MASTERARBEIT

Titel der Masterarbeit

Ubiquitin- and Proteasome-dependent Protein Turnover in *Arabidopsis thaliana*

Verfasser

Bulut Hamali

angestrebter akademischer Grad

Master of Science (MSc)

Wien, 2013

Studienkennzahl lt. Studienblatt: A 0969082

Studienrichtung lt. Studienblatt: A 066 877 Masterstudium Genetik und Entwicklungsbiologie

Betreuerin / Betreuer: Ao. Univ. - Prof. Dr. Andreas Bachmair

"In generosity and helping others be like a river.

In compassion and grace be like sun.

In concealing others' faults be like night.

In anger and fury be like dead.

In modesty and humility be like earth.

In tolerance be like a sea.

Either exist as you are or be as you look."

Mewlana Jalaluddin Rumi

Contents

ZUSAMMENFASSUNG

Stickstoffmonoxid (NO) ist ein kleines Molekül, welches in allen höheren Organismen die eine Rolle als Signalmolekül spielt. Es ist bereits bekannt, dass NO in Pflanzen nicht nur die Antwort auf Umweltstress, sondern auch Entwicklungsprozesse reguliert. Einige Mechanismen, wie die S-Nitrosylierung von regulatorischen Proteinen in Pflanzen, wurden bereits beschrieben, viele molekulare Mechanismen sind aber immer noch rätselhaft. Hier wurde gezeigt, dass NO Proteine mit N-terminalem Cystein in den Ubiquitin-abhängigen Proteinabbaubbau einschleust und dass dieser Prozess O_2 erfordert. Diese Experimente deuten darauf hin, dass der durch NO eingeleitete Abbau von Proteinen eine Rolle bei der NO-vermittelten Signaltransduktion spielt.

Die Expression einer Ubiquitin-Variante mit Arg anstelle von Lys in Position 48 (ubK48R) führt in der Arabidopsis Linie RV86-5 zum Zelltod. Um diesen Zelltodprozess zu verstehen, muss ubK48R Expression in RV86-5 frei von Effekten sein, die auf das Expressionssystem zurückzuführen sind. Da vom verwendeten Aktivierungssystem unerwünschte Effekte berichtet worden waren, wurde versucht, herauszufinden, ob solche Effekte die Zelltodprozesse in RV96-5 Pflanzen beeinflussen. Die Ergebnisse lieferten Informationen über die funktionelle Relevanz des Systems.

Ein weiterer Teil der Diplomarbeit knüpfte an das Thema Zelltod an mit der Untersuchung von Suppressormutanten, welche die Expression von ubK48R überleben. 5 Mutantenlinien waren zuvor durch EMS-Mutagenese erzeugt worden, *sud2* (SUPPRESSOR OF UBIQUITIN UBK48R-INDUCED CELL DEATH 2) war die interessanteste dieser Mutanten. Die Mutation war auf Chromosom 3 kartiert worden, aber weitere Experimente waren notwendig, um nach der partiellen Genomsequenzierung Mutationen in der relevanten Region des Genoms zu bestätigen. Basierend auf einer neuen Validierungsmethode (Sedlazeck et al. 2013) wurden Kandidatenmutationen mit der Sanger-Sequenzierungsmethode bestätigt. Diese Bemühungen führten zu potentiellen Kandidaten für das SUD2 Gen.

SUMMARY

Nitric Oxide (NO) is a small molecule present in all higher organisms that plays a role as a significant signaling compound. It is already known that plants use NO as a regulatory compound not only in response to the stress coming from the environment, but also in developmental processes. Although some mechanisms in plants have been reported, such as S-nitrosylation of regulatory proteins, many molecular mechanisms are still an enigma. Here we show that NO targets protein substrates with N-terminal Cysteine for ubiquitin-dependent degradation and that this process requires O_2. These experiments suggest that NO-mediated protein turnover plays a role in plant NO sensing and signaling.

Expression of a ubiquitin variant with Arg instead of Lys at position 48 (ubK48R) in the *Arabidopsis* plant line RV86-5 leads to cell death. In order to understand the downstream effects of this process in a precise manner, ubK48R expression in line RV86-5 has to be devoid of any side effects due to the expression system. Since the GVG activation system, which is present in line RV86-5, has already been reported as having side effects, we tried to find out whether it has any effect on cell death in RV86-5. These results provided reliable information about the functional relevance of the system.

Another part of the Thesis is related to the previous topic; analyzing a suppressor mutant that survives the lethal effects of ubK48R. 5 mutant lines had previously been generated by EMS mutagenesis and *sud2* (SUPPRESSOR OF UBIQUITIN UBK48R-INDUCED CELL DEATH 2) was the most promising among them. The position of SUD2 had been mapped to chromosome 3, but there were still experiments necessary for validation after next generation sequencing of the relevant region. Based on scoring developed by Sedlazeck et al. (2013), we tried to validate candidate mutations using Sanger sequencing methods for confirmation. These efforts provided us with potential candidate(s) for the SUD2 gene.

1. INTRODUCTION

To maintain homeostasis in the whole body, cellular life has to be taken under control by several processes. One of the ways for keeping homeostasis is to eliminate damaged or misfolded proteins from the cellular environment in a highly selective and precise manner (Goldberg, 2003). For this purpose, the cellular machinery has two major intracellular proteolytic pathways: the lysosomal pathway and the ubiquitin–proteasome pathway (UPP) (Mizushima et al., 2008, Ciechanover, 2007). The lysosomal pathway appears to be a major pathway for degradation of long-lived bulk proteins and organelles, whereas the UPP plays a crucial role for cytosolic protein degradation (Glickman and Ciechanover, 2002).

In recent years, there has been an increasing amount of literature on ubiquitin and its role . Although the preliminary studies on ubiquitin date back to 1970s (Goldstein et al., 1975), the field as we know it today has been co-founded by the discoveries in the 1980s (reviewed in Varshavsky, 2006, Wilkinson, 2005). Subsequently, those groundbreaking works on ubiquitin have been awarded by Nobel Prize Committee (Ciechanover, 2007).

As the stem of word suggests, ubiquitin has been found in almost all tissues (ubiquitously) of eukaryotic organisms. Among other functions such as vesicular trafficking, regulation of histone modification, DNA repair (see reviews Grillari et al., 2006, Schnell and Hicke, 2003), the major role of ubiquitin is targeting cellular proteins for degradation by the 26S proteasome (Smalle and Vierstra, 2004). Cell cycle progression, signal transduction, transcriptional regulation, receptor down-regulation, and endocytosis are just some examples of processes that ubiquitin-mediated degradation of regulatory proteins is involved in. In addition, the ubiquitin system has also critical

roles in a number of processes such as immune response, development, and programmed cell death.

1.1 Some Components of the Ubiquitin 26S Proteasome Pathway (UPP)

1.1.1 Ubiquitin

Ubiquitin is a 76-amino acid protein highly conserved across phyla that is conjugated post-translationally to a wide variety of substrates and has a molecular mass of about 8.5 kDa (Goldstein et al., 1975). However, in plants it differs by two and three residues from the yeast and animal proteins, respectively (Burke et al., 1988, Callis et al., 1995). Ubiquitin is encoded as a precursor protein by several genes and needs to be processed to release functional monoubiquitin into cell cytoplasm. In Arabidopsis thaliana, there are 14 different ubiquitin genes present (AtUBQ1-14).
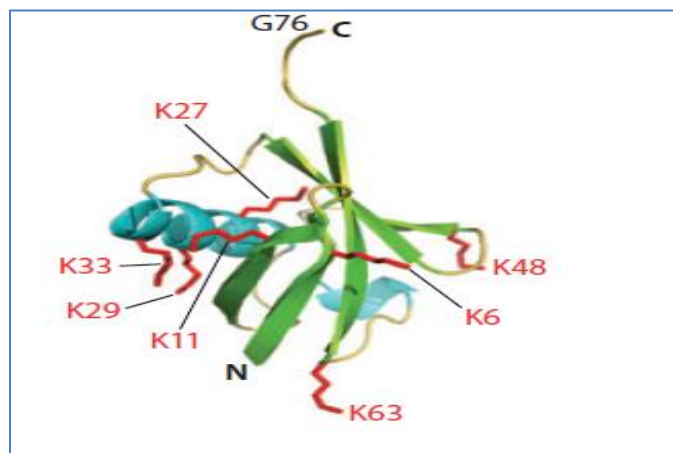
Figure 1.Three-dimensional ribbon model of plant ubiquitin. The seven lysine residues shown are possible linkage sites for ubiquitin (Figure taken from Hua and Vierstra (2011)).

Ubiquitin, as seen in Fig. 1, has seven lysine residues that may serve as sites of ubiquitination; K6, K11, K27, K29, K33, K48 and K63[1]. All of these linkages may have different meanings for cell signaling and are recognized by ubiquitin-binding proteins which have a ubiquitin binding motif among their domains (Peng et al., 2003). For instance, if a substrate is modified by a Lys48 poly-ubiquitin chain , this means the substrate is targeted for degradation by the 26S proteasome (Smalle and Vierstra, 2004), whereas mono-ubiquitin tagging appears to be involved in DNA repair (Schnell and Hicke, 2003).
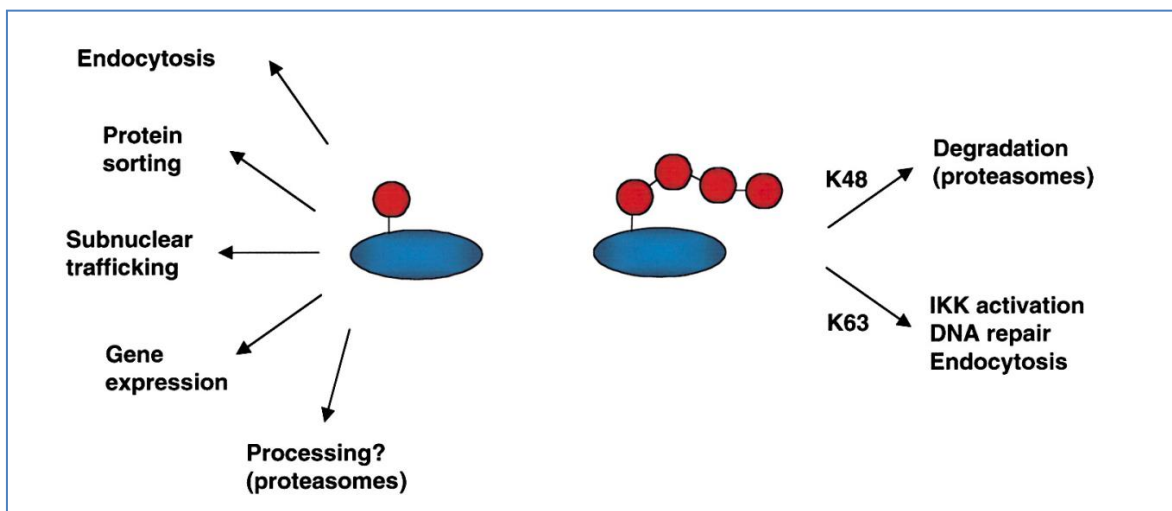
Figure 2. Differing ubiquitin modification resulting in distinct functions. Left, some of the known functions of monoubiquitination; right, some of the known functions of different polyubiquitin chains (Figure taken and slightly modified from Pickart (2001b)).

---

[1] Recent studies show that there are other types of ubiquitin chains, can also be recognized by the 26S proteasome. Ubiquitin can also be conjugated to a threonine or serine residue in the substrate by an ester-based linkage or to a cysteine residue by a thiolester-based linkage (see a review Kravtsova-Ivantsiv and Ciechanover, 2012).

1.1.2   Ubiquitination

The ubiquitination pathway consists of a three-step process requiring three different types of enzymes; they are E1 (the ubiquitin activating enzyme), E2 (the ubiquitin conjugating enzyme) and E3 (the ubiquitin ligase).
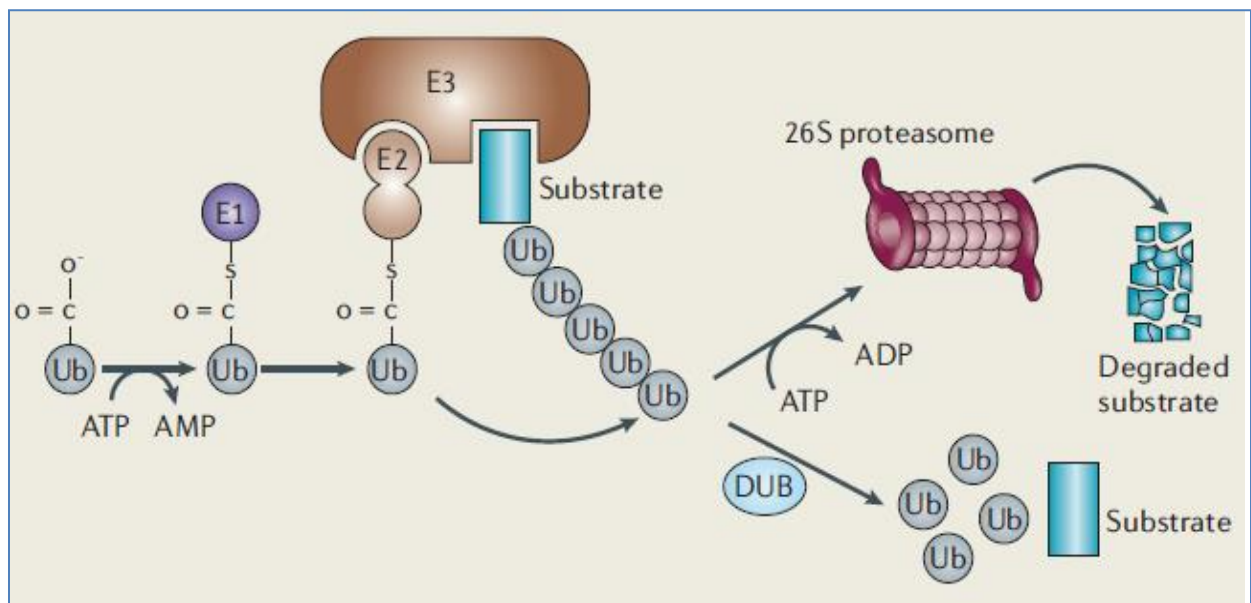
Figure 3.The Ubiquitin-Proteasome Pathway. Polyubiquitination of proteins targets them for degradation by the 26S proteasome. This process is carried out by the E1 (ubiquitin activating enzyme)-E2 (ubiquitin conjugating enzyme)-E3 (ubiquitin ligase) enzymatic cascade (Figure taken from Hoeller et al (2006)).

Ubiquitin modification is an ATP-dependent process and starts with hydrolysis of ATP. This activation by E1 results in formation of a thio-ester bond between ubiquitin and E1. Subsequently, this ubiquitin-enzyme complex is transferred to E2 through a thioester bond. At that time, E3 appears in scene and attaches ubiquitin to a lysine residue in the target protein. By doing so, E3 marks the substrates for degradation in the 26S proteasome. In the whole process, E3 plays a most important role by making degradation selective. Therefore, the number of E3s is larger than of E2s

and E1s in a cell (Pickart, 2001a, Moon et al., 2004, Sullivan et al., 2003). The ubiquitination processes by de-ubiquitinating enzymes (DUBs) have the function to release attached ubiquitin moieties for another ubiquitination cycle. Ubiquitins can be added several times to attach new ubiquitins on substrates which have already been modified by ubiquitin addition to the substrate protein (Reyes-Turcu et al., 2009). This modification allows establishing poly-ubiqutination, which is essential for recognition by the 26S proteasome.

1.1.3   The 26S Proteasome

The 26S proteasome is a 2-MDa ATP-dependent proteolytic complex that degrades Ub conjugates and is present in both cytosol and nucleus of all eukaryotes (Voges et al., 1999, Yang et al., 2004).

The 26S proteasome contains 31 principal subunits arranged into two sub complexes, the 20S core protease (CP) and the 19S regulatory particle (RP). According to Fu et. al (1999) and Groll et. al (1997), the 20S core is made up of 4 rings of 7 different β and 7 different α subunits, making the whole structure a barrel shape. The main substrate destruction site is in the β-subunits and has chymotryptic, tryptic and caspase-like proteolytic activity. The 19S regulatory part is present at both ends of the core particle, and it is divided into base and lid. Both parts play different roles in degradation, the 19S lid part recognizes poly-ubiquitylated proteins, while the base consists of 6 RP triple A (AAA$^+$) ATPases (RPTs 1-6) that are involved in unfolding of substrates and transferring them into the 20S core subunit for degradation.

1.1.4   De-ubiquitinating Enzymes (DUBs)

As proteases, deubiquitinating enzymes (DUBs) cleave ubiquitin proteins from pro-proteins or target proteins. They play essential and multiple roles in the UPP. Firstly, they are involved in the process of maturation of ubiquitin precursors; secondly, they act as a proofreading enzyme, which recycles ubiquitins from the substrates that are ubiquitinated by mistake. Thirdly, similar to phosphatases in a kinase/phosphatase regulatory pathway, DUBs simply reverse the ubiquitination of target proteins.

The recent studies show that de-ubiquitination is crucial for numerous cellular functions including cell cycle regulation, proteasome and lysosome-dependent protein degradation, gene expression, DNA repair, kinase activation, microbial pathogenesis, and more (Reyes-Turcu et al., 2009). Additionally, defects in deubiquitinating enzymes relate to a number of diseases such as cancer and neurological disorders (Fischer, 2003, Jiang and Beaudet, 2004, Shanmugham and Ovaa, 2008). However, although a few substrates have been identified until now, there is still a huge gap in knowledge about the substrates and physiological role of most DUBs. As a general classification, DUBs can be categorized into two main categories, "Cysteine proteases" and "Metalloproteases".

Similar to other eukaryotic organisms, plants also contain a number of putative DUBs (Weissman, 2001, Wing, 2003, Yan et al., 2003). The Arabidopsis genome contains nearly 30 genes that encode putative de-ubiquitinating cysteine proteases (DUBs). They are subdivided into two main categories, namely: the ubiquitin carboxyl-terminal hydrolase (UCH) class that includes two enzymes, and the ubiquitin specific processing protease (UBP) class, which has 27 members (Johnston et al., 1999).

1.2   The N-end Rule Pathway

The N-end rule pathway (NERP) is a ubiquitin-dependent selective protein degradation pathway, which relates the in vivo half-life of a protein to the identity of its N-terminal residue. In other words, the N-terminal sequence of a protein substrate impacts on its stability in the cell; this phenomenon was termed the N-end rule.

The discovery of this pathway originally came from an unexpected observation that a stable chimeric protein such as *Escherichia coli* β-galactosidase, bearing ubiquitin fused to an *Escherichia coli* Lac repressor derived N-terminal extension, can be destabilized in *Saccharomyces cerevisiae* cells when its N-terminal methionine (Met) is mutated to other residues. The result of a number of experiments indicated that protein substrates having particular amino acids such as Met, Thr, Ser, Gly, Val, Cys[2] at the N terminus appear to be relatively stable compared to other proteins which have Lys, Arg, His, Phe, Tyr, Trp, Ile, Leu, Asp, Asn, Glu and Gln at their N-terminus. There are two ways of degradation, either direct degradation or modification before degradation.

Subsequent genetic screening in *S. cerevisiae* for proteins involved in N-degron-dependent proteolysis indicated that E1 E2 and E3 enzymatic cascades are involved in degradation of such proteins.

The amino acids at the N-terminus which delay the degradation are called stabilizing residues, whereas the ones causing degradation are called destabilizing residues and can be divided into three

---

[2] However, the classification of amino acids regarding stabilization is not always as in yeast. For example, Cysteine, in contrast to yeast, is a secondary destabilizing residue in both plants and mammals (Gonda, 1989).

major classes, primary, secondary and tertiary. Only substrates with primary residues at the N-terminus are directed to degradation in 26S proteasome without any modification, to which substrates with tertiary or secondary residues have to be exposed (Fig. 4) (Turner et al., 2000, Varshavsky, 1997). Apart from other amino acids, specifically, the degradation based on the tertiary type of Cys residue involves a non-enzymatic NO and O_2-mediated modification process in animals. This modification process is already well known in mammals but more studies are needed in plants, such studies are among my project topics.

1.2.1   Role of the Arginylation in the Eukaryotic N-End Rule Pathway

The N-terminal Arg is recognized as a substrate for the UBR box of N-recognins in eukaryotes (Baker and Varshavsky, 1995, Cojocaru et al., 2011, Dantuma et al., 2000). The enzymes producing "degron Arg" are ATE1-encoded arginyl (R)-transferases, provides Arg for the N-terminal α-amino group of acceptor substrates (Fig. 4) (Carpio et al., 2010, Choi et al., 2009, Davydov and Varshavsky, 2000).

In yeast there is the only a single R-transferase; encoded by Ate1 (Chiu et al., 2007). Conversely, although the mammalian genome has one copy of ATE1 gene, it expresses several isoforms via alternative splicing. (Choi et al., 2010, Ditzel et al., 2008, Ditzel et al., 2003). In contrast to mammals, the plant Arabidopsis genome has two distinct R-transferases, called as AtATE1 and AtATE2 genes. To date, PRT1 and PRT6 are the N- recognins discovered in *Arabidopsis thaliana* (Garzon et al., 2007, Bachmair et al., 1993, Stary et al., 2003).

Although the molecular mechanisms of Cys as an arginylation substrate is not well characterized, it is suggested that N-terminal Cys is not a direct target of arginylation, rather that it is converted to an arginylation-permissive acceptor after oxidation (Hu et al., 2005). Intriguingly, the experiments carried out in mice suggest that N- terminal Cys-dependent degradation is inhibited in the absence of nitric oxide and oxygen (Dohmen et al., 1994, DeMasi et al., 2007, Dougan et al., 2010). Additionally, the recent findings about N-end rule pathway of the plant Arabidopsis (Driessen et al., 1985, Du et al., 2002) strengthens an idea that pro-N-degron Cys can play a role as an oxygen sensor.

The Arabidopsis genome encodes more than 206 proteins with the Met-Cys motif (Driessen et al., 1985, Du et al., 2002, Apel et al., 2010), which can be considered as having function related to sensing oxygen and other cell signaling pathways.
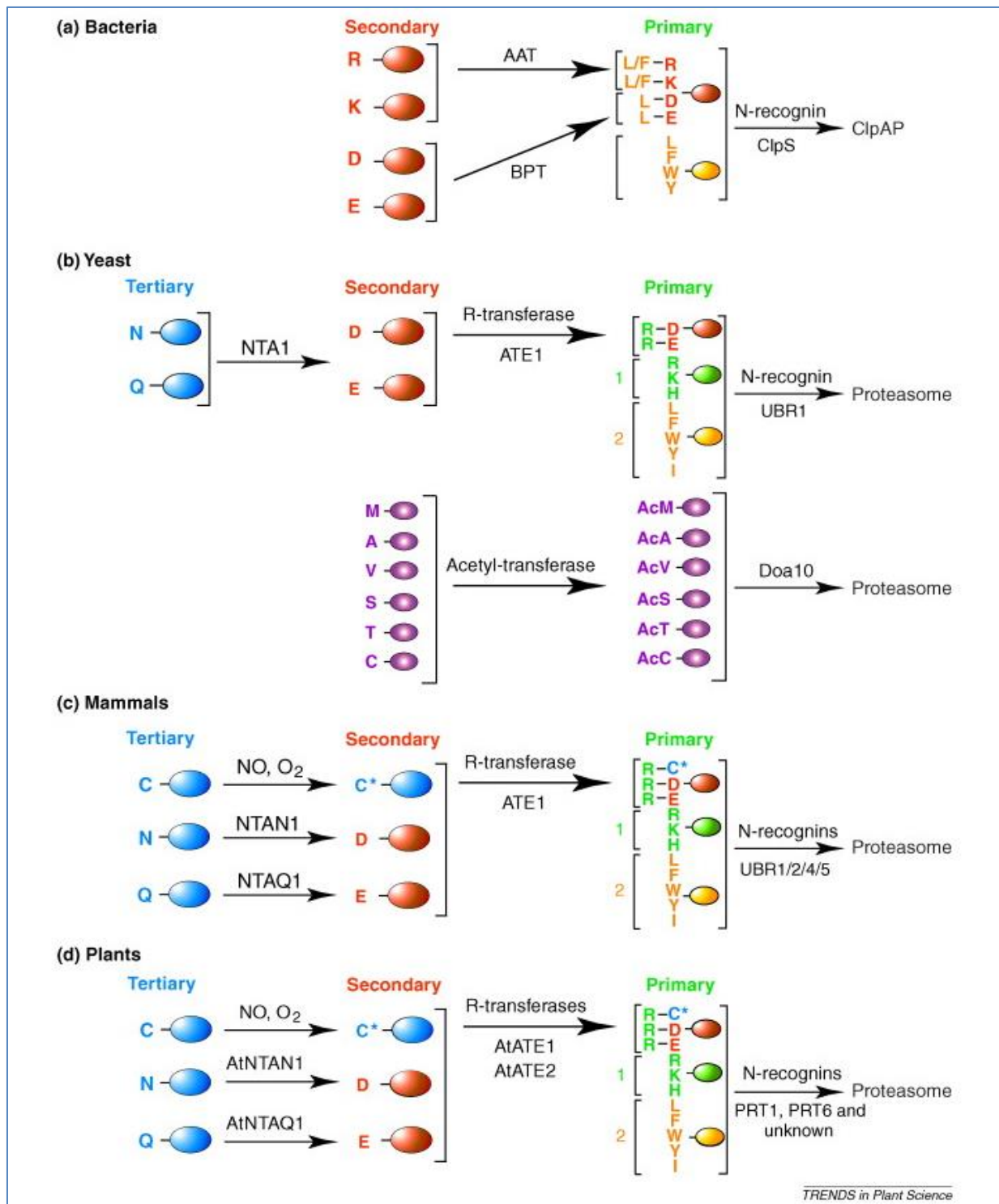
Figure 4. **The N-end rule pathway in bacteria, fungi, mammals and plants.** (Figure taken from Graciet and Wellmer, 2010)

1.3   Cell Death in Response to Malfunction in the UPP

Experiments done by Bachmair et al. (1989) and Schlogelhofer et al. (2006) indicated that a ubiquitin variant that contains Arg instead of Lys at position 48 (so called UbK48R) causes cell death, revealing a link between cell death and UPP. In order to study ubiquitin-proteasome dependent processes, a transgene inducible by dexamethasone has been introduced into Arabidopsis plants for expression of a ubiquitin variant with Arg instead of Lys at position 48 (ubK48R). However the induction system has already been speculated of having off-target effects because of the transcription factor GVG (Kang et al., 1999). This formed the basis for one of the research topics of this thesis work.

In Arabidopsis, efforts were made to find a suppressor of this phenotype and therefore a line carrying ubK48R was mutagenized by Ethyl methanesulfonate (EMS) to isolate survivors of ubK48R lethality. Out of 5 complementation groups , sud2 (suppressor of ubiquitin UbK48R-induced cell death 2) was the most promising line and therefore was used for mapping to identify responsible candidate gene(s) responsible for cell death in ubK48R background. Due to the low recombination frequency concerning region and difficulty of phenotype detection in mixed Col-Ler backgrounds, it was difficult to narrow down the position of the mutation on chromosome 3 closer than between the markers MUO22 and CIW4. Here, a next generation technology, Solexa Sequencing, was used to detect SNPs in a time-saver manner. This is the point where I took over a part of this continuing project as one of my Thesis topics.

1.4   Aim and Strategy of the Project Work

The goal of this project work is to shed light on the molecular links between ubiquitin dependent protein degradation pathways (UPP/NERP) and programmed cell death by using the model plant Arabidopsis. To this end, several independent experiments were carried out to decipher molecular mechanisms of the N-end rule pathway, a ubiquitin dependent protein degradation pathway.

The first project aimed at identifying the role of NO as a signaling compound in the N-end rule pathway.

To date, it is well known for mammals that nitric oxide (NO) is involved in degradation of proteins which have Cys at their N-terminus (Hu et al., 2005). This N-end rule pathway works by converting the tertiary Cys residue into ox-Cys and then directs its degradation. I studied whether NO has any role in degradation of proteins that have Cys in their N-terminus in the plant *Arabidopsis thaliana*. To find out, our experiments were based on photometric analysis of a genetic construct, which will be explained in detail in the "Materials and Methods" part.

The second project is aimed to find out whether the GVG activation system functions properly in ubK48R line without having side effects. Thus, to test, we used pTA-CK2, a pleiotropic Ser/Thr kinase expressed with the same induction system, and pTAGUS2 and pTAGUS13 lines expressing *E. coli* β-Glucuronidase under control of GVG, and compared these to RV86-5, which is a line expressing ubK48R. The conclusion will be significant if we can only see the effect of having Arg instead of Lys at position 48, which would allow studying the relationship between UPP and cell death. In this context, we also validated possible SNPs, as already mentioned in "1.3" section, using Sanger sequencing method.

2. MATERIALS AND METHODS

2.1   MATERIALS

2.1.1   Oligonucleotides, Markers, Enzymes, Kits

Oligonucleotides

Oligos were purchased from Microsynth AG (Balgach, Switzerland)

DNA and Protein Markers

GeneRulerTM 100 bp DNA Ladder (Thermo Fisher Scientific)

GeneRulerTM 1kb DNA Ladder (Thermo Fisher Scientific)

DNA polymerase enzymes

GoTaq (Promega)

LA TaqTM (TaKaRa)

DreamTaq (Thermo Scientific)

Phusion Hot Start High-Fidelity (Thermo Scientific)

Reverse Transcriptase Enzymes

SuperScript TM II Reverse Transcriptase (Invitrogen)

M-MLV Reverse Transcriptase (Promega)

Kits

Illustra DNA extraction kit PHYTOPURE (GE Healthcare)

RNeasy Mini Kit (Qiagen)

Wizard® SV Gel and PCR Clean-Up System (Promega)

2.1.2   Vectors

pER8

This vector is a binary vector, has a β-estradiol-inducible promoter, and provides resistance against

Spectinomycin for Agrobacteria and against Hygromycin for plants (Zuo et al., 2000).

p3

This vector is a binary vector, has a constitutive 35S promoter with three enhancer regions and has

two selection markers to provide resistance against Kanamycin for Agrobacteria and Hygromycin for

plants (Schlogelhofer et al., 2006).

2.1.3   Plants

Table 1. *Arabidopsis thaliana* mutant genotypes that were used in this thesis work.

Name of line	Transgene	Genetic Background	Reference
pER-M-GUS	Estradiol R. Promoter-Met - β gluc.	Col-0	Talloji, (2011)
pER-C-GUS	Estradiol R. Promoter-Cys - β gluc.	Col-0	Talloji, (2011)
p3-C-GUS	35S Pro-Cys- β gluc.	Col-0	Talloji, (2011)
RV86-5/sud2	poly ubK48R expression	Col-0	Schlogelhofer et al., (2006)

ck2	Insertion Ser/Thr kinase	Ler-0	Marques-Bueno et al.(2011)
pTAGUS-2	35S Pro - β gluc	Col-0	Bachmair et. al. (unpublished)
pTAGUS-13	35S Pro - β gluc	Col-0	Bachmair et. al. (unpublished)

2.1.4   Buffers and Solutions

Seed sterilization solution

5 g of Ca(ClO)$_2$ and 1 μl/ml of 20% Triton X-100 was added before use in 100 ml of dH$_2$O.

Plant DNA extraction buffer

Tris 200 mM (pH 7.5), EDTA 25 mM, NaCl 250 mM, and SDS 0.5 %.

Plant protein extraction buffer

50mM NaPO$_4$ pH=7, 10 mM 2-mercaptoethanol, 10 mM EDTA, 0.1% SDS, 0,1% Triton-X-100.

Vitamin mix (500X)

Biotin 10 mg, thiamine 1 g, myo-inositol 5 g, nicotinic acid 50 mg, dissolved in a final volume of 100

ml ddH$_2$O.

GUS buffer

50mM NaPO$_4$ pH=7, 5 mM DTT (add fresh from 1M stock), 1mM EDTA, 2mM PNPG (add fresh from

100mM stock).

GUS staining buffer

2mM X-Gluc (add fresh from 40mM stock), 50mM Na-P, pH=7, 0.5 % Triton X-100.

Fixation solution

2% paraformaldehyde in PBS, 100 mM Na-Phosphate (pH=7) and 1 mM EDTA.

Running buffer

50x TAE stock: 242g Tris base, 57.1ml glacial acetic acid, 37.2g Na_2EDTA x $2H_2O$, and diluted before use.

Carboxy-PTIO potassium salt (C-PTIO)

To have 50mM C-PTIO, 634 µl Dimethyl sulfoxide (DMSO) was added onto 10 mg of solid C-PTIO and stored at -20°C.

S-Nitroso-N-Acetyl-D,L-Penicillamine (SNAP)

To have 50mM SNAP, 908 µl was added onto 10 mg of solid SNAP and stored at -20°C.

4-Nitrophenyl β-D-glucuronide (PNPG)

31.5 milligrams of PNPG has been dissolved of DMSO to reach 50mM concentration of β-estradiol in 2 ml.

β-estradiol

2.7 mg of β-estradiol has been dissolved in DMSO to reach 5mM concentration of β-estradiol in 2ml.

Dithiothreitol (DTT)

2.31 mg of DTT has been dissolved in dH_2O to reach 1mM concentration of DTT in 15ml and then filter sterilized.

Sodium phosphate (NaPO₄) buffer

To prepare 500 mM $NaPO_4$ buffer at pH 7 in 1 L, 390 ml of 1 M NaH_2PO_4 (monobasic) and 610 ml of 1 M Na_2HPO_4 (dibasic) stock solutions have been mixed according to the table shown at the concerning protocol (2006). For each stock solutions, 138 g of Na_2HPO_4 x H_2O (monobasic; m.w. = 138 g) and 142 g of Na_2HPO_4 (dibasic; m.w. = 142 g) have been dissolved separately in 1 L of, which makes final concentration 1 mM for each solution.

5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc)

For 40 mM X- Gluc, 17 mg of solid X-Gluc has been dissolved in 1 ml DMSO.

2.1.5   Media

½ x strength Arabidopsis medium

This medium is prepared using 1/2 of the concentration of the common MS (Murashige-Skoog) salt. The common composition for 1L is: Murashige-Skoog (MS) salt: 2.15 g, Sucrose 10 g, MES 0.5g. After adding these components, pH is adjusted to 5.7 with 1M KOH, then the solution is filled with dH_2O up to 500ml. For plates, 8 g/L plant agar Merck or 4.5g/L Gelrite was added before autoclaving. After the autoclave step 2ml 500x vitamin-mixes per liter medium were added, (final concentration 1x). In our experiments, Hygromycin was added to the media at the concentration of 20 mg/l to allow the selection of transgenic plants, and 10µM β-estradiol was added to induce the transgenic system.

2.2   METHODS

2.2.1   Plant Nucleic Acid Purification and Manipulation

Plant genomic DNA extraction (Illustra PHYTOPURE automated DNA extraction)

For PCR amplification of a very long and fragile DNA fragment, high quality DNA purification was needed; therefore the Illustra DNA extraction kit PHYTOPURE (GE Healthcare) was performed to meet the high quality expectations. Fresh plant leaf material (2 to 3 week old) of 30-50 mg (less than or equal to 100mg) was used as starting material and collected into 2 ml micro-tubes which had been frozen in liquid nitrogen. Before starting the tissue lysis process, the tubes were filled with two 3 mm tungsten-carbide beads to ensure proper disruption of starting material. The racks of the TissueLyser II (Qiagen, Germany) were filled with some liquid nitrogen to prevent leaf material from possible melting activity, which might decrease the quality of extraction. Later on, the racks of the TissueLyser II were placed between the adapters and fixed firmly into the TissueLyser II clamps and then the frozen samples were homogenized about 4-5 mins at 30 Hz until the samples became free flowing powder. Subsequently, the same microfuge tube is kept for following process. Further steps were performed according to "Illustra Nucleon Phytopure Genomic DNA Extraction Kits Product Booklet Codes: RPN8510, RPN8511. For the final DNA elution in 1.5ml micro-centrifuge tubes, 50-60 μl of dH$_2$O was used and stored at -20°C.

Quick and Dirty (QND) small scale plant genomic DNA isolation by homogenizer IKA-Mixer

30–50mg of plant material (leaves, flower buds, or entire plantlets approximately 2 weeks old) was frozen in liquid nitrogen. After adding one micro spoon of quartz sand and 400 µl isolation buffer (200mM Tris-Cl pH7.5, 250mM NaCl, 0.5% SDS, 25mM EDTA), the samples were homogenized using a glass rod and an IKA-Mixer. After centrifugation (14000rpm at room temperature for 5min), the supernatant was mixed with 1 volume isopropanol at room temperature for 5min, followed by another centrifugation step (14000 rpm at room temperature for 5min). The pellet was washed with ice-cold 70% ethanol and dried. Next the DNA was dissolved in 30–50ul 1x TE (10mM Tris-Cl pH7.5, 1mM EDTA) by incubating the sample at 65°C for 5min (and gently stirring the pellet in between). 2–5µl of this DNA was used in PCR reactions.

Quick and Dirty (QND) small scale plant genomic DNA isolation by TissueLyser II Qiagen

As a starting material, fresh plant leaves (2 to 3 week old) of 30-50 mg were collected in 2 ml tubes containing two tungsten beads and then immediately frozen in liquid N2. The tubes with plant tissue were inserted in the adapter plates of the TissueLyser II and the samples were homogenized for 1 min at 30 Hz. The downstream steps were kept mostly as in the DNA isolation by IKA mixer.

Plant RNA isolation

Three to four week old seedlings grown in soil were used as starting material for isolation of total RNA. Fresh seedlings of maximum of 100 mg were used for RNA isolation by using the RNeasy Plant Mini Kit (Qiagen). The Protocol for Isolation of Total RNA from Plant Cells mentioned in RNeasy Mini

Handbook 06/2001 was used. Digestion of DNA during RNA isolation was performed by on-membrane DNase digestion with RNase free DNase as mentioned in the protocol. To elute, 40µl of RNase-free water was used and eluted RNA was used for downstream applications such as control on agarose gel and RT-PCR.

cDNA synthesis

Extracted total RNA from transgenic lines was used for synthesis of complementary DNA (cDNA). The SuperScript TM II Reverse Transcriptase (Invitrogen) or M-MLV Reverse Transcriptase (Promega) enzyme were used in this experiment.

In case of using the SuperScript TM II, for each sample, the amount of RNA was calculated according to the concentration of RNA in the product. Following components have been calculated and mixed together: 2 pmol of gene-specific primer, 10 pg–500 ng mRNA, 1 µl 10 mM dNTP Mix (10 mM each dATP, dGTP, dCTP and dTTP at neutral pH), and filled up to 13 µl distilled water. Then the mixture is kept at 65°C for 5 min. The reaction tubes were placed immediately on ice. The reverse transcription enzyme mixture of 8.6 µl (RT buffer (5 x) 4 µl, RNasin 0.6 µl, dNTP (10 mM) 2 µl, oligo dT (100 µM) 1 µl and reverse transcriptase 1 µl) was added. This final 20 µl reaction volume containing micro tubes were incubated at 55°C for 1 h and from this cDNA 1-2 µl were used as template for subsequent PCR reactions.

As for M-MLV Reverse Transcriptase (Promega), similar to SuperScript TM II, the manufacturers' protocol has been followed.

Quantification of RNA

Purified total RNA from plant lines of induced or un-induced origin was quantified using PEQ-LAB

NanoDrop 1000 Spectrophotometer machine.

Quantification of DNA

Purified DNA was quantified using PEQ-LAB NanoDrop 1000 Spectrophotometer machine.

Quantification of DNA using gel densitometry technique by Gel-Doc System

To compare the relative quantities of bands after gel visualization process done, we followed the

instructions given by user manual (BioRad Molecular Imager User Guide). We first clicked on the

Quantity Tools Relative tab, and then clicked on a band to use as a reference band from our image,

which is tubulin in our case, and quantitate all other bands based on tubulin density. The relative

quantity is measured by dividing the band volume by the reference band volume.

We also used absolute quantification method based on known standards band by using a calibration

curve. To determine the absolute quantities of bands, from the Quantity Tools Absolute tab, we

clicked on Select button to choose at least two known standard (which are Gene Ruler DNA ladder

bands in our case) and then entered quantity values. By doing so, the molecular weight values for

the remaining bands had been automatically calculated and displayed. After all, we also had chance

to choose our unit of measure from the list in the Units menu.

Agarose gel electrophoresis

The samples are usually loaded on a gel submersed in a saline "running buffer" that allows the

separation of the fragments when currency is applied. The running buffer used in all experiments

was 1x TAE. On the one hand, low concentration gels such as 0.8% and high voltage (i.e. 120 V) were used for a better resolution of large DNA fragments, on the other hand, for the case of high concentrated gels (e.g 1.5 and 2.0) and low voltage (40 V-80V) were used to separate fragments less than 500 bp. The required amount of agarose was molten in 1x TAE, by boiling the mixture in a microwave oven. After cooling down, ethidium bromide to a final concentration of 5mg/l was added to visualize the nucleic acids under UV-light.The samples were mixed with 1/6 vol. of 6x loading buffer (50% glycerol, 0.2M EDTA pH 8.0, 0.005% bromophenol Blue or Orange G) or 10x loading buffer.

Purification of DNA fragments from agarose gel or PCR reaction mix

DNA fragments with a certain length were isolated and purified for further applications such as sequencing or genotyping. The DNA fragments of choice were isolated from an agarose gel or purified from a PCR reaction using the Wizard SV Gel and PCR Clean-Up System (Promega, Cat.No. A9282) according to the manufacturer's protocol.

2.2.2   Protein Quantification

Quantification of Proteins with Bradford Assay

The Quick Start Bradford protein assay (BioRad Bradford Assay) has been taken out from 4°C storage and then we prepare a Bradford assay diluting one volume Dye Reagent with four volumes dH_2O. Afterwards, the mix was filtered through Whatman filter to remove particulates. On the one hand, for standards to calculate protein amount, we prepared different dilutions of a protein standard in a 1ml solution (0.2 to 1 mg/ml of BSA) and then pipetted 50 µl of dilutions of a protein standard and

950 µl of diluted dye reagent to each cuvette and did vortex gently. On the other hand, for each sample, 5 µl sample solution has been added onto 45 µl of water, which is in a clean, dry disposable cuvette. Later on, 950 µl of diluted dye reagent was added to each cuvette. Alternatively, to mix well, in some cases all components were poured into 1.5 ml micro centrifuge tubes together. After homogeneity was reached, the solution was incubated for at least 5 min but not longer than 1h at room temperature. To measure, the spectrophotometer machine has been set to 595 nm. Before starting to measure samples, the machine has to be zeroed. However, since the spectrophotometer machine has both reference and sample holder, it was zeroed with two blank samples.

2.2.3 Other Plant Related Methods

Spectrophotometric GUS measurement

30-50 mg of fresh plant material was placed in a 1.5 ml reaction tube as a starting material for protein extraction. From now on, samples were kept on ice during the experiment. The plant material was mixed with one micro spoon of fine quality quartz (sea sand), 200µl of DNA isolation buffer and thoroughly homogenized with an IKA-Mixer/glass pestle. The homogenized solution was centrifuged for 15min at 14000 rpm at 4°C. The supernatant was transferred into a fresh sterile 1.5ml reaction tube. Of the samples, 40µl has been added into 500µl of GUS assay buffer, which had been prepared before, and then the mixed samples were incubated at 37°C. At specific time points (e.g., 00:00h, 00:15h, 2:30h, 5:00h), 100µl of sample-GUS assay buffer mix was transferred to 800µl of 0.4M Na_2CO_3 which is used to stop the activity of β-Glucuronidase (Gallagher, 1992). This stop solution-GUS assay mix was used to screen visual change during time courses that is done by spectrophotometer at 405nm. The GUS assay calculations were done based on the results. If not otherwise stated, all the steps were performed on ice.

Histochemical GUS staining

Plant materials to be examined for histochemical GUS staining were germinated on selection MS solid medium with β-estradiol added for inducible promoters or without β-estradiol for constitutive promoters. Seedlings were grown on solid medium for less than 1 week and then transferred into fresh fixation solution and incubated for 30 min on ice. Later on, plants are washed once in 100mM Na-P (pH=7) solution. Next, the plants were submerged in 2mM X-Gluc, 50mM Na-P (pH=7) and 0.5 % Triton X-100. The medium was vacuum infiltrated for 10 sec by placement into a desiccator and application of a vacuum and then put overnight incubation at 37 °C. After incubation, the material was washed once in dH$_2$O and chlorophyll was extracted by washing in 100% and then 70% EtOH.

Nitric oxide (NO) treatment

For treatment of Arabidopsis seedlings with NO donor S-Nitroso-N-Acetyl-D,L-Penicillamine (SNAP), seedlings were grown for up to at least 2 weeks in liquid MS medium with β-estradiol for inducible promoters or without β-estradiol for constitutive promoters. These seedlings were transferred into fresh medium supplemented with β-estradiol and were treated with 1mM SNAP concentrations. After 5h, seedlings were taken up for further analysis.

Plant anoxic treatment

To expose Arabidopsis seedlings to anoxic conditions, seedlings were grown up to at least 2 weeks in liquid MS medium with β-estradiol for inducible promoters or without β-estradiol for constitutive promoters. On the day of treatment, fresh MS medium replaced the old one. For the degassing process, a noble gas was used; argon which is heavy enough to replace oxygen in the medium. The argon treatment was carried out twice and each process took around 2-3 min with a medium

pressure unit. The first treatment was done after preparation of the fresh medium, the second one was done after the two weeks old seedlings were transferred into 1.5 ml reaction tubes and submerged totally by filling tubes with the degassed medium. These micro-centrifuge tubes were kept in darkness for 5 h.

Seed sterilization

Seeds of each plant line were subjected to seed sterilization mix (see methods section) and kept on a shaker for 10 min at room temperature. They were centrifuged briefly and the supernatant was discarded. Seeds were washed 3 times with 350 µl sterile dH$_2$O or EtOH. In case of using EtOH, these seeds were left under the laminar hood to be dried. In case of using water; 600 µl water was added into each eppendorf tube and the seeds were resuspended carefully before spreading onto plates. For the first at least two days, the plates were kept at 4°C and then transferred to a 23°C light incubation room.

2.2.4   Statistical Analysis and Calculations

Mean

The mean is the arithmetic average of a set of values, or distribution, which is equal to the sum of the values divided by the number of values. To find a representative average values for each treatment and line we calculated arithmetic mean for the quantitative GUS analyses.

Standard deviation of the mean

Standard deviation (denoted by the symbol sigma: σ) is a method used in statistics which indicates how much variation or dispersion exists from the average, or expected value (Gauss, 1816). This method is formulated as shown below:

$$\sigma(r) = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(x_i - r)^2}.$$

Where $r = \overline{x}.$, which means arithmetic average of the samples, and N= sample size, meaning that number of samples, X_i = each elements of the sample collection.

We used this method to define how much our quantitative assay varies from the average and used for finding standard error of the mean.

Standard error of the mean

The standard error of the mean (SEM) is the standard deviation of the mean's estimate of a population mean (Everitt, 2006). It is formulated as shown below:

$$SD_{\bar{x}} = \frac{\sigma}{\sqrt{n}}$$

Where n= sample size, and σ = standard deviation of the mean.

We used standard error of the mean to draw a "confidence interval", which is an indication of the reliability of our average estimates for GUS assay values, on our charts.

Student's t-test

Student's t-test is a statistical test used to determine if two sets of data are significantly different from each other (Fadem, 2009). However, it is commonly used when the variances of two normal distributions are unknown and when an experiment uses a small sample size such as two different groups. In case of number of variables more than two, analysis of variance (ANOVA) is applied.

Although there are various statistical t-test methods used, they all have the same principle that is described in the following. It is based on a null hypothesis, which states that there is no effective difference between the observed sample mean and the hypothesized or stated population mean, meaning that observed difference is just because of chance. After calculating t value as shown below, one rejects the null hypothesis if observed t-statistic is more than the critical value determined by the appropriate reference distribution.

The appropriate reference distribution for the t-statistic is the t distribution. The critical value is based on the significance level of the t- test (usually the 0.10, the 0.05, or 0.01 level).

$$t = \frac{\overline{x} - \mu_0}{s/\sqrt{n}}$$

where $\overline{x}$ = sample mean, s = sample standard deviation of the sample and n = sample size. The degrees of freedom used in this test is n − 1.

We used t-test to say if groups of quantitative GUS assays are different or not. To give detail, we also conferred statistical significance of the difference with the graph (Fig. 6).

Maximum normed residual test (Grubbs' test)

The maximum normed residual test, also known as Grubbs' test, is a statistical test used to detect outliers from a normally distributed population (Grubbs, 1969, Stefansky, 1972).

Grubbs' test suggests two hypotheses:

H_0: There are no outliers in the data set

H_a: There is at least one outlier in the data set

The Grubbs' test statistic is defined as:

$$G = \frac{\max\limits_{i=1,\ldots,N} \left| Y_i - \bar{Y} \right|}{s}$$

with $\bar{Y}$ and s denoting the sample mean and standard deviation, respectively. For the two-sided test, the hypothesis of no outliers is rejected at significance level α if

$$G > \frac{N-1}{\sqrt{N}} \sqrt{\frac{t_{\alpha/(2N),N-2}^2}{N-2+t_{\alpha/(2N),N-2}^2}}$$

where $t_{\alpha/(2N),N-2}$ means the upper critical value of the t-distribution with N – 2 degrees of freedom and a significance level of α/(2N). One should replace α/(2N) with α/N for the one-sided tests.

In our experiments, we used this test to eliminate some data points, which are far beyond from the other members of sample population in which it occurs that are present in quantitative GUS assay. By doing so, we minimized false interpretations due to incidental systematic error.

Coefficient of determination

The coefficient of determination, represented by R^2, is a test used to determine how well observed outcomes are replicated by the model (Steel and Torrie, 1960). The most general definition as follows:

$$R^2 \equiv 1 - \frac{SS_{\text{err}}}{SS_{\text{tot}}}.$$

We used this test to check the reliability of a drawn standard curve for the Bradford standard samples.

Rate of reaction

The rate of reaction R (in nanomoles product per minute per OD_{600} unit) for each sample as follows:

$$R = \frac{S}{0.02 \times V \times OD_{600}}$$

Where S= Slope is calculated by a graph of OD_{405} (y-axis) against time (x-axis) per minute. V = the volume assayed in ml. The 0.02 in the denominator has been derived from the molar extinction coefficient of p-nitrophenol (Gallagher, 1992).

We used this formula to find relative enzyme activity of quantitative GUS assay measurement for each line and treatment.

2.2.5   Genotyping

Plant genotyping

Genomic DNA of at least two weeks old mutant and wild type plant lines was purified according to the protocols mentioned in the methods for DNA isolation. To find proper primers, several primers have been tested for suitability. Oligonucleotides used for PCR-based amplification of the genomic region of interest are shown in Appendix 1. The positive PCR samples have been sent for sequencing for validation, which is explained in Results.

2.2.6   Standard Enzymatic Reactions

Polymerase Chain Reaction

Go-Taq

Table 2. **Go-Taq PCR components.** This table shows the volumes designated from stock solutions to carry out polymerase chain reaction by GoTaq.

Components	[Stock] µM	[Work] µM	Vol, µl
H₂O			34,25
Buffer	5x	1x	10
dNTPS	2500	200	4

Primer 1	100	0,5	**0,25**
Primer 2	100	0,5	**0,25**
Taq	5U/µl	1.25U	**0,25**
Template			**1**
Total			**50**

TaKaRa La taq

Table 3. TaKaRa La taq PCR components. This table shows the volumes designated from stock solutions to carry out polymerase chain reaction by TaKaRa La taq.

Components	[Stock] µM	[Work] µM	Vol, µl
H_2O			**35,3**
Buffer	10x	X	**5**
dNTPS	2500	400	**8**
Primer1	100	0,2	**0,1**
Primer2	100	0,2	**0,1**
Taq	5U/µl	2.5U	**0,5**
Template			**1**
Total			**50**

Dream Taq

Table 4. Dream taq PCR components. This table shows the volumes designated from stock solutions to carry out polymerase chain reaction by Dream Taq.

Components	[Stock] µM	[Work] µM	Vol, µl
H_2O			**37,55**
Buffer	10x	1x	**5**
dNTPS	2000	200	**5**

Primer 1	100	0,2	0,1
Primer 2	100	0,2	0,1
Taq	5U /µl	6,25	1,25
Template			1
Total			50

Hot Start

Table 5. Hot Start PCR components. This table shows the volumes designated from stock solutions to carry out polymerase chain reaction by Hot Start.

Components	[Stock] µM	[Work] µM	Vol, µl
H$_2$O			37,3
Buffer	5x	1x	10
dNTPS	10000	200	1
Primer 1	100	0,2	0,1
Primer 2	100	0,2	0,1
Taq	5U /µl	2.5U	0,5
Template			1
Total			50

Amplification programs of PCR

Table 6. This table documents the programs used in PCR to amplify fragments. *:represent "35 times repeated" for both konczdip and ml-mmz and hot-start program but "30 times repeated" for dreamtaq and "27 times repeated" for rt-pcr program.

PCR Programs	First Dena.	Dena.*	Annealing*	Extension*	Final Extension
koncz-dip	3' at 95°C	30'' at 95°C	30'' at 48°C	6' at 68°C	10'' at 68°C
ml-mmz	4' at 94°C	30'' at 94°C	20'' at 48°C	3' at 72°C	6' at 72°C

dream taq	3' at 95°C	30'' at 95°C	30'' at 55°C	8' at 72°C	10' at 72°C
hot start	30'' at 98°C	10'' at 98°C	30'' at 55°C	12' at 72°C	7' at 72°C
rt- pcr	5' at 94°C	30'' at 94°C	30'' at 55°C	1.5' at 72°C	10' at 72°C

3. RESULTS

3.1   Reporter Lines Expressing Test Substrates

In order to study non-enzymatic conversion of Cys into Cys-sulphinic acid ($CysO_2H$) or Cys-sulphonic

acid ($CysO_3H$) in N-end rule pathway by Nitric Oxide (NO), we used inducible reporter lines carrying

ubiquitin fusion protein constructs which were generated by Talloji (2011). This genetic construct

has an open reading frame (ORF) consisting of DHFR-HA-UB-X-lac-3HA-GUS parts that were inserted

into a plant binary vector pER8 (constructs called pER-X-GUS) (Fig. 5B). As seen in Fig. 5B, "X" letter

represents single amino acid, in our case only Cys (C), or Met (M). The expression of these

constructs was kept under a β-estradiol inducible promoter (pER-X-GUS) or a constituve promoter

(p3-X-GUS). After successful transformation and induction (depending on promoter) in *A. thaliana*,

the protein product of the transgene is cleaved into two proteins, dihydrofolate reductase (DHFR)-

ubiquitin and β-glucuronidase (GUS) by de-ubiquitinating enzymes (DUBs) (Fig. 5A). This cleavage

exposes the amino acid X at the N-terminus and allows us to observe the stability of protein in

different experimental approaches. That is to say, if the tested GUS protein is present in the tested

plant line background depending on N-terminal residue, that plant line gives positive result in a GUS

assay and if not, the GUS assay is negative. These GUS assay results can be assessed by both

spectrophotometric analyses and Western blotting technique.
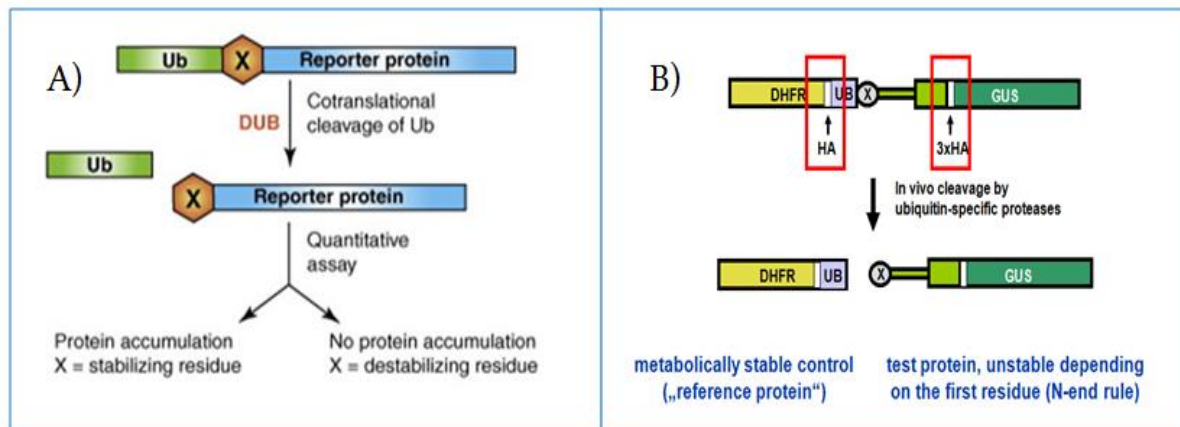
Figure 5.**Reporter lines for expressing test substrates.** **A)** This figure shows the underlying principle how artificial N-end rule substrates are generated through the Ub fusion technique. After cleavage by deubiquitinating enzymes (DUBs) and the release of an X-reporter protein bearing a specific residue X at its N-terminus, measurement of reporter protein is possible in a quantitative manner (Figure taken from Graciet and Wellmer, 2010). **B)** The DHFR-Ub contains a single HA tag and is a stable protein, serves as reference protein. In contrast, GUS contains a 3xHA tag and specific N-terminal residues, is either stable or unstable depending on the type of N-terminal residue, and serves as test protein. The flexible spacer between N-terminal residue and GUS protein helps GUS protein to expose residue X in the folded GUS protein (Figure taken from Talloji, 2011).

3.2   NO-mediated Modification in the N-end Rule Pathway of Arabidopsis

As mentioned in the "Introduction" section, the N-end rule pathway of mammals is known to respond to a non-enzymatic process. This modification results in generation of substrates to be degraded in a proteasome-dependent manner. This non-enzymatic modification involves NO and O_2 in mammals and converts Cys into Cys-sulphinic acid ($CysO_2H$) or Cys-sulphonic acid ($CysO_3H$) which then leads to arginylation, which is already mentioned in "Introduction". However, this modification is not well characterized in Arabidopsis. Since this reaction is a non-enzymatic process, one might expect this process in plants as well. Therefore, to learn more about its presence, we used both a β-

estradiol inducible and a constitutive Cys-GUS line under different NO concentrations. Moreover, as

a control we used Met-GUS which has nothing to do with N-end rule pathway under NO-treatment.

Table 7. Quantitative GUS assay values with standard error of the mean for each treatment.

Column1	M-GUS	C-GUS	C-GUS dark	C-GUS anoxic
Control	1 ± 0.45	1 ± 0.23	1 ± 0.14	1 ± 0.22
SNAP	1.03 ± 0.31	1.08 ± 0.08	0.81 ± 0.21	1.07±0.3
C-PTIO	1.06 ± 0.35	1.8 ± 0.2	1.51 ± 0.09	1.07 ± 0.25

We performed two types of GUS assay to prove our hypothesis, one is quantitative GUS assay,

where we looked for enzyme activity by disrupting tissue into a homogenate, the other one is

histochemical GUS staining, where GUS reporter activity is visualized in the whole plant by

histochemical staining.

As for quantitative GUS assay, once we obtained the raw data from each treatment and line, we

used the Grubbs' outlier test to minimize incidental systematic error. Depending on Grubbs' test

report, we eliminated the values out of experimental analyses if any. Then GUS relative enzyme

activity formula was applied out to see real relative values regarding each lines and treatment.

Based on our methods and experimental results, Cys-GUS shows reasonably different behavior

under light conditions in comparison to Met-GUS (Fig. 6 and Table 7). The statistical difference

between untreated plants and C-PTIO treated plants was significant: ($P<0.01$). However, C-GUS

reporter amounts under dark condition appear to be closer to M-GUS reporter amount, although

there is a statistically significant difference between untreated control and C-PTIO treated plants

($P<0.05$). Implementation of both dark and anoxic conditions makes C-GUS reporter amount almost

similar with M-GUS under light condition.
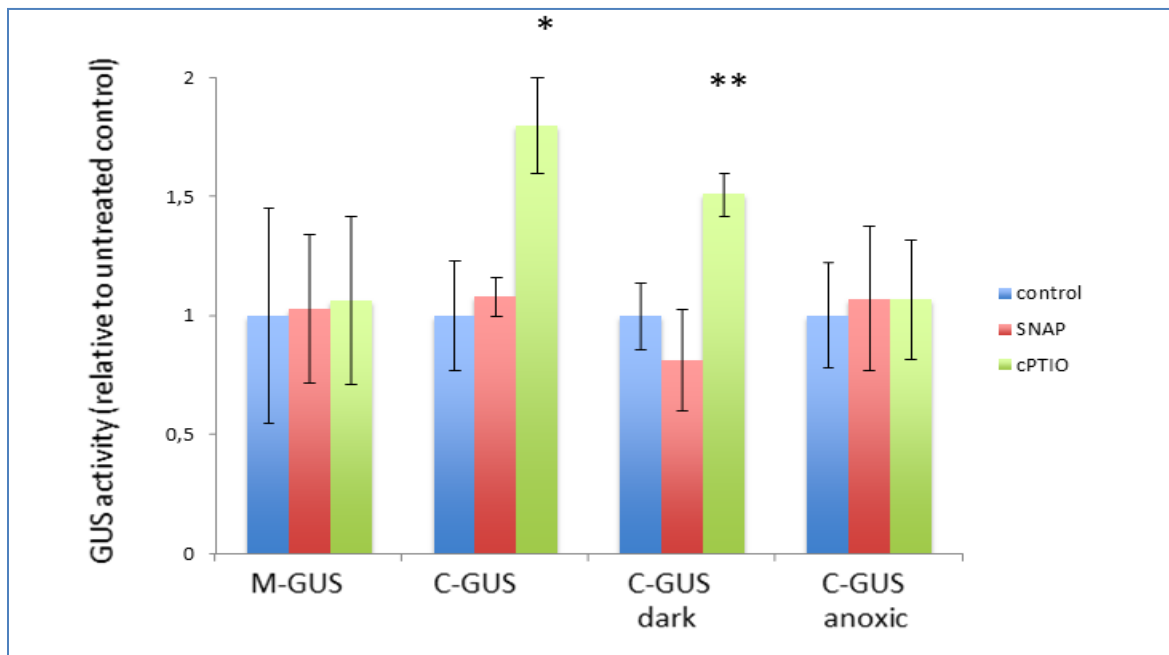
Figure 6. Quantitative analysis of M-GUS and C-GUS reporter under different conditions. The graph basically shows relative GUS activity values to untreated control of reporters in different conditions. M-GUS: under light for 5 hours; C-GUS: under light for 5 hours; C-GUS dark: in darkness for 5 hours; C-GUS anoxic: darkness, and medium has been degassed by Argon gas. Control: untreated, SNAP: 1mM; C-PTIO: 1mM. *: differs significantly from control (P<0.01), **: differs significantly from control (P<0.05).

We also performed histochemical GUS staining for the plants both untreated and treated with 1mM C-PTIO to compare staining density between M-GUS and C-GUS lines (Fig. 7). The staining photographs appear to be consistent with the spectrophotometric data analysis; M-GUS quantity is similar in both cases, whereas 1mM C-PTIO treated C-GUS line stabilized much higher reporter than untreated C-GUS line.
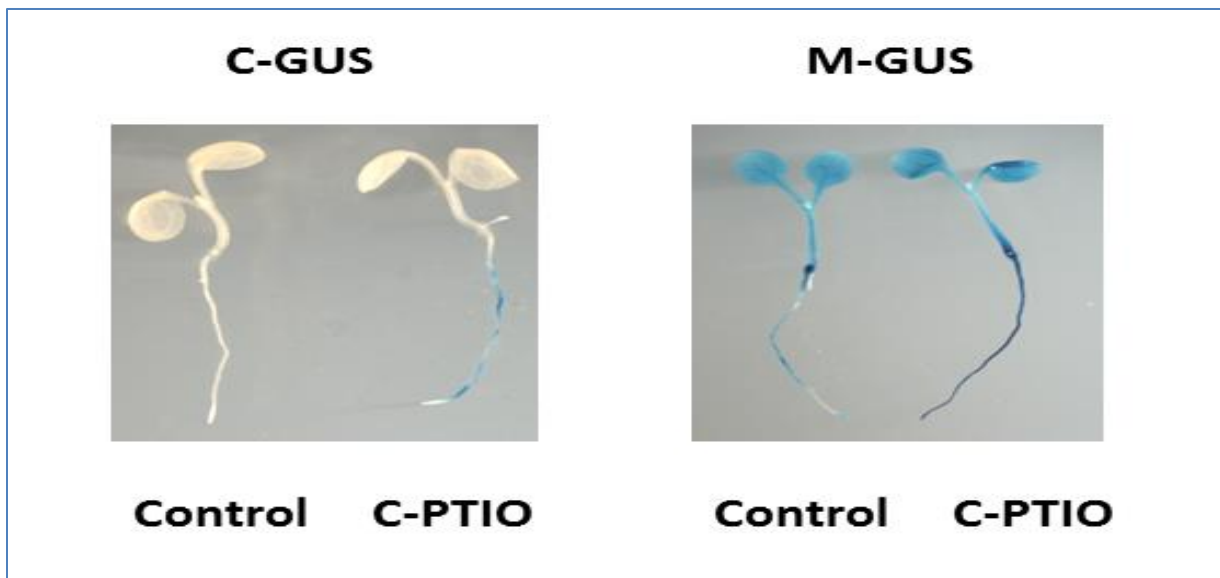
Figure 7. Histochemical GUS staining of C-GUS and M-GUS seedlings incubated with or without C-PTIO. Although M-GUS plants show staining for both shoots and roots, there is little difference between different conditions. C-GUS plants show only root staining, only in plants treated with C-PTIO. Control: untreated seedlings; C-PTIO: 1mM

3.3   Validation of a Candidate *sud2* SNP

According to the benefit of doubt (BOD) scoring invented by Sedlazeck et. al (2013), an SNP caller aligns sequences of mutated genome corresponding sequence of the WT, and then the output in terms of local read coverage and its difference converted into a score. By doing so, six-representative SNP candidates have been chosen for further validation. To experimentally validate, we used Sanger sequencing method on PCR fragments amplified from those candidates, with the predicted mutant spots lying on the fragment amplified. As seen in the Table 9, we found a small insertion/deletion compared to the reference sequence. Yet, this difference appears to be present

in the non-mutagenized progenitor line, which means that this sequence change cannot be responsible of any phenotypic differences between progenitor and mutant plant lines.

Table 8. Sequence verification of selected candidate single nucleotide polymorphism regions. Selected genomic region was amplified by PCR from mutated and progenitor line and subjected to conventional sequencing, with the result as listed.

BOD Score	Coordinate on chromosome 3	Sequence context	Annotation
0.628107	16 374 619	CCGAAG TGACAAC CCGAAGGTGACAAC	Before the stop codon of ORF At3g44850
0.617491	16 362 587	CTACTA A TCGCCA CTACTAGCTTCGCCA	Within ORF At3g44820

3.4   Comparative Levels of Transcription Factor GVG

As mentioned in the "Introduction" section, RV86-5 plant line is a dexamethasone-inducible line expressing ubiquitin variant ubK48R. However, the experiments carried out by Kang et. al. (1999) indicated that the induction system via vector pTA7002 appears to have side effects, namely induction of misguided target genes, depending on the level of transcription factor GVG. In case of overexpression of GVG protein, plants show developmental defects and expression of defense genes. Therefore, we compared the RV86-5 line with two other lines that express different proteins from the same inducible promoter. The first one expresses an activated Ser/Thr kinase, CK2. The reports show that transcriptional changes in the published plant line after induction does not share high similarity with induced line RV86-5 (Marques-Bueno et al., 2011). The second one, pTAGUS13, expresses *E. coli* β-Glucuronidase under control of the GVG induction system and was also tested to detect side effects of induction.

As seen in Fig 8, an RT-PCR experiment was done to compare semi-quantitatively the levels of transcription factor GVG in these three lines. We showed that the expression of GVG is slightly higher in the cK2 line than in the RV86-5 line. However, both the GVG levels of pTA-GUS2 and pTA-GUS13 appear to be lower than in the RV86-5 line.
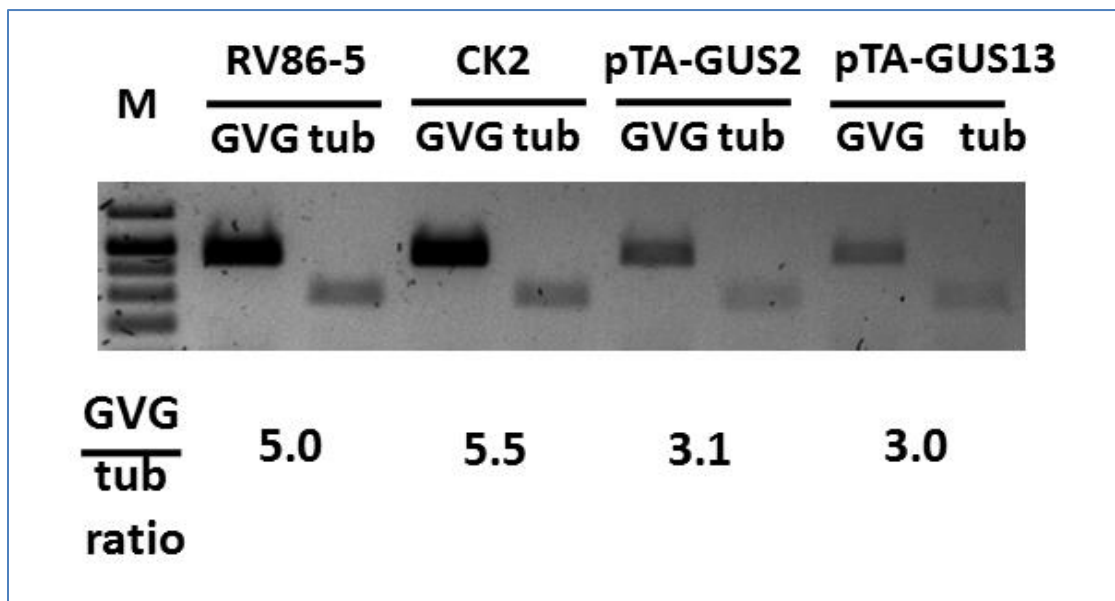
Figure 8. RT-PCR semi-quantitative measurement of the level of Dexamethasone-activated transcription factor GVG. RV86-5 was compared to line cK2, and to pTA-GUS lines. Beta tubulin TUB2 served as a standard. Transcript levels were calculated from gel images.

We checked for any developmental side effect as mentioned in Kang et al. (1999) of tested plants. Fig. 9 illustrates the developmental behavior of some tested plants. Although pTA-GUS transgenic plants appear to be green and continue to grow, RV86-5 shows arrested growth.

As a conclusion, we suggest that developmental arrest and death upon Dex-application to line RV86-5

are due to the induced ubiquitin variant, and not to excess amount GVG expression as reported before.

Figure 9. Comparing of the growth of RV86-5, pTA-GUS2 and pTA-GUS13. Seeds of lines RV86-5, pTA-GUS2 and pTA-

GIS13 were germinated in absence of inducer Dexamethasone, and transferred to Dex-containing medium after germination.

Whereas RV86-5 plants uniformly arrested growth, pTA-GUS transgenic plants stayed green and continued to grow.

4. DISCUSSION

4.1 Role of the N-end Rule Pathway in Nitric oxide (NO) Response of Plants

Degradation processes are essential processes to maintain homeostasis in living organisms. As mentioned in the "Introduction", in the N-end rule pathway, degradation of proteins is based on the identity of N-terminus that provides information to cell about protein half-life. In other words, the first amino acid at the N-terminus has a crucial effect on the stability of a protein, which is called N-end rule degradation pathway.

Apart from different and specific modifications for those single amino acids, S-Nitrosylation of Cys converts Cys into Cys-sulphinic acid ($CysO_2H$) or Cys-sulphonic acid ($CysO_3H$), which then becomes a substrate for arginylation (Boehning and Snyder, 2003, Hess et al., 2005, Hu et al., 2005). It is confirmed in mammalians that nitric oxide (NO) plays crucial role in this transformation. However, in plants, this pathway needs detailed investigation to be proven. In this work, this was tried by using a reporter line expressing a test substrate with an N-terminal Cys.

As experimental design, we performed four different settings to find effects of NO in the cell. At the beginning, to really ensure that this effect is caused by Cys-specific modification, we used a control line, which has the same genetic construct in the genome except containing a Met instead of Cys, so called M-GUS. Since the stability of Met-substrate was not affected by NO treatment, this line is a control for any changes due to Cys modification. Therefore, our first setting was incubating M-GUS plants under light and treating them with SNAP and C-PTIO afterwards using the same treatment for C-GUS in a second setting. Then, we carried out the same treatments for only the C-GUS line except

that no light was present during incubation. In addition, as a final setting we applied both darkness and anoxic condition for plants to see the difference.

Although it is not direct, our quantitative GUS assay results confirmed expectations about Cys amino acid. Regarding SNAP, when it was added to medium, the quantity of our reporter protein appeared to be in the range of untreated groups' mean value in all cases. Possible explanations for this result may be that the NO concentration might be not enough to trigger a degradation process in the whole tissue, or cells might be not permeable enough due to the chemical structure of SNAP. Another reasonable explanation could be that the plant cells might be already saturated for NO, meaning that C-GUS degradation was already at the maximum level; hence any external injection of NO would be denied or not have any effect.

Regarding C-PTIO as NO scavenger, the difference is between untreated and C-PTIO treated plants are highly significant (P<0.01) even in the dark condition, there is also a significant increase for those groups (P<0.05), yet it is not as high as in the light. However, blocking light and oxygen for C-GUS plants makes them behave like M-GUS. The reason why we used dark conditions for plants was to simply block photosynthesis as an oxygen source, which allows us to find out how important oxygen is in the process. According to our results, in addition to the confirmation that NO is important for the degradation of proteins with Cystein at the amino terminus, we concluded that oxygen is also necessary for the degradation process.

As a supportive validation, histochemical staining proves that a difference is present between C-GUS plants treated with C-PTIO or not treated, but the staining is not as high as for M-GUS. We could not manage to stain leaves of C-GUS lines, whereas M-GUS leaves appeared to be stained. The response appears to be different to C-PTIO chemical, although we could not say anything reasonable for those

results. We concluded that quantitative enzymatic analysis is much more reliable than any histochemical analysis.

4.2   *sud2* Genotyping

In this work, as mentioned before, the *sud2* (suppressor of ubiquitin UbK48R-induced cell death) mutant was the most promising line surviving the lethal effect of UbK48R; a ubiquitin variant with Arg instead of Lys at position 48. Therefore, the *sud2* line was used for mapping to identify the responsible candidate gene necessary for cell death in the ubK48R background.

Using different mathematical approaches, Sedlazeck et. al (2013) developed a simple method to evaluate predicted deviations from the reference sequence (SNPs and small indels) by using model organism Arabidopsis thaliana. Since the already established SNP callers were not well suited for our data, a method has been developed asking whether the position contains the base of the reference sequence. Since this caller questions each reference nucleotide at the given position, it is called benefit-of-doubt (BOD) scoring. Depending on BOD scoring, some regions have to be confirmed by conventional sequencing. Although sequencing of our region interest does not show any indels that might cause phenotypic differences, the BOD allows the identification of high confidence SNP loci through complete reference genome as Arabidopsis accession Col-0.

4.3 Comparative Levels of Transcription Factor GVG

In this work, we tried to find out functionality of GVG activation system in Arabidopsis thaliana. Expression of a ubiquitin variant, ubK48R, might influence a large number of regulatory processes carried out in cell. To experimentally test, an inducible system based on transcription factor GVG was used in plant cells. However, a report presented by Kang et. al (1999) suggesting that overexpression of GVG protein in plants leads to aberrant development and induction of defense genes. Thus, to test this hypothesis, we used pTA-CK2, a pleiotropic Ser/Thr kinase expressed with the same induction system, pTAGUS2 and pTAGUS13 lines expressing *E. coli* β-Glucuronidase under control of GVG, and compared these to RV86-5, which is a line expressing ubK48R.

As shown in the results section, and by comparison to published work (Marques-Bueno et al., 2011), CK2 and RV86-5 appears to have a different spectrum of transcriptional changes upon dexamethasone induction, which means that differences are caused by the induced transgene, and not by off-target effects due to GVG. Similarly, although pTA-GUS13 shows similar levels of GVG with RV86-5, plant growth seems to be uninhibited after induction (Fig. 9). Hence, we concluded that overexpression of GVG is not responsible for the growth inhibition and death upon Dex-application to line RV86-5, rather this is the consequence of the induced ubiquitin variant expression.

5. LIST OF FIGURES

6. LIST OF TABLES

7. REFERENCES

2006. Sodium phosphate. *Cold Spring Harbor Protocols,* 2006**,** pdb.rec8303.

BioRad Molecular Imager User Guide. Molecular Imager.® Gel Doc™ XR+ and ChemiDoc™ XRS+ Systems with Image Lab™ 2.0 Software. 64-66.

APEL, W., SCHULZE, W. X. & BOCK, R. 2010. Identification of protein stability determinants in chloroplasts. *Plant Journal,* 63**,** 636-650.

BACHMAIR, A., BECKER, F. & SCHELL, J. 1993. Use of a reporter transgene to generate arabidopsis mutants in ubiquitin-dependent protein degradation. *Proc Natl Acad Sci U S A,* 90**,** 418-21.

BACHMAIR, A. & VARSHAVSKY, A. 1989. The degradation signal in a short-lived protein. *Cell,* 56**,** 1019-32.

BAKER, R. T. & VARSHAVSKY, A. 1995. YEAST N-TERMINAL AMIDASE - A NEW ENZYME AND COMPONENT OF THE N-END RULE PATHWAY. *Journal of Biological Chemistry,* 270**,** 12065-12074.

BOEHNING, D. & SNYDER, S. H. 2003. Novel neural modulators. *Annu Rev Neurosci,* 26**,** 105-31.

BURKE, T. J., CALLIS, J. & VIERSTRA, R. D. 1988. Characterization of a polyubiquitin gene from Arabidopsis thaliana. *Mol Gen Genet,* 213**,** 435-43.

CALLIS, J., CARPENTER, T., SUN, C. W. & VIERSTRA, R. D. 1995. Structure and evolution of genes encoding polyubiquitin and ubiquitin-like proteins in Arabidopsis thaliana ecotype Columbia. *Genetics,* 139**,** 921-39.

CARPIO, M. A., LOPEZ SAMBROOKS, C., DURAND, E. S. & HALLAK, M. E. 2010. The arginylation-dependent association of calreticulin with stress granules is regulated by calcium. *Biochemical Journal,* 429**,** 63-72.

CHIU, Y.-H., SUN, Q. & CHEN, Z. J. 2007. E1-L2 activates both ubiquitin and FAT10. *Molecular Cell,* 27**,** 1014-1023.

CHOI, W. S., JEONG, B.-C., JOO, Y. J., LEE, M.-R., KIM, J., ECK, M. J. & SONG, H. K. 2010. Structural basis for the recognition of N-end rule substrates by the UBR box of ubiquitin ligases. *Nature Structural & Molecular Biology,* 17**,** 1175-+.

CHOI, Y. E., BUTTERWORTH, M., MALLADI, S., DUCKETT, C. S., COHEN, G. M. & BRATTON, S. B. 2009. The E3 Ubiquitin Ligase cIAP1 Binds and Ubiquitinates Caspase-3 and-7 via Unique Mechanisms at Distinct Steps in Their Processing. *Journal of Biological Chemistry,* 284**,** 12772-12782.

CIECHANOVER, A. 2007. Intracellular protein degradation from a vague idea through the lysosome and the ubiquitin-proteasome system and on to human diseases and drug targeting: Nobel Lecture, December 8, 2004. *Ann N Y Acad Sci,* 1116**,** 1-28.

COJOCARU, M., BOUCHARD, A., CLOUTIER, P., COOPER, J. J., VARZAVAND, K., PRICE, D. H. & COULOMBE, B. 2011. Transcription Factor IIS Cooperates with the E3 Ligase UBR5 to Ubiquitinate the CDK9 Subunit of the Positive Transcription Elongation Factor B. *Journal of Biological Chemistry,* 286**,** 5012-5022.

DANTUMA, N. P., LINDSTEN, K., GLAS, R., JELLNE, M. & MASUCCI, M. G. 2000. Short-lived green fluorescent proteins for quantifying ubiquitin/proteasome-dependent proteolysis in living cells. *Nature Biotechnology,* 18**,** 538-543.

DAVYDOV, I. V. & VARSHAVSKY, A. 2000. RGS4 is arginylated and degraded by the N-end rule pathway in vitro. *Journal of Biological Chemistry,* 275**,** 22931-22941.

DEMASI, J., CHAO, M. C., KUMAR, A. S. & HOWLEY, P. M. 2007. Bovine papillomavirus E7 oncoprotein inhibits anoikis. *Journal of Virology,* 81**,** 9419-9425.

DITZEL, M., BROEMER, M., TENEV, T., BOIDUC, C., LEE, T. V., RIGBOLT, K. T. G., ELLIOT, R., ZVELEBIL, M., BLAGOEV, B., BERGMANN, A. & MEIER, P. 2008. Inactivation of Effector Caspases through Nondegradative Polyubiquitylation. *Molecular Cell,* 32**,** 540-553.

DITZEL, M., WILSON, R., TENEV, T., ZACHARIOU, A., PAUL, A., DEAS, E. & MEIER, P. 2003. Degradation of DIAP1 by the N-end rule pathway is essential for regulating apoptosis. *Nature Cell Biology,* 5**,** 467-473.

DOHMEN, R. J., WU, P. P. & VARSHAVSKY, A. 1994. HEAT-INDUCIBLE DEGRON - A METHOD FOR CONSTRUCTING TEMPERATURE-SENSITIVE MUTANTS. *Science,* 263**,** 1273-1276.

DOUGAN, D. A., TRUSCOTT, K. N. & ZETH, K. 2010. The bacterial N-end rule pathway: expect the unexpected. *Molecular Microbiology,* 76**,** 545-558.

DRIESSEN, H. P. C., DEJONG, W. W., TESSER, G. I. & BLOEMENDAL, H. 1985. THE MECHANISM OF N-TERMINAL ACETYLATION OF PROTEINS. *Crc Critical Reviews in Biochemistry,* 18**,** 281-325.

DU, F. Y., NAVARRO-GARCIA, F., XIA, Z. X., TASAKI, T. & VARSHAVSKY, A. 2002. Pairs of dipeptides synergistically activate the binding of substrate by ubiquitin ligase through dissociation of its autoinhibitory domain. *Proceedings of the National Academy of Sciences of the United States of America,* 99**,** 14110-14115.

EVERITT, B. S. 2006. *The Cambridge dictionary of statistics*, Cambridge University Press.

FADEM, B. 2009. *High-yield Behavioral Science*, Wolters Kluwer Health/Lippincott Williams & Wilkins.

FISCHER, J. A. 2003. Deubiquitinating enzymes: their roles in development, differentiation, and disease. *Int Rev Cytol,* 229**,** 43-72.

FU, H., GIROD, P. A., DOELLING, J. H., VAN NOCKER, S., HOCHSTRASSER, M., FINLEY, D. & VIERSTRA, R. D. 1999. Structure and functional analysis of the 26S proteasome subunits from plants. *Mol Biol Rep,* 26**,** 137-46.

GALLAGHER, S. R. 1992. *GUS protocols: using the GUS gene as a reporter of gene expression*, Academic Press.

GARZON, M., EIFLER, K., FAUST, A., SCHEEL, H., HOFMANN, K., KONCZ, C., YEPHREMOV, A. & BACHMAIR, A. 2007. PRT6/At5g02310 encodes an Arabidopsis ubiquitin ligase of the N-end rule pathway with arginine specificity and is not the CER3 locus. *FEBS Lett,* 581**,** 3189-96.

GAUSS, C. F. 1816. Bestimmung der Genauigkeit der Beobachtungen". Zeitschrift für Astronomie und verwandt Wissenschaften. 1**,** 187–197.

GLICKMAN, M. H. & CIECHANOVER, A. 2002. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev,* 82**,** 373-428.

GOLDBERG, A. L. 2003. Protein degradation and protection against misfolded or damaged proteins. *Nature,* 426**,** 895-899.

GOLDSTEIN, G., SCHEID, M., HAMMERLING, U., SCHLESINGER, D. H., NIALL, H. D. & BOYSE, E. A. 1975. Isolation of a polypeptide that has lymphocyte-differentiating properties and is probably represented universally in living cells. *Proc Natl Acad Sci U S A,* 72**,** 11-5.

GRACIET, E. & WELLMER, F. 2010. The plant N-end rule pathway: structure and functions. *Trends Plant Sci,* 15**,** 447-53.

GRILLARI, J., KATINGER, H. & VOGLAUER, R. 2006. Aging and the ubiquitinome: traditional and non-traditional functions of ubiquitin in aging cells and tissues. *Exp Gerontol,* 41**,** 1067-79.

GROLL, M., DITZEL, L., LOWE, J., STOCK, D., BOCHTLER, M., BARTUNIK, H. D. & HUBER, R. 1997. Structure of 20S proteasome from yeast at 2.4 A resolution. *Nature,* 386**,** 463-71.

GRUBBS, F. 1969. Procedures for Detecting Outlying Observations in Samples. *Technometrics,* 11**,** 1-21.

HESS, D. T., MATSUMOTO, A., KIM, S.-O., MARSHALL, H. E. & STAMLER, J. S. 2005. Protein S-nitrosylation: purview and parameters. *Nat Rev Mol Cell Biol.,* 2**,** 150-66.

HOELLER, D., HECKER, C. M. & DIKIC, I. 2006. Ubiquitin and ubiquitin-like proteins in cancer pathogenesis. *Nat Rev Cancer,* 6**,** 776-88.

HU, R. G., SHENG, J., QI, X., XU, Z., TAKAHASHI, T. T. & VARSHAVSKY, A. 2005. The N-end rule pathway as a nitric oxide sensor controlling the levels of multiple regulators. *Nature,* 437**,** 981-6.

HUA, Z. & VIERSTRA, R. D. 2011. The cullin-RING ubiquitin-protein ligases. *Annu Rev Plant Biol,* 62**,** 299-334.

JIANG, Y. H. & BEAUDET, A. L. 2004. Human disorders of ubiquitination and proteasomal degradation. *Curr Opin Pediatr,* 16**,** 419-26.

JOHNSTON, S. C., RIDDLE, S. M., COHEN, R. E. & HILL, C. P. 1999. Structural basis for the specificity of ubiquitin C-terminal hydrolases. *EMBO J,* 18**,** 3877-87.

KANG, H. G., FANG, Y. & SINGH, K. B. 1999. A glucocorticoid-inducible transcription system causes severe growth defects in Arabidopsis and induces defense-related genes. *Plant J,* 20**,** 127-33.

KRAVTSOVA-IVANTSIV, Y. & CIECHANOVER, A. 2012. Non-canonical ubiquitin-based signals for proteasomal degradation. *Journal of Cell Science,* 125**,** 539-548.

MARQUES-BUENO, M. M., MORENO-ROMERO, J., ABAS, L., DE MICHELE, R. & MARTINEZ, M. C. 2011. A dominant negative mutant of protein kinase CK2 exhibits altered auxin responses in Arabidopsis. *Plant J,* 67**,** 169-80.

MIZUSHIMA, N., LEVINE, B., CUERVO, A. M. & KLIONSKY, D. J. 2008. Autophagy fights disease through cellular self-digestion. *Nature,* 451**,** 1069-75.

MOON, J., PARRY, G. & ESTELLE, M. 2004. The ubiquitin-proteasome pathway and plant development. *Plant Cell,* 16**,** 3181-95.

PENG, J., SCHWARTZ, D., ELIAS, J. E., THOREEN, C. C., CHENG, D., MARSISCHKY, G., ROELOFS, J., FINLEY, D. & GYGI, S. P. 2003. A proteomics approach to understanding protein ubiquitination. *Nat Biotechnol,* 21**,** 921-6.

PICKART, C. M. 2001a. Mechanisms underlying ubiquitination. *Annu Rev Biochem,* 70**,** 503-33.

PICKART, C. M. 2001b. Ubiquitin enters the new millennium. *Mol Cell,* 8**,** 499-504.

REYES-TURCU, F. E., VENTII, K. H. & WILKINSON, K. D. 2009. Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes. *Annu Rev Biochem,* 78**,** 363-97.

SCHLOGELHOFER, P., GARZON, M., KERZENDORFER, C., NIZHYNSKA, V. & BACHMAIR, A. 2006. Expression of the ubiquitin variant ubR48 decreases proteolytic activity in Arabidopsis and induces cell death. *Planta,* 223**,** 684-97.

SCHNELL, J. D. & HICKE, L. 2003. Non-traditional functions of ubiquitin and ubiquitin-binding proteins. *J Biol Chem,* 278**,** 35857-60.

SEDLAZECK, F. J., TALLOJI, P., VON HAESELER, A. & BACHMAIR, A. 2013. Benefit-of-doubt (BOD) scoring: a sequencing-based method for SNP candidate assessment from high to medium read number data sets. *Genomics,* 101**,** 204-9.

SHANMUGHAM, A. & OVAA, H. 2008. DUBs and disease: activity assays for inhibitor development. *Curr Opin Drug Discov Devel,* 11**,** 688-96.

SMALLE, J. & VIERSTRA, R. D. 2004. The ubiquitin 26S proteasome proteolytic pathway. *Annu Rev Plant Biol,* 55**,** 555-90.

STARY, S., YIN, X. J., POTUSCHAK, T., SCHLOGELHOFER, P., NIZHYNSKA, V. & BACHMAIR, A. 2003. PRT1 of Arabidopsis is a ubiquitin protein ligase of the plant N-end rule pathway with specificity for aromatic amino-terminal residues. *Plant Physiol,* 133**,** 1360-6.

STEEL, R. G. D. & TORRIE, J. H. 1960. *Principles and procedures of statistics: with special reference to the biological sciences*, McGraw-Hill.

STEFANSKY, W. 1972. Rejecting Outliers in Factorial Designs. *Technometrics,* 14**,** 469-479.

SULLIVAN, J. A., SHIRASU, K. & DENG, X. W. 2003. The diverse roles of ubiquitin and the 26S proteasome in the life of plants. *Nat Rev Genet,* 4**,** 948-58.

TALLOJI, P. 2011. Identification of novel components and links in ubiquitin dependent protein degradation pathways of Arabidopsis thaliana. *PhD Thesis, University of Cologne, Germany*.

TURNER, G. C., DU, F. & VARSHAVSKY, A. 2000. Peptides accelerate their uptake by activating a ubiquitin-dependent proteolytic pathway. *Nature,* 405**,** 579-83.

VARSHAVSKY, A. 1997. The N-end rule pathway of protein degradation. *Genes Cells,* 2**,** 13-28.

VARSHAVSKY, A. 2006. The early history of the ubiquitin field. *Protein Sci,* 15**,** 647-54.

VOGES, D., ZWICKL, P. & BAUMEISTER, W. 1999. The 26S proteasome: a molecular machine designed for controlled proteolysis. *Annu Rev Biochem,* 68**,** 1015-68.

WEISSMAN, A. M. 2001. Themes and variations on ubiquitylation. *Nat Rev Mol Cell Biol,* 2**,** 169-78.

WILKINSON, K. D. 2005. The discovery of ubiquitin-dependent proteolysis. *Proc Natl Acad Sci U S A,* 102**,** 15280-2.

WING, S. S. 2003. Deubiquitinating enzymes-The importance of driving in reverse along the ubiquitin-proteasome pathway. *Int J Biochem Cell Biol,* 35**,** 590-605.

YAN, J., WANG, J., LI, Q., HWANG, J. R., PATTERSON, C. & ZHANG, H. 2003. AtCHIP, a U-box-containing E3 ubiquitin ligase, plays a critical role in temperature stress tolerance in Arabidopsis. *Plant Physiol,* 132**,** 861-9.

YANG, P., FU, H., WALKER, J., PAPA, C. M., SMALLE, J., JU, Y. M. & VIERSTRA, R. D. 2004. Purification of the Arabidopsis 26 S proteasome: biochemical and molecular analyses revealed the presence of multiple isoforms. *J Biol Chem,* 279**,** 6401-13.

ZUO, J., NIU, Q. W. & CHUA, N. H. 2000. Technical advance: An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. *Plant J,* 24**,** 265-73.

8. APPENDIX

8.1   Genotyping of *sud2*

Oligonucleotides used for PCR-based amplification of genomic region of interest of sud2 are shown

below. "?" represents uncertainty about the presence of band.

Fragment no.	Primers for amplification	Fragment size(bp)	Comments
1	CGA CCA AAC CAT CTT CTC GGA CTC T - ATC AGA GAT TCC GGA AGC CCA TT	1755	OK
2	ATG CGT GTG GTA TCT ATG TCT ATC - ATC AGA GAT TCC GGA AGC CCA TT	1872	OK
3	CGA CCA AAC CAT CTT CTC GGA CTC T - ATC TCA TCG TCT TCT TCT CCA TAC T	2302	OK
4	ATG CGT GTG GTA TCT ATG TCT ATC - ATC TCA TCG TCT TCT TCT CCA TAC T	2419	OK
5	CGA TGC AGC GAA TCA ACG AAA TTC C - GAG TCT CGA GCT CTG GAG GGT T	3237	Failed
6	CGA TGC AGC GAA TCA ACG AAA TTC C - GTT TGC AGA TCT TCT AGC TCA GGA TC	3304	Failed
7	CGA TGC AGC GAA TCA ACG AAA TTC C- CAC AAA TTA TTA GAA ACA CTT GGC ATT	3456	Failed
8	GGA GGA GAA CTC TTC ATG CTC CT- GAG TCT CGA GCT CTG GAG GGT T	1237	OK
9	GGA GGA GAA CTC TTC ATG CTC CT- GTT TGC AGA TCT TCT AGC TCA GGA TC	1304	OK
10	GGA GGA GAA CTC TTC ATG CTC CT- CAC AAA TTA TTA GAA ACA CTT GGC ATT	1456	Failed
11	AAG CAT TTC AAA CCG GTG AAA CCT T- GTT TGC AGA TCT TCT AGC TCA GGA TC	1839	Failed
12	AAT GTC CGA AAA TGT TGT TCC GTC - GAT ACT CGA GTG CAA CGA CAA CTT	2245	Failed
13	AAT GTC CGA AAA TGT TGT TCC GTC - ACA AAG AGA TAT CGC CAT TGC CTT	2423	Failed
14	AAT GTC CGA AAA TGT TGT TCC GTC - GTT TGC AGA TCT TCT AGC TCA GGA TC	3363	Failed
15	AAT GTC CGA AAA TGT TGT TCC GTC - TGA AGA TTG GAG TTT GTT GAC TCT T	2631	Failed

16	AAG AAA CTC GAT GCA GCG AAT C - GAT ACT CGA GTG CAA CGA CAA CTT	2194	Failed
17	AAG AAA CTC GAT GCA GCG AAT C - ACA AAG AGA TAT CGC CAT TGC CTT	2372	Failed
18	AAG AAA CTC GAT GCA GCG AAT C - TGA AGA TTG GAG TTT GTT GAC TCT T	2580	?
19	TTC CGT CAG GCC GAA GAA ACT CT - GAT ACT CGA GTG CAA CGA CAA CTT	2228	Failed
20	TTC CGT CAG GCC GAA GAA ACT CT - ACA AAG AGA TAT CGC CAT TGC CTT	2406	Failed
21	TTC CGT CAG GCC GAA GAA ACT CT - TGA AGA TTG GAG TTT GTT GAC TCT T	2614	Failed
22	CGA ATC GAC GAA TCT CCA TCC CTT - GAT ACT CGA GTG CAA CGA CAA CTT	2317	Failed
23	CGA ATC GAC GAA TCT CCA TCC CTT - ACA AAG AGA TAT CGC CAT TGC CTT	2495	Failed
24	CGA ATC GAC GAA TCT CCA TCC CTT - TGA AGA TTG GAG TTT GTT GAC TCT T	2703	Failed
25	ATG CGT GTG GTA TCT ATG TCT ATC - GTT TGC AGA TCT TCT AGC TCA GGA TC	5486	Failed
26	CAC AGT GAA GAA GAT TCG AAA TGC T - CGA CCA AAC CAT CTT CTC GGA CTC T	-	Failed
27	AAG CAT TTC AAA CCG GTG AAA CCT T - GTT TGC AGA TCT TCT AGC TCA GGA TC	1839	Failed
28	AAG AAA CTC GAT GCA GCG AAT C - GTT TGC AGA TCT TCT AGC TCA GGA TC	3312	OK
29	ATG CGT GTG GTA TCT ATG TCT ATC - TGA AGA TTG GAG TTT GTT GAC TCT T	4754	Failed
30	GGA GGA GAA CTC TTC ATG CTC CT - TGA AGA TTG GAG TTT GTT GAC TCT T	572	Failed
31	AAG AAA CTC GAT GCA GCG AAT C - ATA CCT TCT GAT CAC GCA TAG GTT	890	OK
32	CAC AGT GAA GAA GAT TCG AAA TGC T - CAT CAG GAA GTT CTC GAA CCG CTT	573	OK
33	CGT AGA ACC AGT TCG CAA TGT CAT T - CAT CAG GAA GTT CTC GAA CCG CTT	193	OK
34	CGT AGA ACC AGT TCG CAA TGT CAT T - GCA TAA CAG CCT TGT CCA TTG CTT	628	OK
35	AAG CAT TTC AAA CCG GTG AAA CCT T - GCA TAA CAG CCT TGT CCA TTG CTT	211	OK
36	AAG CAT TTC AAA CCG GTG AAA CCT T - GTT GGC ACT GAA GAG TAC ATT GCT	-	Failed

37	AAG CAT TTC AAA CCG GTG AAA CCT T - GAT ACT CGA GTG CAA CGA CAA CTT	721	OK
38	AAG CAT TTC AAA CCG GTG AAA CCT T - ACA AAG AGA TAT CGC CAT TGC CTT	899	OK
39	AAG CAT TTC AAA CCG GTG AAA CCT T - TGA AGA TTG GAG TTT GTT GAC TCT T	1107	OK
40	CGT AGA ACC AGT TCG CAA TGT CAT T - GAT ACT CGA GTG CAA CGA CAA CTT	1138	OK(?)
41	CGT AGA ACC AGT TCG CAA TGT CAT T - ACA AAG AGA TAT CGC CAT TGC CTT	1316	OK(?)
42	CGT AGA ACC AGT TCG CAA TGT CAT T - TGA AGA TTG GAG TTT GTT GAC TCT T	1524	OK(?)

8.2   GUS Assay Raw Data

These data represent raw GUS assay values at OD_{405} (GUS measurement) or at OD_{590} (Bradford

analyses) for each line and treatment.

8.2.1   PER-M-GUS (light and oxic)

GUS assay values OD405 vs. time (hh:mm:ss)				
Control				
Control	**00:00:00**	**00:15:00**	**02:30:00**	**05:00:00**
#1	0.117	0.122	0.118	0.121
#2	0.066	0.088	0.222	0.338
#3	0.058	0.054	0.068	0.086
#4	0.095	0.104	0.129	0.165
#5	0.094	0.113	0.122	0.13
SNAP				
#1	0.081	0.083	0.114	0.14
#2	0.064	0.067	0.093	0.122
#3	0.093	0.093	0.097	0.097
#4	0.066	0.07	0.088	0.104
#5	0.096	0.088	0.101	0.106
C-PTIO				
#1	0.121	0.124	0.123	0.13
#2	0.11	0.108	0.11	0.115
#3	0.082	0.084	0.111	0.136
#4	0.073	0.079	0.1	0.118
#5	0.096	0.104	0.142	0.168
Bradford assay values at OD590				
	Control	**SNAP**	**C-PTIO**	
#1	0.498	0.541	0.641	
#2	0.586	0.537	0.561	
#3	0.463	0.479	0.549	
#4	0.497	0.484	0.444	
#5	-		0.595	

8.2.2 P3-C-GUS (Light and oxic condition)

GUS assay values OD405 vs. time (hh:mm:ss)				
Control				
Control	**00:00:00**	**00:15:00**	**02:30:00**	**05:00:00**
#1	0.044	0.045	0.058	0.068
#2	0.06	0.062	0.07	0.084
#3	0.053	0.059	0.068	0.084
#4	0.053	0.05	0.067	0.08
#5	0.06	0.059	0.067	0.075
#6	0.07	0.072	0.072	0.073
SNAP				
#1	0.043	0.044	0.054	0.061
#2	0.039	0.039	0.049	0.056
#3	0.038	0.039	0.062	0.081
#4	0.063	0.066	0.082	0.097
#5	0.06	0.062	0.077	0.091
#6	0.04	0.042	0.058	0.072
C-PTIO				
#1	0.055	0.062	0.073	0.097
#2	0.059	0.062	0.088	0.115
#3	0.06	0.063	0.08	0.099
#4	0.052	0.056	0.088	0.121
#5	0.057	0.062	0.087	0.117
#6	0.053	0.053	0.054	0.06
Bradford assay values at OD590				
	Control	**SNAP**	**C-PTIO**	
#1	0.337	0.4	0.497	
#2	0.478	0.433	0.665	
#3	0.485	0.362	0.496	
#4	0.413	0.635	0.493	
#5	0.501	0.555	0.473	
#6	0.642	0.475	0.45	

8.2.3 P3-C-GUS (in darkness and oxic condition)

GUS assay values OD405 vs. time (hh:mm:ss)				
Control				
Control	**00:00:00**	**00:15:00**	**02:30:00**	**05:00:00**
#1	0.053	0.06	0.103	0.141
#2	0.071	0.079	0.122	0.158
#3	0.073	0.077	0.112	0.144
#4	0.068	0.074	0.1	0.128
#5	0.073	0.083	0.114	0.148
#6	0.064	0.077	0.123	0.178
SNAP				
#1	0.053	0.059	0.082	0.103
#2	0.062	0.062	0.109	0.149
#3	0.062	0.066	0.08	0.095
#4	0.053	0.065	0.105	0.161
#5	0.061	0.067	0.094	0.112
#6	0.069	0.071	0.091	0.109
C-PTIO				
#1	0.073	0.085	0.147	0.204
#2	0.062	0.077	0.14	0.208
#3	0.067	0.075	0.115	0.151
#4	0.065	0.075	0.125	0.162
#5	0.083	0.095	0.16	0.224
#6	0.062	0.068	0.124	0.18
Bradford assay values at OD590				
	Control	**SNAP**	**C-PTIO**	
#1	0.52	0.631	0.84	
#2	0.841	0.589	0.802	
#3	0.876	0.901	0.684	
#4	0.75	0.749	0.857	
#5	0.88	0.673	0.644	
#6	0.798	0.813	0.596	

8.2.4 P3-C-GUS (in darkness and anoxic condition)

GUS assay values OD405 vs. time (hh:mm:ss)				
Control				
Control	00:00:00	00:15:00	02:30:00	05:00:00
#1	0.063	0.064	0.086	0.096
#2	0.072	0.078	0.117	0.145
#3	0.045	0.049	0.059	0.07
#4	0.057	0.059	0.095	0.123
#5	0.055	0.062	0.068	0.073
#6	0.076	0.074	0.09	0.101
SNAP				
#1	0.038	0.037	0.043	0.042
#2	0.054	0.057	0.088	0.111
#3	0.056	0.061	0.098	0.124
#4	0.051	0.054	0.066	0.068
#5	0.065	0.067	0.106	0.131
C-PTIO				
#1	0.061	0.063	0.088	0.107
#2	0.063	0.062	0.067	0.07
#3	0.071	0.071	0.101	0.117
#4	0.054	0.056	0.093	0.125
#5	0.057	0.061	0.079	0.097
#6	0.08	0.078	0.099	0.113
Bradford assay values at OD590				
	Control	SNAP	C-PTIO	
#1	0.639	0.472	0.597	
#2	0.823	0.66	0.631	
#3	0.449	0.667	0.736	
#4	0.598	0.627	0.545	
#5	0.584	0.633	0.631	
#6	0.682	-	0.73	

9. Curriculum Vitae

Bulut Hamali, Bsc

Personal Contact Information

Work: Max F. Perutz Laboratories,
A joint venture of the University Vienna
and the Medical University of Vienna, Department
of Biochemistry and Department of Cell Biology,
Dr. Bohr-Gasse 9, A-1030,
Vienna,
Austria

Home: Verein Wonder,
Kreitnergasse 4-6, A-1160,
Vienna,
Austria

E-mail: bulut.hamali@univie.ac.at

Date of Birth: July 30th, 1987
Nationality: Turkish

Research Interests

Chromatin Biology, Transcriptional Regulation, RNA Biology, Neuroscience

Education and Research Experience

Since 02/2013 Research Assistant in the Laboratory of Andreas Bachmair
Additional experiments for publications

04/2012-01/2013 Master Thesis in the Laboratory of Andreas Bachmair
Thesis on " Protein turnover by N-end rule pathway in Arabidopsis Thaliana"
MFPL- Max F. Perutz Laboratories, Vienna, AT

11/2011 - 12/2011	Practical internship in the Laboratory of Kazufumi Mochuziki Project on "Small RNA-directed DNA elimination in Tetrahymena" IMBA - Institute of Molecular Biotechnology GmbH, Vienna, AT
9/2011 - 9 /2011	Practical internship in the Laboratory of Nafiseh Sabri Project on "Nuclear transport function in cell signalling" University of Gothenburg, Gothenburg, SE
03/2010	Registration for MSc in Genetics and Developmental Biology Vienna University, Vienna, AT
09/2005 - 07/2009	BSc in Molecular Biology and Genetics Halic University, Istanbul, TR
2005	Bayrampasa Anadolu High School, Graduated with Honor, Istanbul, TR

Awards & Honours

04/2012-01/2013	Master Thesis Scholarship (FWF)
09/2005 - 07/2009	ÖSYM (Turkish Student Selection and Placement Center) BSc Scholarship

Publications

Daniel J. Gibbs, Nurulhikma Md Isa, Mahsa Movahedi, Guillermina Mendiondo, Cristina Sousa Correia, Peter D. Jones, Simon Pearce, Pauline Roubira, **Bulut Hamali**, Prabhavathi Talloji, Daniel Tomé, Alberto Coego, Jim Beynon, Françoise Corbineau, José León, Andreas Bachmair, Julie E. Gray, Frederica L. Theodoulou, Michael J. Holdsworth. Control of Nitric Oxide sensing in plants by Group VII Ethylene Response Factors. **(in preparation)**