3rd	64284.88
	4th	66651.19
Plant 2	1st	13363.89
	2nd	35454.21
	3rd	16918.55
	4th	26247.71
Plant 3	1st	25599.56
	2nd	18676.54
	3rd	24626.06
	4th	21149.11
Plant 4	1st	61911.25
	2nd	6327.89
	3rd	57108.36
	4th	27429.088

TABLE 5.12: Fluorescent measurements obtained for the different quarters of Wild-type (WT) mutant Arabidopsis plant for sampling method 2. The first, second, third and fourth quarter were 12, 16, 20 and 24 weeks old respectively. The emission wavelength was 460 nm.

Proteasome sample		Fluorescent measurement
Plant number	Age (month)	
Plant 1	1	4266.53
Plant 2	2	10426.136
Plant 3	3	26185.33
Plant 4	4	30146.20

TABLE 5.13: A 1:5 dilution of extract to buffer to determine the total protein concentration and absorbance values for the quartets obtained from E46K12 mutant *Arabidopsis thaliana* plant for sampling method 2 using Thermo Scientific BCA protein assay kit. Absorbance was read at 562 nm.

| Proteasome sample		Absorbance,	concentration	mass,
Replicate	quarter		mg/mL	mg protein per g leaves
1	1st	1.5552	4.161	8.322
	2nd	0.9297	2.028	4.056
	3rd	1.2458	2.782	5.564
	4th	1.3402	3.016	6.032
2	1st	1.551	2.560	5.120
	2nd	0.2508	0.541	1.082
	3rd	1.1930	2.652	5.304
	4th	0.9643	2.459	4.918
3	1st	1.4125	3.198	6.396
	2nd	1.0426	2.292	4.584
	3rd	0.4491	0.956	1.912
	4th	0.7283	1.569	3.138
4	1st	0.2684	0.578	1.156
	2nd	0.6033	1.293	2.586
	3rd	0.6506	1.397	2.794
	4th	1.2583	2.469	4.938

TABLE 5.14: A 1:5 dilution of extract to buffer to determine the total protein concentration and absorbance values for the quartets obtained from ISUSN5 mutant *Arabidopsis thaliana* plant for sampling method 2 using Thermo Scientific BCA protein assay kit. Absorbance was read at 562 nm.

Proteasome sample		Absorbance,	concentration	mass,
Replicate	quarter		mg/mL	mg protein per g leaves
1	1st	0.6080	1.086	2.172
	2nd	1.553	2.134	4.268
	3rd	1.2232	2.272	4.544
	4th	0.5187	0.924	1.848
2	1st	0.5726	1.019	2.038
	2nd	0.7274	1.306	2.612
	3rd	1.1839	2.192	4.384
	4th	0.3728	0.664	1.328
3	1st	0.4023	0.716	1.433
	2nd	0.7059	1.266	2.532
	3rd	0.7991	1.441	2.882
	4th	0.9644	1.757	3.514
4	1st	0.7404	1.331	2.662
	2nd	0.5921	0.944	1.888
	3rd	0.4968	0.885	1.770
	4th	1.1475	2.118	4.236

TABLE 5.15: A 1:5 dilution of extract to buffer to determine the total protein concentration and absorbance values for the quartets obtained from WT *Arabidopsis thaliana* plant for sampling method 2 using Thermo Scientific BCA protein assay kit. Absorbance was read at 562 nm.

Proteasome sample	Absorbance,	concentration,	mass,
Whole plant		mg/mL	mg protein per g leaves
1	1.4997	2.852	5.704
2	1.2408	2.769	5.538
3	1.1876	2.639	5.278
4	1.5985	3.681	7.362

TABLE 5.16: Summary of Total protein concentration obtained for young and old leaves in Sampling method 2.

Plant ID	[average total protein] ± SD, mg/mL	Plant number, age(month)	Average RFU
Wild-type (WT)	2.985±0.41	Plant 1	4266.53
		Plant 2	10426.14
		Plant 3	26185.33
		Plant 4	30146.20
ISUSN 5	1.284± 0.65	Quarter 1	33650.30
		Quarter 2	18455.63
		Quarter 3	31502.64
		Quarter 4	29204.34
E46K 12	2.122±0.97	Quarter 1	13266.84
		Quarter 2	26824.95
		Quarter 3	13600.38
		Quarter 4	20204.88

Both intrinsic and extrinsic factors including ageing, generation of reactive oxygen species (ROS), oxidation of biomolecules and induced-stress respectively generally lead to a reduction in physiologic functions such as protein turn over, degradation of altered protein and clearance. This consequently brings about an elevated rate of morbidity and mortality [239, 240].

In the model transgenic plants and WT, the differential physiologic state is due to the different stress conditions. A measure of proteasomal activity in the leaves of the model plants, thus its ability to specifically break the conjugated substrate (LLVY-AMC), thereby releasing AMC is as a function of the physiologic state. AMC as a fluorophore was then quantized.

Protein degradation and clearance under the conscious control of the proteasome is expected to be higher in young tissues or organs relative to old tissues but with no known fold or magnitude though a substantial difference in activity is expected in both.

In sampling method 1, Table 5.2 and 5.3 showed that, the relative fluorescent unit (RFU) measured for the young leaves were higher as compared to the old leaves. Although there were some few inconsistencies in the number of replicates where RFU recorded in the old leaves were higher than those in the young leaves as observed in Table 5.4.

However, in table 5.8 the average RFU measured for young leaves were higher than those measured in old leaves for the WT, E46K12 and ISUSN5. In addition, the WT and E46K12 had the was highest average total protein concentration as compared to ISUSN5 as sen in Table 5.8 The low RFU in ISUSN5 could be attributed to the influx or transport of α - synuclein in the mitochondria which has the tendency to compromise of

mitochondrial function and integrity due to stress inner mitochondrial membrane where oxidative phosphorylation occurs. Reduced oxidative phosphorylation implies that there is low ATP synthesis.

In a similar work conducted in Fisher 344 rats tissues of (liver, heart and kidney) and neuronal cells by [241] to study the effect of age and stress on multicatalytic proteasome (MCP), proteasomal activity was shown to decrease with age. In their report, proteasome activity in the liver, lung, heart and kidney tissues had showed no significant decrease in proteasome activity after 12 months. This results obtained when compared to week 3 or 3 months old animals did not have much difference. Contrarily, a decline of proteasomal activity was observed after 24 months in lung tissues. There was an early detection of a decline in proteasomal activity in the cortex and hippocampus in the central nervous sysytem. Therefore, it can be inferred that the proteasome degree of susceptibility and function varies within different organs and tissues.

Fluorescent measurements for young and old leaves as seen in Table 5.4 was in this case, a reverse of the expected in sampling method 1. Measurements obtained were low and high in young and old leaves respectively. The observed feature of the leaves, that is, young and old, as a measure of age may not be a true reflection of the actual age of the plants. Hence, this obsevation could be attributed to (i) the sampling and (ii) the inherent behaviour or features of the mutant plant. To ascertain the different RFU measurements recoreded for the different *Arabidopsis thaliana* plants, the protein concentration was determined. The total protein yield of an extract may not necessarily predict the amount of a particular protein since most soluble protein reside in the cytosolic but to some extent inference could be made. In Table 5.8, the RFU unit was manisfested by the value of absorbance or concentration. E46K12 had a maximum cytosolic protein yield. This was followed by WT and lastly ISUSN5.

Interference by chlorophyll a - the most abundunt plant pigment, was accounted for by obtaining a emission spectrum with the starting wavement set at diffrent wavelengths - 400 nm and 490 nm 400 nm . Chlorophyll a has a maximum emission at 673 nm. In figure 5.7 and figure 5.8, there was not any observed peak of emission at 673 nm and hence inference can be made that, interference from chlorophyll was nil.

Results obtained from the sampling method 2 showed some inconsistencies. In general, The relative fluorescent unit (RFU) as a function of the proteasome activity was to expected to have a declining order or fashion from the first quarter to the fourth quarter. In this experiment, conscious effort to have least effect of bias was ensured and hence critical.In the sectioning process, eaxh quarter reprsented or contained an uneven distribution of leaves accounting for the different masses (densities) and hence different volumes of buffer was added as shown in table 5.9. In table 5.16, WT had the

highest averrage total protein. This was followed by E46K12 and ISUSN 5 which had the least average total protein concentration. In both sampling methods, the average total protein concentration is almost similar for the WT and E46K12 mutant *Arabidopsis thaliana* plant. The inconsistent measurements of RFU in the sampling method 2 as seen in Table 5.12, 5.10, 5.11 gave rise to outliers. The effect of the outlier is that an inverse relationship was observed between the RFU and the total protein concentration.

In addition to the above, the low proteosome activity in the young leaves as compared to the old leaves could also be attributed to the following factors: (i) a less demand or low assembly in activity in response to stress at the young stage in the plant growth since stress is associated with ageing. (ii)The developmental stage of the proteosome as a component of the proteome had marked its prime in activity and hence its expression is manifasted by a heightened activity.

The different treatment or storage method following harvesting could also account for the unexpected or differentRFU in sampling method 1 and 2. In sampling method 2, the harvested leaves were stored at -74°C prior to extraction over a period of 4 months unlike sampling method 1 where the leaves were shortly stored in liquid nitrogen four less than an hour prior extraction. Plants' ability to survive freezing temperatures vary hugely owing to their different level of cold acclimatization at approximately below 10°C [242]. Therefore, the extent of injury caused by extreme cold would also vary. Protein denaturation and precipitation could be as a result of freeze-induced dehydration [242].

Chapter 6

Conclusion

Sampling method 1 provided a better and a more consistent results with respect to size as a measure of age and proteasome activity. In general, proteasome activity was higher in the young leaves than the old leaves. A functional proteasome reduces the rate of protein aggregation and hence facilitate its clearance. On the other hand, sampling method 2 gave some hints about protein or $\alpha - synuclein$ aggregation and age. Therefore , it could be inferred that age is one of the major factors that can influence the accumulation of $\alpha - synuclein$.

Chapter 7

Future perspective

1. A number of proteins such chaperones and proteasome involved in protein processing /handling and clearance is hugely dependent on the viability of ATP. Therefore, growing the plants in the presence of a suitable varying concentration of ATP and measuring proteasome activity will provide more direct relationship between proteasome activity and ATP. This could help us establish a possible effect of over /under supplementation of ATP on these proteins.

2. To have a true age of the plants, a specific time period should be set for each plant whiles the plants are grown on different different beds. For example, within a period of 12 months, first, second, third and fourth harvest harvest could be made intermittedly at the 3rd, 6th, 9th and 12th month respectively as reported by [241]. This will avoid the effect of storage conditions on protein activity. This is because, as seen in sampling method 1, extracts prepared shortly after harvest provided a more consistent RFU unlike the second sampling method.

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