Links between the Ubiquitin-Proteasome System and Cell Death Pathways in *Arabidopsis thaliana*

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ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
2iP	6-(3,3-dimethylallylamino)purine (isopentenyladenine)
A. thaliana	*Arabidopsis thaliana*
AAA+	protein with an amino-acid ATP-binding domain (AAA domain)
ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid (ethylene precursor)
Ara	*A. thaliana* modified MS medium
ATE1	arginine-tRNA-protein transferase1 (R-transferase1)
ATP	adenosine triphosphate
bp	base pair
CaMV	cauliflower mosaic virus
CAN	cantharidin
cDNA	complementary DNA
ClpS	*E. coli* gene, subunit of Clp protease
Col-0	Columbia wild type
D. melanogaster	*Drosophila menalogaster*
Da	Dalton
Dex	Dexamethasone
DHFR	murine dihydrofolase reductase
DML	double mutant line
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
DUB	deubiquitinating enzyme
E1	ubiquitin activating enzyme
E2	ubiquitin conjugating enzyme
E3	ubiquitin protein ligase
E. coli	*Escherichia coli*
e. g.	example given
EMS	1-methylsulfonyloxyethane (ethyl methanesulfonate)
Fig.	figure
GAL4	a yeast transcription factor, necessary for galactose utilization
GR	glucocoticoid binding domain of rat glucocorticoid receptor
GUS	β-glucuronidase gene
GVG	Chimeric transcription factor: GAL4 + VP16 + GR
HA	influenza hemagglutinin

HDM	Ara medium containing Hyg, Dex and MTX
HECT	homologuous to the E6 associated protein carboxyl terminus
HPT	hygromycin phosphotransferase
HR	hypersensitive response
Hyg	hygromycin
IAP	inhibitor of apoptosis protein
kb	kilo base pair
kDa	kilo Dalton
lacI	Protein encoded by the gene of the *E. coli* lac operon
L*er*	Landsberg *erecta*
M	molar
M. musculus	*Mus musculus*
Mb	mega base pair
MeJA	methyl jasmonate
MG132	N-Ac-Leu-Leu-norleucinal (proteasome inhibitor)
MP	mapping population
mRNA	messenger RNA
MTX	methotrexate
N-end	amino terminus of a protein
NOS	nopaline synthase
Nt-amidase	amino-terminal amidase
ORF	open reading frame
PCD	programmed cell death
PCR	polymerase chain reaction
pNOS	NOS promotor
PRT1	proteolysis 1 locus of *A. thaliana*
PRT6	proteolysis 6 locus of *A. thaliana*
QTL(s)	quantitative trait locus/loci
RING	really interesting new gene
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcription followed by polymerase chain reaction
RV86-5	*A. thaliana* plant line carrying the UbK48R transgene
S	Svedberg sedimentation coefficient
S. cerevisiae	*Saccharomyces cerevisiae*
SA	salicylic acid
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sud	supressor of ubiquitin UbK48R-induced cell death

T-DNA	transferred DNA of *Agrobacterium tumefaciens* Ti plasmid
tRNA	transfer RNA
UAS	upstream activation sequence
UbK48R	Variant ubiquitin with amino acid 48 changed from Lys to Arg
UBR	ubiquitin (substrate) recognition (factor) – a ubiquitin ligase
UPR	ubiquitin-protein reference (technique)
UV-C	short wave ultraviolet light
vol.	volume
VP16	Herpes virus protein 16
WT	wild type

AMINO ACIDS

Ala	A	Alanine
Arg	R	Arginine
Asn	N	Asparagine
Asp	D	Aspartic acid
Cys	C	Cysteine
Gln	Q	Glutamine
Glu	E	Glutamic acid
Gly	G	Glycine
His	H	Histidine
Ile	I	Isoleucine
Leu	L	Leucine
Lys	K	Lysine
Met	M	Methionine
Phe	F	Phenylalanine
Pro	P	Proline
Ser	S	Serine
Thr	T	Threonine
Trp	W	Tryptophan
Tyr	Y	Tyrosine
Val	V	Valine

SUMMARY

The dominant pathway for protein degradation in eukaryotes is the ubiquitin-proteasome system. In an enzymatic cascade with three different enzymes (E1, E2, E3), the small protein ubiquitin is covalently linked to a target protein, and substrates carrying a multi-ubiquitin chain consisting of at least four ubiquitin moieties are rapidly degraded by the 26S proteasome. Programmed cell death (PCD) is the genetically controlled suicide of cells that occurs in all eukaryotes. Examples for PCD in plants are leaf senescence, the hypersensitive response (HR) after pathogen attack and processes during development such as the formation of tracheary elements. Several proteins involved in PCD in animals are regulated by the ubiquitin-proteasome pathway.

In this work, the ubiquitin-proteasome system was perturbed in the model plant *Arabidopsis* by expression of UbK48R, a variant ubiquitin that was able to induce cell death in plants. Physiological characterization of the UbK48R-induced cell death could not precisely pinpoint the type of PCD observed. However, DNA degradation at a very late time point during PCD indicated that the UbK48R-induced PCD was related to senescence. It could also be shown that exposure to additional, normally harmless stress factors is able to accelerate the cell death process, and the isolation and characterization of suppressor of UbK48R-induced cell death (*sud*) mutants indicated changes in distinct, rather specialized pathways. One dominant mutant (*sud1*) resulted in higher resistance to the proteasome inhibitor MG132, and the recessive *sud2* mutation could be positioned in a first-pass mapping to chromosome III between 10.7 Mbp and 18.9 Mbp.

A second line of research dealt with the N-end rule pathway, a distinct ubiquitin-dependent protein degradation pathway that connects the fate of a protein with the nature of its amino-terminal residue. This pathway is also involved in leaf senescence. By using model substrates with defined amino acids at the amino terminus, it was shown that the *Arabidopsis* gene

At5g02310 (now *PRT6*) encodes a ligase of the N-end rule pathway that recognizes and degrades proteins carrying Arg as amino-terminal residue. Model substrates with Leu as amino-terminal residue were, although also short-lived, not degraded by PRT6 or the second known ligase PRT1. Therefore, the existence of at least one more N-end rule ligase in plants was postulated.

ZUSAMMENFASSUNG

Der vorherrschende Proteinabbauweg in Eukaryoten ist das Ubiquitin-Proteasom-System. In einer enzymatischen Kaskade mit drei verschiedenen Enzymen (E1, E2, E3) wird das kleine Protein Ubiquitin kovalent an das Zielprotein gebunden und Substrate, die eine aus mindestens vier Ubiquitin-Einheiten bestehende Ubiquitinkette tragen, werden schnell vom 26S Proteasom abgebaut. Programmierter Zelltod (PCD) ist der genetisch kontrollierte Selbstmord von Zellen, der in allen Eukaryoten zu finden ist. Beispiele für PCD bei Pflanzen sind Blatt-Seneszenz, die Hypersensitive Antwort (HR) nach Schädlingsbefall und Prozesse während der Entwicklung wie z.B. die Bildung von Tracheen. Mehrere Proteine, die am PCD bei Tieren beteiligt sind, werden durch den Ubiquitin-Proteasom Abbauweg reguliert.

In dieser Arbeit wurde das Ubiquitin-Proteasom-System im pflanzlichen Modellorganismus *Arabidopsis* durch die Expression von UbK48R gestört, einer Ubiquitin-Variante, die in der Lage war, Zelltod in Pflanzen zu verursachen. Physiologische Charakterisierung des durch UbK48R hervorgerufenen Zelltodes konnte nicht genau die Art des eingeleiteten Zelltodes bestimmen. Jedoch zeigte der DNA-Abbau zu einem sehr späten Zeitpunkt während des PCD, dass der UbK48R-induzierte Zelltod mit Seneszenz verwandt war. Es konnte ebenfalls gezeigt werden, dass zusätzliche Stressfaktoren, die für sich alleine genommen harmlos sind, im Zusammenwirken miteinander den Zelltod beschleunigen, und die Isolierung und Charakterisierung von Suppressoren des UbK48R-induzierten Zelltodes (*sud* Mutanten) wies auf distinkte, eher spezialisierte Stoffwechselwege hin. Eine dominante Mutante (*sud1*) zeigte eine höhere Resistenz gegenüber dem Proteasom-Inhibitor MG132, und die rezessive Mutation *sud2* konnte in einem ersten Mapping auf Chromosom III zwischen 10.7 Mbp und 18.9 Mbp positioniert werden.

Ein zweiter Forschungsansatz befasste sich mit dem N-end rule Stoffwechselweg, einem ubiquitinabhängigen Proteinabbauweg, der das

Schicksal eines Proteins mit der Beschaffenheit seiner amino-terminalen Aminosäure verknüpft. Dieser Stoffwechselweg ist ebenfalls an der Blatt-Seneszenz beteiligt. Durch den Gebrauch von Modellsubstraten mit definierter Aminosäure am amino-terminalen Ende konnte gezeigt werden, dass das Arabidopsis-Gen *At5g02310* (jetzt *PRT6*) für eine Ligase des N-end rule Proteolyseweges kodiert, die Proteine, die Arg als amino-terminalen Rest besitzen, erkennt und abbaut. Modellsubstrate mit Leu als amino-terminalem Rest wurden, obwohl ebenfalls kurzlebig, nicht von PRT6 oder der zweiten bislang bekannten Ligase PRT1 abgebaut. Daher wurde die Existenz von mindestens einer weiteren N-end rule Ligase in Pflanzen postuliert.

1 INTRODUCTION

1.1 Plant science

1.1.1 Working with plants

Plants are fascinating organisms. Although plants cannot walk like animals or talk and think like human beings, they have developed a great ability: Photosynthesis. By using the light energy of the sun and transforming it into small packages of transportable units of energy, plants found a way to convert energy. And as a side product they emit oxygen that is used by all animals including human beings for breathing. Without plants, animal life on earth, as we know it, would not be possible.

Plants are autotrophic and are used by heterotrophic animals, including human beings, for nutrition. The cultivation of crops was an essential step for mankind to succeed in survival and to conquer the world. Since the first days of agriculture, plants were selected by humans who tried to improve their

yields. Due to plant breeding, the yield of useful plants could be increased and kept up with the growth of population. Large improvements have been made in the last century, when physiological processes in plants were started to be examined and to be understood. Working with plants ensures the survival of human beings.

Besides these aspects of application, plant research can also help to understand fundamental biological processes that play important roles in all cells. Although plants, animals and fungi are now three independent groups, they all have the same ancestors. The last common ancestor of plants and animals lived 1.5 billion years ago (Dennis and Surridge, 2000), but life on earth started much earlier. Basic cellular processes like mitosis, anabolism, catabolism or proteolysis were already developed by prokaryotes over 3 billion years ago and can now be found both in animals and plants. Comparison of those processes in animals and plants can help to understand the complex mechanisms of life on a molecular level.

1.1.2 *Arabidopsis thaliana* as a model plant

In the last 20 years, *Arabidopsis thaliana* has become the model organism in molecular plant research (Fig. 1.1). Like *Drosophila melanogaster* – a model organism for research in developmental biology – it combines a lot of advantages that are very helpful for efficient research: *Arabidopsis thaliana* is a small plant with low requirements concerning growth conditions, therefore it can be easily cultivated in a growth chamber or a greenhouse. Wild type plants have a short life cycle (six to ten weeks for one generation) and reproduce via self pollination. *Arabidopsis thaliana* is big enough to be handled easily, but it has a small genome (125 Mb) and only five chromosomes so that linkage analysis is much easier than in plants with a higher number of chromosomes (Walbot, 2000; Somerville and Koornneef, 2002). The genome sequence was published in the year 2000 by The Arabidopsis Genome Initiative, which enables comparisons of conserved

Fig. 1.1:* Arabidopsis thaliana*, the model organism for molecular plant research. *The inconspicuous weed (left: plantlets aged four weeks; right: seven-week old flowering plants) concentrates several characteristics that favour the use of this small plant for basic research: It is easy to cultivate and has a short life cycle, its small genome of 125 Mb is distributed over only five chromosomes, and the genome sequence was published in the year 2000.*

processes and the identification of plant specific gene functions. Several different ecotypes with a large number of polymorphisms at DNA level support mapping processes and as it is used by many researchers, thousands of different mutant lines are available.

1.2 Protein degradation by the ubiquitin-proteasome system

1.2.1 Proteolysis

Proteins are the basic modules of cell structure and function, therefore they are essential in life. Their synthesis as well as their degradation is of particular importance for the function of the organism. A disturbance of the

formation of proteins can result in severe damage to the cell up to the collapse of important metabolic processes. In the same way, problems can occur when protein degradation is perturbed, as proteolysis is involved in many cellular and physiological processes. Proteolysis controls the lifespan of a protein, it eliminates misfolded or damaged proteins and it refills the pool of free amino acids (Ciechanover, 2005). A precise and efficient system for protein degradation is therefore essential for the organism.

Eukaryotes possess several specific mechanisms for protein degradation in subcellular compartments. One example is the degradation of proteins by the mammalian lysosome and in vacuoles by cysteine proteases like cathepsins (mammals) and caspases (plants), which is supposed to be linked with senescence and programmed cell death (Turk *et al.*, 2001; Sanmartín *et al.*, 2005). Plant caspases are thought to be responsible for up to 90 % of all proteolytic activity in response to various internal and external stimuli like e. g. dehydration (Wisniewsky and Zagdanska, 2001).

Another very powerful and highly conserved mechanism with a multitude of enzymes that ensures the efficient degradation of nuclear and cytoplasmic proteins is the ubiquitin-proteasome system (Smalle and Vierstra, 2004). This pathway is thought to be the dominant proteolytic system in higher eukaryotes. Protein degradation by the ubiquitin-proteasome pathway is a two-step process: The target protein is first tagged by covalent attachment of ubiquitin, and then the marked protein is recognized and degraded by a multicatalytic protease complex, the 26S proteasome.

1.2.2 Ubiquitin

Besides the 26S proteasome complex, ubiquitin is the central component of the ubiquitin-proteasome pathway for protein degradation. Ubiquitin (Fig. 1.2) is a highly conserved protein with only 76 amino acids that marks proteins for degradation. It was first purified during the isolation of thymopoietin (Schlesinger *et al.*, 1975) and can be found in all tissues of higher organisms

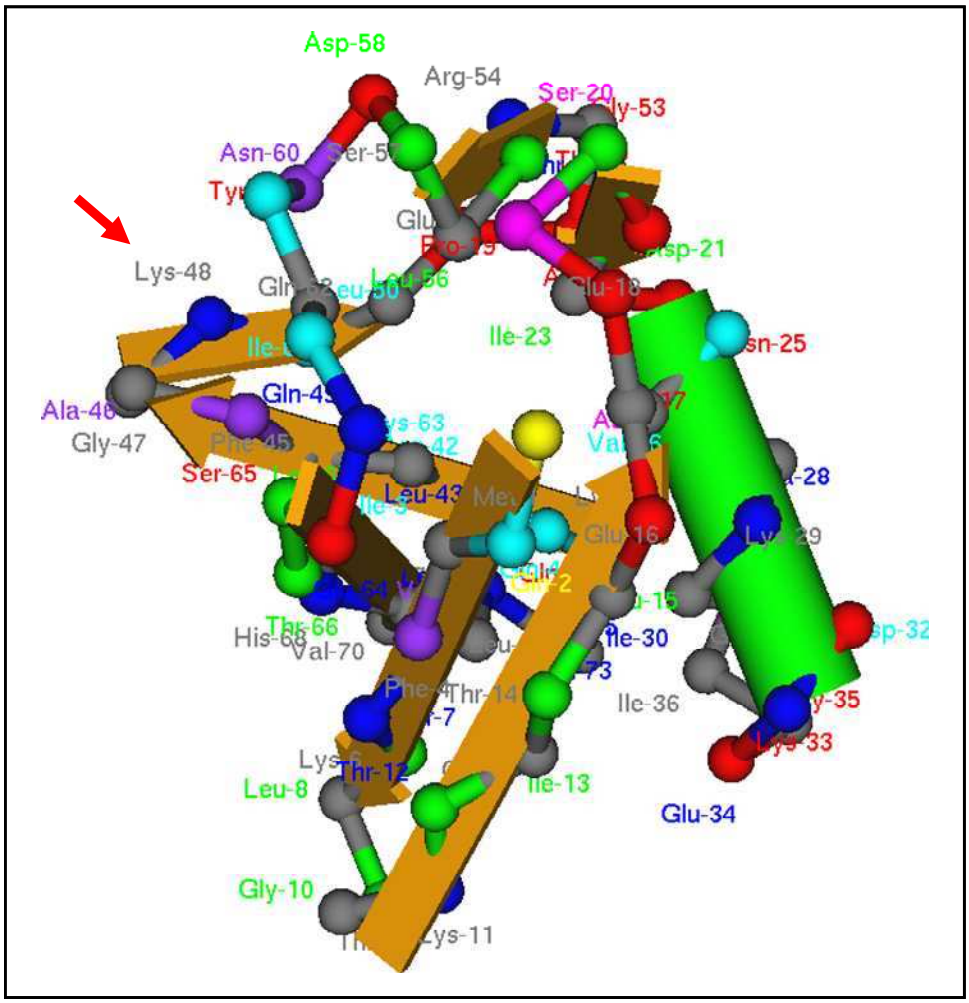

Fig. 1.2: Structure of ubiquitin. *Ubiquitin is a highly conserved molecule with only 76 amino acids that marks proteins for degradation by the 26S proteasome. In an enzymatic cascade with three different enzymes, ubiquitin is covalently linked to the target substrate. The Gly residue at the carboxyl terminus (position 76) forms an isopeptide bond with the ε-amino group of an internal Lys residue of the substrate. A multi-ubiquitin chain consisting of at least four moieties serves as recognition signal for rapid degradation. Ubiquitin itself contains seven different Lys residues (Lys 6, Lys 11, Lys 27, Lys 29, Lys 33, Lys 48 and Lys 63) which are all potential ubiquitin attachment sites involved in chain formation. Chains that are linked via Lys 48 (red arrow) represent a signal for proteasomal degradation of modified substrates. Figure drawn after Vijay-Kumar* et al.*, 1987.*

both in the animal and the plant kingdom. It is highly conserved with only three different amino acids between animals and plants and it exists either free or covalently linked to other proteins. An enzymatic cascade with three different enzymes links ubiquitin covalently to the target substrate. The ubiquitin-proteasome system has been shown to be involved in fundamental processes like the cell cycle and cell division, DNA repair, endocytosis, antigen processing and apoptosis (Di Fiore *et al.*, 2003).

Most, but not all of these processes depend on ubiquitin's role in protein turnover (Ciechanover, 2005).

1.2.3 Ubiquitylation

Ubiquitylation, the process of targeting a substrate with ubiquitin (Fig. 1.3), can occur in different forms. Ubiquitin can be attached to a target protein, or to already attached ubiquitin moieties. The ubiquitylation process is called monoubiquitylation when only one single ubiquitin is attached (Hicke and Dunn, 2003), and multi- or polyubiquitylation when several ubiquitin moieties are added to the target protein (Haglund *et al.*, 2003). In most cases, polyubiquitylation occurs by linking several ubiquitin moieties to each other via an internal Lys residue of ubiquitin (Pickart and Fushman, 2004; review by Haglund and Dikic, 2005). Seven different Lys residues (Lys 6, Lys 11, Lys 27, Lys 29, Lys 33, Lys 48 and Lys 63) can be found in ubiquitin. All Lys residues are potential ubiquitin attachment sites which are involved in chain formation. The best characterized ubiquitin chains are linked via Lys 48 and via Lys 63 of ubiquitin, whereas only chains that are linked via Lys 48 serve as signal for proteasomal degradation of modified substrates. Like phosphorylation, ubiquitylation is also a mechanism of signal transduction (Varshavsky *et al.*, 2000).

In an ATP-dependent enzymatic cascade with three different enzymes – a ubiquitin activating enzyme (UBA or E1), a ubiquitin conjugating enzyme (UBC or E2) and a ubiquitin protein ligase (E3) - one ubiquitin moiety is covalently linked to a target protein (Hershko and Ciechanover, 1998).

In a first step, the Gly residue in position 76 at the carboxyl terminus of ubiquitin forms a thioester bond with a Cys residue of the ubiquitin activating enzyme. This activates the carboxyl terminus of ubiquitin for conjugation to other proteins. In a second step, this activated ubiquitin is transferred from E1 to E2, the ubiquitin conjugating enzyme, and forms again a thioester bond with a Cys residue of E2. The E2 – Ub intermediate and the protein substrate

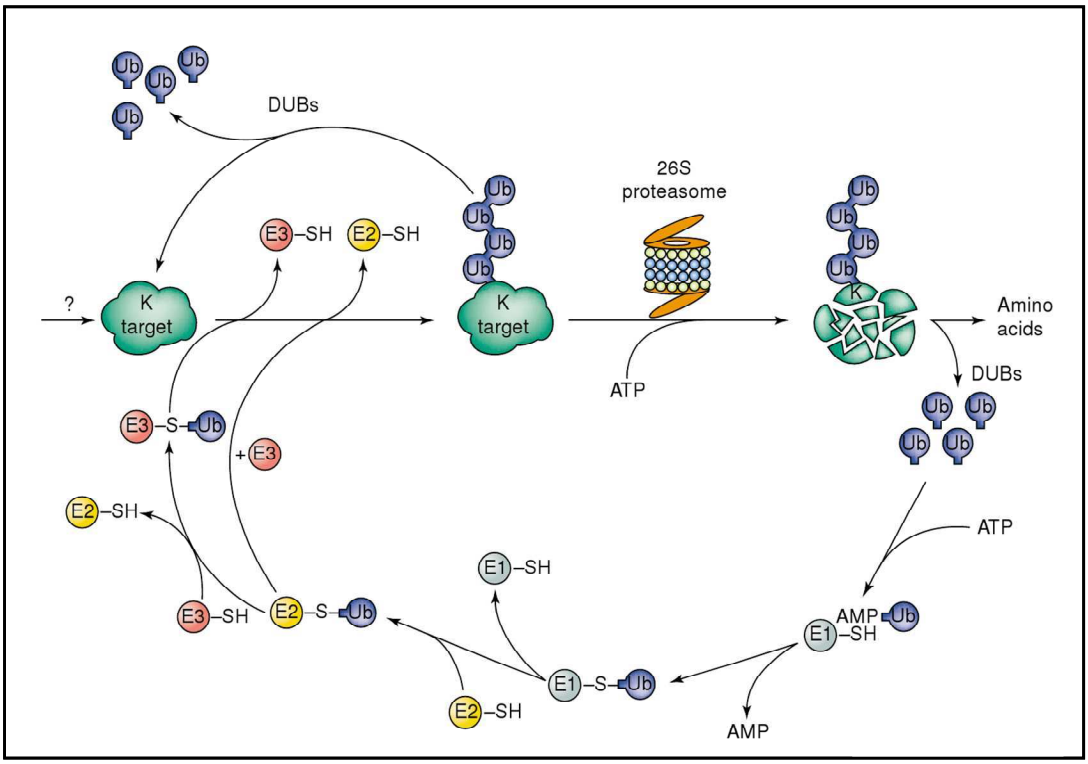

Fig. 1.3: The ubiquitin-proteasome pathway with ubiquitylation and subsequent substrate degradation. *The pathway starts with the ATP-dependent activation of ubiquitin by E1, followed by the conjugation of ubiquitin by E2 and the final attachment of the ubiquitin moiety to the Lys residue of a target substrate, mediated by an E3 ligase. This ubiquitylation cascade is repeated until the substrate carries a multi-ubiquitin chain of at least four ubiquitin moieties. Now the ubiquitin-protein conjugate is recognized by the 26S proteasome and degraded in an ATP-dependent process, whereas ubiquitin is not degraded but recycled after cleavage by deubiquitinating enzymes (DUBs) and can be used again for targeting. Alternatively, the whole conjugate can be disassembled by DUBs which release both the complete target protein and the intact ubiquitin moieties. Abbreviations: E1 - ubiquitin activating enzyme; E2 - ubiquitin conjugating enzyme; E3 - ubiquitin protein ligase; Ub – ubiquitin; DUB – deubiquitinating enzyme; ATP – adenosine triphosphate; AMP – adenosine monophosphate; K – internal lysine residue of a target protein. Figure modified from Vierstra, 2003.*

then both bind specifically to a particular ubiquitin protein ligase E3, where finally the ubiquitin is transferred to the target protein. An isopeptide bond is formed between the Gly residue of the ubiquitin and the ε-amino group of an internal Lys residue of the substrate. The E3 ligase is the specific substrate-binding component of the system and depending on the type of the E3, ubiquitin is either transferred directly to the target protein or via a catalytic Cys residue located on the E3 ligase itself. Further ubiquitin moieties are conjugated to the first ubiquitin in the same cascade, whereas the second

ubiquitin moiety is linked via an internal Lys residue (Lys 48) of the previously attached ubiquitin. Several cycles of the E1-E2-E3-cascade result in a polyubiquitin chain. A ubiquitin chain of at least four moieties serves as a signal for degradation. The tagged protein is recognized for degradation by the 26S proteasome and subsequently degraded whereas the ubiquitin moieties are reused again for ubiquitylation. The release of attached ubiquitin moieties is mediated by deubiquitinating enzymes (DUBs). DUBs cleave the ubiquitin monomers from the precursor protein, recycle ubiquitin moieties during the breakdown of the ubiquitin-protein conjugates and reverse the effects of ubiquitylation in signal transduction (Smalle and Vierstra, 2004).

1.2.4 Enzymes of the ubiquitin pathway

More than 1400 genes in *A. thaliana* encode components of the ubiquitin/26S proteasome pathway, which represents around 5 % of the proteome (Smalle and Vierstra, 2004). Approximately 90 % of these genes encode subunits of the E3 ubiquitin ligases that confer substrate specifity to the pathway (Moon *et al.*, 2004).

The specific activity of the three enzymes E1, E2 and E3 involved in the ubiquitylation cascade varies to a great extent and therefore also their number varies from only a few to several hundreds. As ubiquitin activation does not seem to have any regulatory function, E1 shows nearly no specificity. One E1 was found in yeast (*S. cerevisiae*) and lower animals, and only recently a second E1 in vertebrates has been identified that interacts with a specific subset of E2s (Jin *et al.*, 2007). In *A. thaliana*, two E1 enzymes were identified (Hatfield *et al.*, 1997) but were expected to have the same specificity (Bachmair *et al.*, 2001). The group of the E2s includes at least 45 E2 or E2-like proteins. All E2s share a conserved core domain consisting of around 150 amino acids (Pickart, 2001). The E3 ubiquitin ligase family is the largest group of the three enzymes and contains a great diversity of specific ligases. All E3 ubiquitin ligases in plants possess either a RING/U-box domain or a HECT domain (Fig. 1.4). The RING (Really Interesting New Gene) domain

ligases form the biggest group and contain a zinc binding motiv (RING finger) as an interaction domain for E2s. The RING domain E3s can be further divided into single subunit E3s and multisubunit RING E3s including the SCF, CUL3-BTB and APC complexes. The HECT (Homologous to the E6 associated protein Carboxyl Terminus) E3s are large proteins that are not part of a complex. Unlike the RING domain-containing family, the HECT E3 ligases contain besides the ubiquitin E2 binding site an additional ubiquitin binding site where the conjugated ubiquitin is first bound temporarily before it is

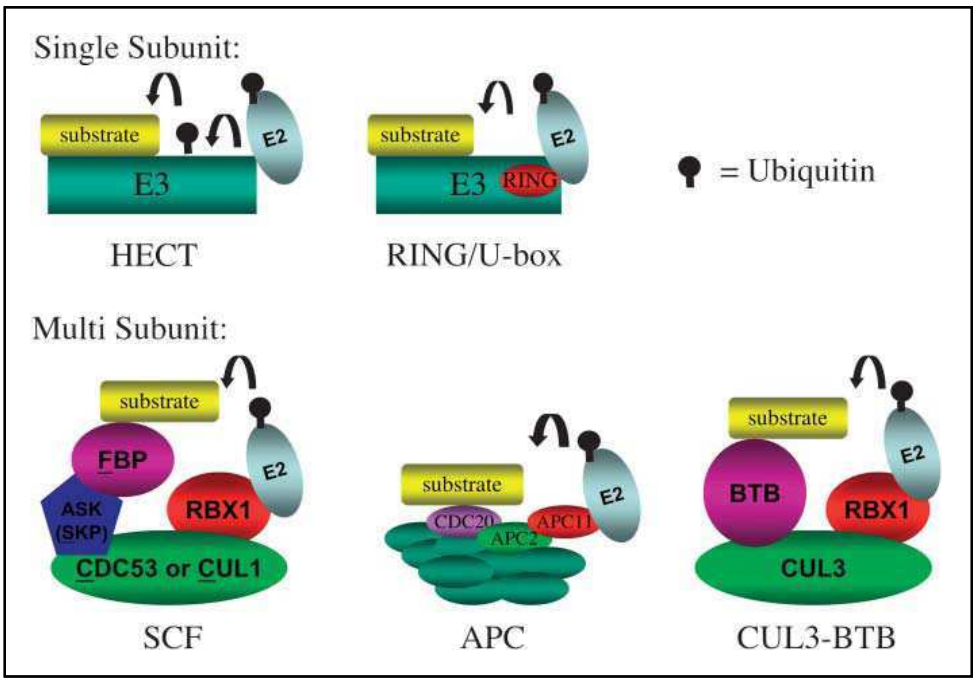

Fig. 1.4: Classes of different E3 ubiquitin ligases in plants. *The E3 ubiquitin ligases in plants comprise a large and diverse family of proteins or protein complexes. Approximately 90 % of the genes in* Arabidopsis *that are involved in the ubiquitin-proteasome pathway encode subunits of the E3 ligases that confer substrate specificity to the pathway. All E3 ligases share as common motif either a HECT (Homologous to the E6 associated protein Carboxyl Terminus) domain or a RING (Really Interesting New Gene)/U-box domain. The latter can be found both in single subunit RING/U-box E3s and in multisubunit RING E3s including the SCF, APC and CUL3-BTB complexes. The HECT motif has only been identified in single subunit E3s. In* Arabidopsis*, several hundreds of proteins with a RING domain have been found, whereas only seven HECT E3s are encoded in the* Arabidopsis *genome. HECT E3s first form a covalent bond with the conjugated ubiquitin from the E2 at an internal ubiquitin binding site before transferring it to the substrate protein. E3s with RING domain transfer the conjugated ubiquitin directly to the target substrate. Colour coding: Green – cullin and cullin-like proteins; red – RING proteins; purple – substrate specificity factors. Abbreviations: SCF – Skp-Cullin-F-box; APC – Anaphase Promoting Complex; BTB – Broad-complex-Tramtrack-Bric-a-Brac. Figure taken from Moon* et al.*, 2004.*

transferred to the final target substrate (Pickart, 2001). Although there are many HECT E3s in animals, only seven HECT E3s have been identified in *Arabidopsis* so far, whereas there are more than 400 different RING finger genes in *Arabidopsis* (Moon *et al.*, 2004).

1.2.5 The proteasome

The proteasome is a large protein complex that degrades polyubiquitylated proteins to small peptides so that their amino acids can be reused by the organism. The proteasome can be found in all eukaryotes where it is located in the nucleus as well as in the cytoplasm (Peters *et al.*, 1994). The most common form is the 26S proteasome (Fig. 1.5), a multisubunit complex that is composed of the two subcomplexes 20S and 19S.

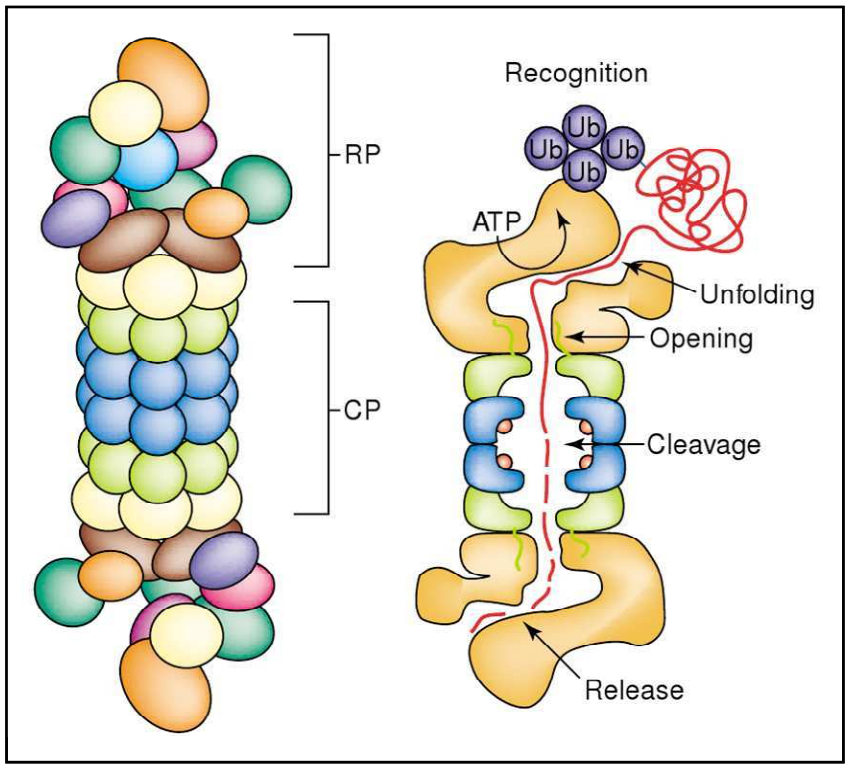

Fig. 1.5: The 26S proteasome. *The 26S proteasome is the proteolytic complex that degrades ubiquitylated proteins and it can be sub-divided in the 20S core protease (CP) and the 19S regulatory particle (RP). The CP is the central part of the complex and responsible for the cleavage of the degrading protein. The RP is associated with one or both ends of the CP and confers both ATP dependence and specificity for Lys 48-linked multi-ubiquitin chains to the particle. Figure modified from Vierstra, 2003.*

The cylindrical 20S core protease (CP) is capped on each end by a 19S regulatory particle (RP) (Groll and Huber, 2003). The 20S core particle is the central unit created by four stacked rings, two identical outer α-rings and two identical inner β-rings. Each ring is composed of seven distinct subunits so that the general structure of the 20S complex can be described as α1-7β1-7β1-7α1-7. The proteolytic activity is located on the inside of the barrel-shaped structure formed by three of the β-subunits (Vierstra, 2003; Ciechanover, 2005).

The 19S regulatory particle can be found like a cap on both ends of the 20S barrel and serves as a gate to get access to the central CP. The RP is composed of 17 different subunits that form a lid (9 subunits) and a base component (8 subunits) (Ciechanover, 2005). Whereas the lid recognizes polyubiquitylated proteins and also removes attached ubiquitin chains, the base is responsible for unfolding the substrate (Yang *et al.*, 2004), as only unfolded proteins are allowed to enter the central CP. The access to the central chamber is regulated by a narrow gated channel that is created by the α-subunit rings of the CP (Glickman, 2000).

1.2.6 Programmed cell death

Programmed cell death (PCD) is also an integral part of life and has been defined as a sequence of (potentially interruptible) events that lead to the death of the cell (Lockshin and Zakeri, 2004). PCD can occur at all developmental stages of the life cycle. It is a self-destructing cellular process, triggered by external or internal factors and mediated through an active genetic program (Lim *et al.*, 2007). It occurs in all eukaryotic kingdoms, both in unicellular and in multicellular organisms and is involved in many aspects of development as well as in responses to external stimuli (Sanmartín *et al.*, 2005). The investigation of PCD in animals is much more advanced than in plants (Della Mea *et al.*, 2007), however it has been shown that some features of PCD in plants are different from the ones found in animals.

In animals, three different categories exist: Apoptosis, autophagy and non-lysosomal PCD, whereas apoptosis is the best studied PCD. In plants, PCD can be found during leaf senescence (Buchanan-Wollaston *et al.*, 2003), as reaction after pathogen attack (where it is called hypersensitive response (HR; Morel and Dangl, 1997) and in several developmental processes as developmental PCD like e.g. the formation of tracheary elements (Fukuda, 1996) or germination-related degeneration of aleuron layer cells (Jones and Dangl, 1996).

1.2.7 The ubiquitin-proteasome system and programmed cell death

In the early 1990s, first studies detected links between the ubiquitin-proteasome system and PCD: Investigations of the hawk moth *Manduca sexta* showed increased ubiquitin RNA levels as well as an increased number of ubiquitylated proteins in the intersegmental muscles cells undergoing PCD (Schwartz *et al.*, 1990). Similar to these findings it could be shown in plants that the inhibition of the ubiquitin-proteasome pathway induced cell death (Bachmair *et al.*, 1990). A correlation between PCD and increased ubiquitin or ubiquitylated proteins was later shown in several other model organisms (Orlowski, 1999). It has been shown in animals that proteins that are involved in cell death like the tumour suppressor p53 (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997) and members of the Bcl-2 family (Cory and Adams, 2002) are regulated by the ubiquitin-proteasome system (Yang and Yu, 2003). In plants, analyses of the delayed senescence mutant *Ore9* showed that ubiquitin-dependent proteolysis is likely to be also involved in the regulation of leaf senescence, one form of plant PCD. The *ORE9* gene encodes an F-box protein, which is a component of the SCF complex that in turn is part of an E3 ligase. As leaf senescence was delayed in the *oresara9* (*ore9*) mutant it can be assumed that the ubiquitin-proteasome pathway plays also an important role in the control of (leaf) senescence (Woo *et al.*, 2001).

1.2.8 The N-end rule pathway

One distinct pathway of the ubiquitin-proteasome system is the so-called N-end rule pathway. The N-end rule pathway was discovered in 1986 by Varshavsky and co-workers when it was found that the half-life of a protein also depends on the nature of the amino acid at the amino terminus (Bachmair *et al.*, 1986). This phenomenon – a protein is degraded or not according to its amino-terminal amino acid – is called the N-end rule pathway (Varshavsky, 1996). The N-end rule can be found both in prokaryotes and in eukaryotes (Mogk *et al.*, 2007), even though prokaryotes do not contain the ubiquitin-proteasome system for protein degradation (Tasaki and Kwon, 2007). Components of the N-end rule pathway have been identified in the model bacterium *E. coli* (Tobias *et al.*, 1991), in the yeast *S. cerevisiae* (Bachmair and Varshavsky, 1989; Varsharvsky, 1996), in mammals (Tasaki and Kwon, 2007) and in plants (Bachmair *et al.*, 1993), where the pathway has been shown to also be involved in leaf senescence (Yoshida *et al.*, 2002) and pathogen defense (Takemoto and Jones, 2005). Prokaryotes and Eukaryotes share common principles of substrate recognition, although the degradation of the N-end rule substrates differs in prokaryotes and eukaryotes as distinct proteolytic machineries are used for degrading the targeted substrates (Mogk *et al.*, 2007).

According to the nature of the amino acid at the amino terminus, there are stabilizing and destabilizing amino acids. The features of proteins that are responsible for metabolic instability are called degradation signals or degrons (Varshavsky, 1991). The degron of the N-end rule pathway (N-degron) is an (unacetylated) bulky first amino acid that is recognized as a signal for ubiquitylation, which leads to proteasomal degradation of the protein. The N-degron is characterized by two fundamental determinants: a destabilizing amino-terminal residue and one or more internal lysines where the substrate gets ubiquitylated (Bachmair and Varshavsky, 1989; Suzuki *et al.*, 1999). The ubiquitylated protein is subsequently recognized and degraded by the 26S proteasome (Varshavsky, 1996). Although the N-end rule pathway can be

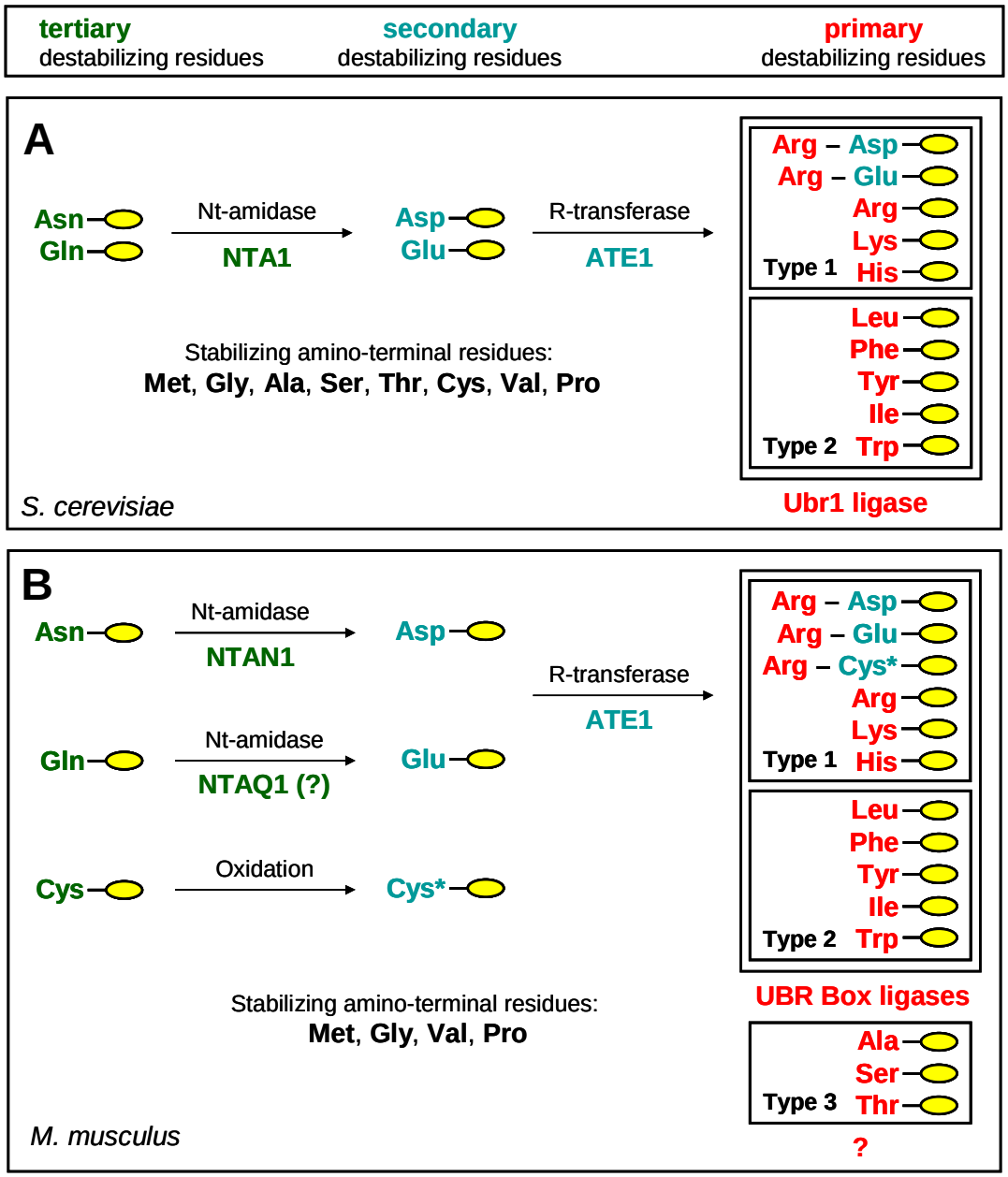

Fig. 1.6: The N-end rule pathway in (A) yeast (S.cerevisiae) and (B) mouse (M. musculus) *- Amino-terminal residues of proteins (yellow) can be divided into stabilizing (black) and destabilizing residues; the latter can be subdivided into primary (red), secondary (blue) and tertiary (green) destabilizing residues. Primary destabilizing residues are classified as type 1 (basic) and type 2 (bulky hydrophobic); type 3 can be found in mammals but not in yeast. Primary destabilizing residues are recognized by special E3 ligases, so-called N-recognins. In yeast, only one N-recognin was identified (Ubr1), whereas in mammals several N-recognins could be found. Secondary destabilizing residues are stable; to be degraded, the primary destabilizing amino acid Arg needs to be conjugated to the residue, which is mediated by the R-transferase ATE1. Similar to this process, tertiary destabilizing residues first have to be deamidated (Asn, Gln) or oxidized (Cys in mammalian cells) to secondary destabilizing residues; subsequently they are processed like secondary destabilising residues. In yeast, the Nt-amidase NTA1 converts both Asn and Gln to Asp and Glu, respectively, whereas in mammals, NTAN1 de-amidates only Asn. The mammalian Nt-amidase for Gln remains unknown. - Figure redrawn from Varshavsky* et al.*, 2000.*

found in all eukaryotes, it is best understood in yeast and mammals whereas the plant N-end rule pathway has only been partly investigated so far. In general, one can distinguish between destabilizing and stabilizing residues, and the destabilizing residues can be divided into primary, secondary and tertiary destabilizing residues (Varshavsky, 1996; Tasaki and Kwon, 2007; Fig 1.6).

Primary destabilizing residues bind directly to an E3 ubiquitin ligase. They can be subdivided into three types of primary destabilizing residues. Type 1 and type 2 can be found both in yeast and in mammals, whereas type 3 occurs only in mammals. Type 1 covers basic amino acids like Arg, Lys and His and type 2 bulky hydrophobic ones like Phe, Leu, Tyr, Trp and Ile. Type 3 comprises Ala, Ser and Thr and can be found in mammals but not in yeast. In yeast these three amino acids have stabilizing effects.

Secondary destabilizing residues are Asp and Glu. To be degraded by the proteasome, proteins with secondary destabilizing residues require modification by the Arg-tRNA-protein transferase (R-transferase) where an Arg moiety is transferred to the amino-terminal amino acid. As Arg is a primary destabilizing residue, the protein is degraded after this ligation like proteins with Arg as the original amino acid at the amino terminus. In yeast, the R-transferase is encoded by *ATE1*, just as in vertebrates. In mammals, alternative splicing of the *ATE1* gene produces at least six isoforms (*ATE1-1* through to *ATE1-6*). In plants, one *ATE1* gene has been isolated so far. A second gene, *ATE2*, has been identified that shares 58 % homology with *ATE1*, although it has not been shown yet that *ATE2* is indeed involved in the N-end rule pathway (Yoshida *et al.*, 2002).

In a similar manner to the secondary destabilising residues Asp and Glu, the tertiary destabilizing residues Asn and Gln are first converted into Asp and Glu by an amino-terminal amidohydrolase (Nt-amidase). This deamidation step turns Asn to Asp and Gln to Glu, the two secondary destabilizing residues. The protein has now a secondary destabilizing residue at the amino terminus

and is degraded as explained before. Whereas yeast has again only one Nt-amidase for both amino acids called NTA1, the deamidation of Asn and Gln in mammalian cells is mediated by two distinct enzymes called NTAN1 and NTAQ1 (Kwon *et al.*, 2000). In spite of intensive research, only one enzyme (NTAN1) has been identified so far whereas the second one, assumed to be responsible for proteins with Gln as last amino acid at the amino terminus, is currently still hypothetical (Tasaki and Kwon, 2007). Besides Asn and Gln, also Cys has been found to be a tertiary destabilizing residue, but only in vertebrates. In yeast, Cys is a stabilizing residue. Like Asn and Gln, the Cys residue has to be converted before it can serve as substrate for the R-transferase. Cys is oxidized with the help of nitric oxide (Hu *et al.*, 2005). In plants, no homologues of the Nt-deamidase gene have been identified so far.

Besides these destabilizing residues, there also exists a group of stabilizing amino-terminal residues including Met, Gly, Val and Pro. Proteins with these amino-terminal residues are metabolically stable and possess a half-life above 20 hours unless they do contain another degron. In yeast, there are also Ala, Ser, Thr and Cys which confer stability to proteins. In mammals, Ala, Ser and Thr are primary destabilizing residues of type 3 (Varshavsky, 1996; Tasaki and Kwon, 2007).

In eukaryotes, primary destabilizing amino acids at the amino terminus are recognized and bound by special ubiquitin ligases called N-recognins (Varshavsky, 1996). In prokaryotes, the selection of N-degrons is mediated by the adaptor protein ClpS (Fig. 1.7). Both specificity factors possess only a limited homology that is restricted to the site of substrate interaction (Mogk *et al.*, 2007). In bacteria, the adaptor protein ClpS shares homology with the substrate binding site of N-recognins and it was shown that ClpS binds to the destabilizing amino-termini of N-end rule substrates that are directly transferred to the ClpAP protease (Mogk *et al.*, 2007). In yeast, only one single N-recognin, the ubiquitin ligase Ubr1, has been identified to be involved in the N-end rule pathway (Bartel *et al.*, 1990; Varshavsky, 2003), whereas in mammals, several different isoforms are expressed from distinct

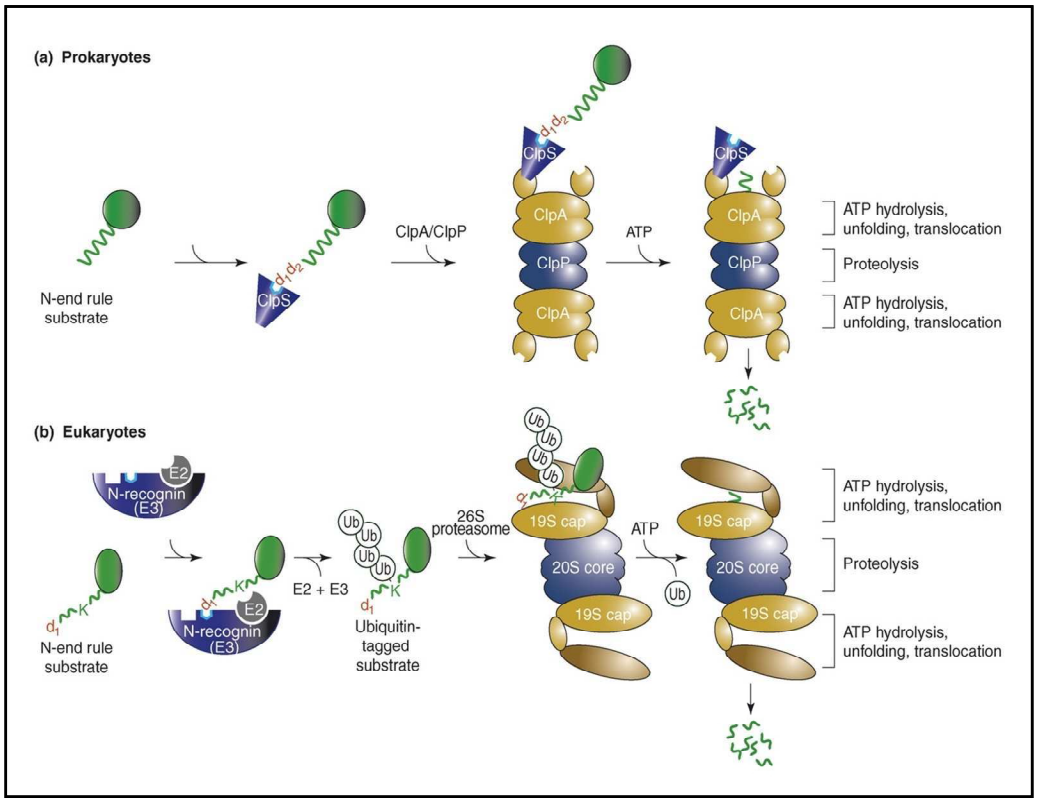

Fig. 1.7: Protein degradation mediated by the N-end rule pathway in prokaryotes (a) and eukaryotes (b). *(a) In bacteria, the four bulky hydrophobic amino acids Phe, Leu, Trp and Tyr are primary destabilizing residues (d1), whereas the basic amino acids Arg and Lys have secondary destabilizing character (d2). Here, the adaptor protein ClpS serves as a functional homolog of a eukaryotic N-recognin but no ubiquitylation takes place. ClpS binds to the target substrate and during a specific interaction with the amino-terminal extra domain of ClpA, the bound substrate is directly delivered to the AAA+ chaperone ClpA for degradation. (b) In eukaryotes, the N-end rule substrate is recognized by the N-recognin, bound and ubiquitylated. As soon as the target protein carries a polyubiquitin chain of at least four moieties, it is recognized by the 19S cap complex of the 26S proteasome and subsequently unfolded and translocated by AAA+ proteins into the 20S core, where the proteolysis takes place. Unlike ClpS in prokaryotes, the eukaryotic N-recognin releases the target protein after ubiquitylation and is not directly involved in its recognition by the proteasome. Figure modified from Mogk* et al.*, 2007.*

genes, all with the common motif of a so-called UBR box, a ~70-residue zinc-finger-like domain (Tasaki *et al.*, 2005). In plants, only two ubiquitin ligases, one for substrates with aromatic amino acids at the amino terminus called PRT1 (Stary *et al.*, 2003) and a second one, responsible for proteins with Arg at the amino terminus called PRT6 (Garzón *et al.*, 2007) have been identified so far to be part of the N-end rule pathway. As it was shown that neither PRT1 nor PRT6 degrades aliphatic amino-terminal residues, the existence of a least one additional N-end rule ligase in plants has been hypothesized (Garzón *et al.*, 2007).

1.2.9 The N-end rule pathway and programmed cell death

It has been shown that the N-end rule pathway is involved in the regulation of peptide import (Turner *et al.*, 2000) and the maintenance of chromosome stability (Rao *et al.*, 2001). In mammals, the N-end rule pathway is also required for cardiovascular development (Kwon *et al.*, 2002) and is involved in the regulation of meiosis (Kwon *et al.*, 2003). Besides these functions, it has also been demonstrated that the N-end rule pathway is involved in controlling the regulation of apoptosis through the degradation of the *D. melanogaster* DIAP1 protein (Ditzel *et al.*, 2003; Varshavsky, 2003). Caspases that are (negatively) controlled by inhibitors of apoptosis (IAPs) have been known to produce protein fragments carrying destabilizing residues at the amino terminus (Varshavsky, 1996). Drosophila DIAP1 is one of those protein fragments that has been shown to be a physiological N-end rule substrate and the degradation of this fragment via the N-end rule pathway is important for the regulation of apoptosis (Ditzel *et al.*, 2003). The major function of IAPs including DIAP1 in eukaryotes is caspase inhibition (Varshavsky, 2003), and although no orthologs of caspases have been identified in plants so far, so-called metacaspases have been discovered (Uren *et al.*, 2000), and were hypothesized to play a role in plant PCD (Koonin and Aravind, 2002), similar to the caspases found in mammals. So far, nine metacaspase genes have been identified in *Arabidopsis*, and one of these genes, metacaspase-8 (*At*MC8), was recently shown to be strongly up-regulated by oxidative stress caused by UVC, H_2O_2 or methyl viologen, which leads to PCD. It was therefore proposed that metacaspase-8 is part of a PCD pathway (He *et al.*, 2008). However, it needs to be analyzed whether metacaspases serve like *Drosophila* DIAP1 as substrates for the N-end rule pathway.

Besides this contribution of the N-end rule pathway to the regulation of apoptosis, it has also been shown that the plant *ATE1* gene, encoding for the R-transferase that mediates the Arg transfer to secondary destabilising residues of N-end rule substrates, is involved in leaf senescence. Analyses of a delayed leaf senescence mutant (*dls1*) revealed that this mutant is

defective in arginyl-tRNA:protein arginyltransferase which is required for the normal progression of leaf senescence. Correct proteolysis by the N-end rule pathway has hence an important physiological function also in the process of leaf senescence in plants (Yoshida *et al.*, 2002).

1.3 Tools for investigations

1.3.1 The UbK48R transgene

One way to improve the understanding of ubiquitin-dependent proteolysis in plants is to manipulate the ubiquitylation process, e.g. the formation of the ubiquitin chain that is necessary to target proteins for degradation. Ubiquitin moieties are linked via Lys 48 to each other and at least four ubiquitin moieties have to be linked in a chain to mark proteins for degradation. To disturb this process, a transgene was designed and introduced into *Arabidopsis thaliana*. This transgene expressed four units of a variant ubiquitin protein where the Lys residue of position 48 was replaced by Arg. In addition to the four repeats of the modified ubiquitin (called UbK48R), a murine dihydrofolate reductase gene (DHFR) was added to the transgene. DHFR confers resistance to Methotrexate (MTX), a chemical normally toxic for plants. If MTX is added to the plant media, it cannot intoxicate the transgenic plant any more; therefore, MTX can be used to select for high transgene expression. When one of the modified ubiquitin moieties is added to a target protein or an already existing chain, no further ubiquitin can be added to position 48 of this variant ubiquitin as the internal Lys for chain elongation is modified, thereby disrupting the chain elongation process (Fig. 1.8).

The variant ubiquitin moiety can be linked normally to a target protein or an already existing ubiquitin chain, but no further ubiquitin moieties can be added to it. This leads to a partial inhibition of the ubiquitin dependent proteolysis in vivo. Experiments with a transgenic plant line that contains a constitutively expressed ubiquitin variant UbK48R showed that continuous

expression of UbK48R leads to cell death at earlier or later stages of development. High mortality rates at various developmental stages resulted in only 40 % surviving plants until seed set, which made it difficult to further investigate the ubiquitylation process.

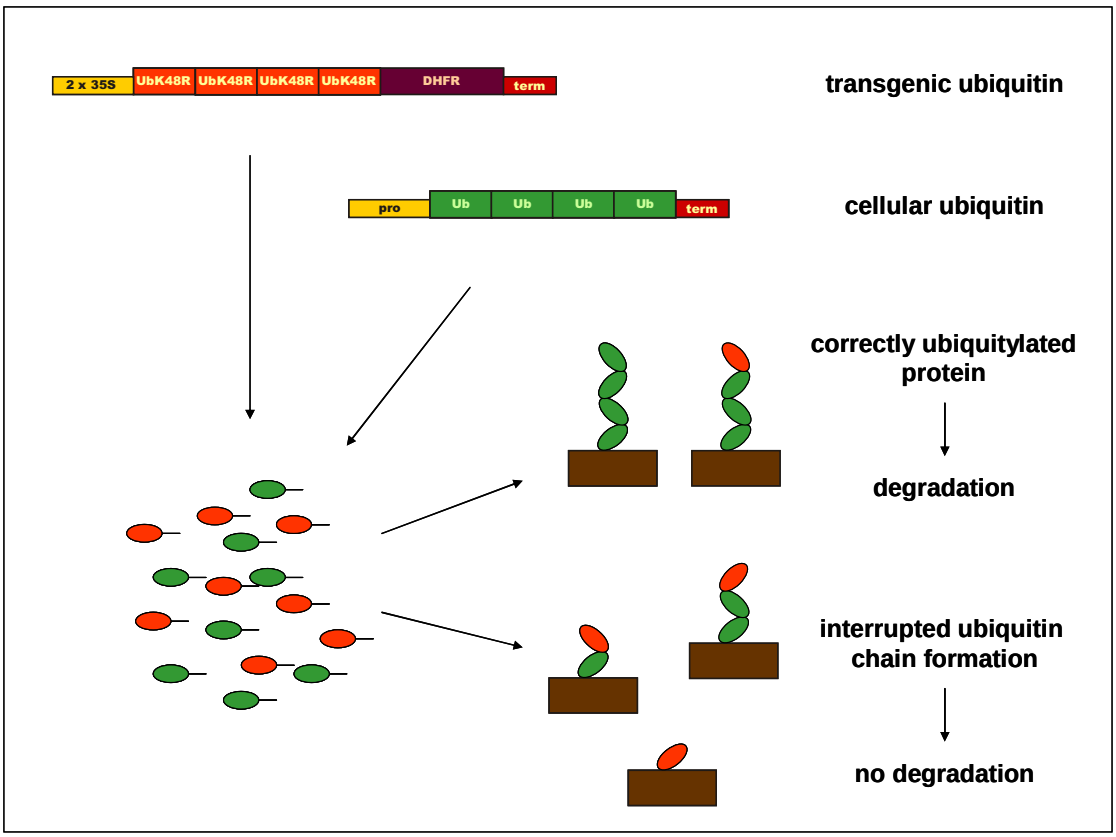

Fig. 1.8: Perturbation of proteolysis in the presence of UbK48R. *In transgenic plants, both modified ubiquitin that is expressed by the transgene and cellular ubiquitin are expressed and randomly chosen for substrate ubiquitylation. As a protein needs a ubiquitin chain of at least four moieties to be recognized for degradation, only proteins that used cellular ubiquitin in the first three ubiquitylation rounds are marked for proteolysis. The protein is not degraded if a modified ubiquitin has been used in one of the first three ubiquitylation rounds. Colour coding: Brown – protein substrate; green – cellular ubiquitin; red – ubiquitin variant UbK48R. Abbreviations: 2x35S – CaMV 35S promoter with doubled enhancer; pro – promoter; term – terminator; DHFR – mouse dihydrofolate reductase; UbK48R – variant ubiquitin; Ub - Ubiquitin.*

1.3.2 The UbK48R-inducible transgene

In order to avoid that transgenic plants die because of the uncontrolled expression of the UbK48R transgene, an inducible vector was constructed to

control the UbK48R expression. With this solution, plants can be grown without damaging transgene expression, and potential effects can be studied at any desired time point after induction, because the starting point of the expression of UbK48R can be chosen by experimental design.

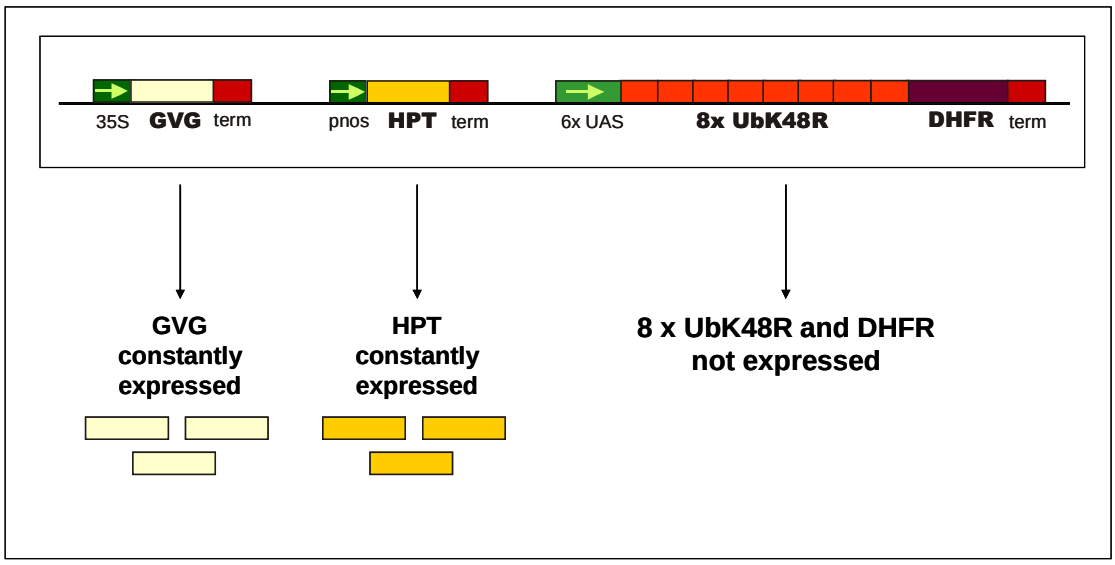

Fig. 1.9: Inducible transgene: Expression in the absence of Dex. *In the absence of Dex, only GVG and HPT are expressed but not the variant ubiquitin UbK48R and DHFR. Therefore, the presence of the transgene can be assessed by adding Hyg to the growth media although UbK48R expression is not taking place. Abbreviations: 35S – CaMV 35S promoter; term – terminator; pnos – nos promoter; 6xUAS – GAL4 upstream activating sequence; DHFR – mouse dihydrofolate reductase; GVG – chimeric transcription factor, consisting of GAL4, VP16 and GR (see text); HPT – hygromycin phosphotransferase; UbK48R – variant ubiquitin.*

The new transgene consists of an inducible part and a constantly expressed part (Fig. 1.9). The inducible part encodes 8 repeats of UbK48R and a DHFR fragment and is under control of a promoter with six copies of the GAL4 upstream activating sequence (6xUAS). Besides this, the transgene constantly expresses a hygromycin phosphotransferase (HPT) and the chimeric transcription factor GVG. HPT confers resistance to hygromycin (Hyg) and can be used for selection of the transgene. The GVG consists of the DNA-binding domain of the yeast transcription factor GAL4, the transactivating domain of the herpes simplex viral protein VP16 and the receptor domain of the rat glucocorticoid receptor (GR). It is transcribed from a cauliflower mosaic virus (CaMV) 35S promoter (Aoyama and Chua, 1997). When Dexamethasone (Dex), a synthetic glucocorticoid, is added, it binds to

the GVG transcription factor so that the GVG-Dex complex can now bind to the 6xUAS promoter of the inducible part and activate the transcription of the octa-ubiquitin UbK48R and the DHFR (Fig. 1.10).

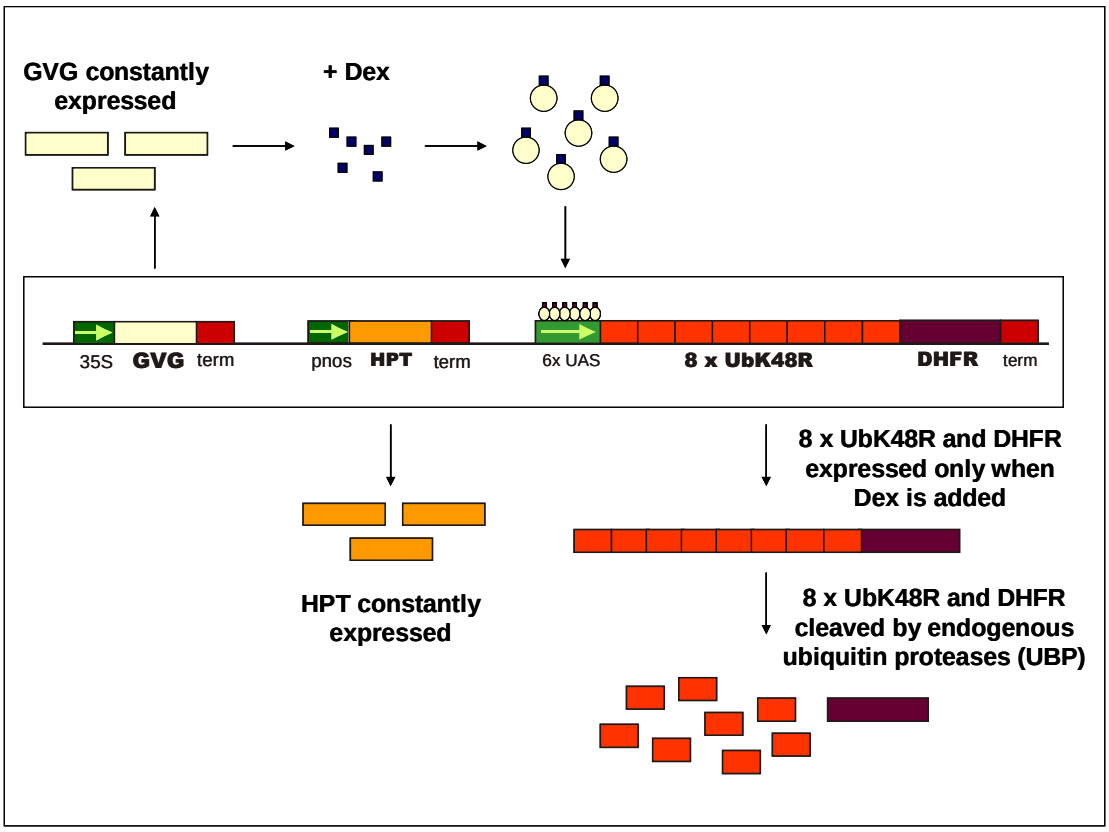

Fig. 1.10: Inducible transgene: Expression in the presence of Dex. *Like in the absence of Dex, GVG and HPT are expressed. Due to the presence of Dex, GVG forms now a complex with the glucocorticoid and binds to the 6xUAS promoter that initiates also the expression of UbK48R and DHFR. Abbreviations: 35S – CaMV 35S promoter; term – terminator; pnos – nos promoter; 6xUAS – GAL4 upstream activating sequence; DHFR – mouse dihydrofolate reductase; GVG – chimeric transcription factor, consisting of GAL4, VP16 and GR (see text); HPT – hygromycin phosphotransferase; UbK48R – variant ubiquitin.*

The inducible transgene was transformed into *Arabidopsis thaliana* Columbia WT plants. Plant line RV86-5 showed best results and was therefore selected as main transgenic line for further investigations (Schlögelhofer, 2002). All experiments in this work were carried out with RV86-5.

1.3.3 The ubiquitin fusion degradation technique

To study the role of different N-terminal amino acids in protein degradation and to identify potential ubiquitin ligases, the ubiquitin fusion degradation technique was used for the experimental work. This method, developed by Varshavsky and co-workers (Lévy *et al.*, 1996; Varshavsky, 2005), allows investigating the stability of test proteins. For these purposes, a fusion protein consisting of a potentially unstable test protein and a metabolically stable reference protein is transformed into the plant. After its synthesis, it is cleaved into two independent proteins. Whereas the reference protein is stable and serves as control of the correct expression of the fusion protein, the test protein can be examined for its stability. The ubiquitin fusion degradation technique has already been applied successfully in different variations in plants (Stary *et al.*, 2003; Karsies *et al.*, 2001).

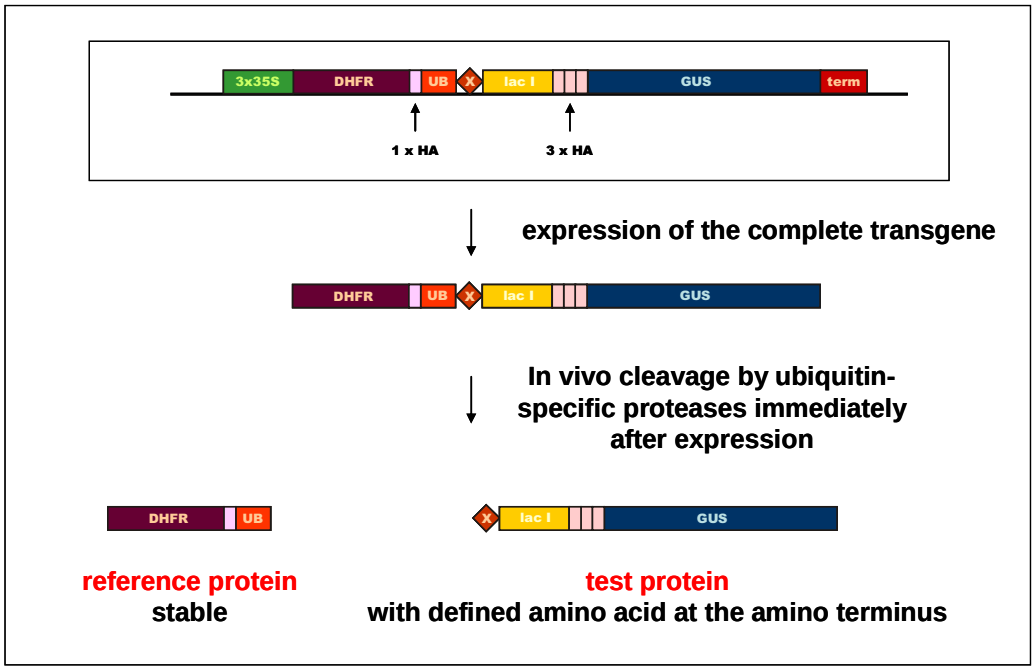

Fig. 1.11: The ubiquitin protein fusion technique. *A fusion protein is expressed and shortly after translation cleaved into two independent proteins by ubiquitin-specific intracellular proteases that cut the fusion protein in such a way that a defined amino acid is exposed at the amino terminus of the test protein. The reference protein is stable; an accumulation of the test protein can be visualized with GUS staining. Abbreviations: 3x35S – triple CaMV 35S promoter; term – terminator; DHFR – mouse dihydrofolate reductase; HA – influenza hemagglutinin epitope; UB – UbK48R variant ubiquitin with defined amino acid at the carboxyl terminus; lac I – part of the* E. coli *lac I protein; GUS – β-glucuronidase; X – defined amino acid (Phe, Leu, Met or Arg).*

The fusion protein that was used for studying the plant N-end rule proteolysis in this work consisted of a stable monomeric protein (the murine dihydrofolate reductase DHFR), a UbK48R variant ubiquitin with a defined amino acid on its carboxyl terminus (Phe, Leu, Met or Arg), an *E. coli* β-glucuronidase (GUS) protein and influenza hemagglutinin (HA) epitopes. One HA epitope tag was inserted between the DHFR and the variant ubiquitin, and a triple HA epitope tag was fused to the GUS protein. The GUS ORF was extended by unstructured amino acids (from the *E. coli* lac I protein) to amplify the effect of destabilizing amino-terminal residues (Fig. 1.11).

The fusion protein is expressed as one continuous reading frame. Its cleavage takes place during or shortly after its translation. Cleavage occurs by ubiquitin-specific intracellular proteases that recognize the ubiquitin moiety at the carboxyl end of the reference protein. The fusion protein is cleaved into two independent proteins: One stable reference protein with DHFR, UbK48R and one single HA tag, and a test protein containing the β-GUS cassette, a triple HA epitope and a defined amino acid at the amino terminus.

Due to the design, the HA epitope tags can be used for qualitative and quantitative analyses: As they appear in both the reference and the test protein, the presence of both proteins can be detected immunologically with the same antibody. In addition to this, also the quantity of each protein can be calculated with the different intensity of protein amount due to the different number of HA tags present in the two proteins.

1.4 Aim of work

The aim of this work was to learn more about the role of ubiquitin in cell death programs of plants. Therefore, the *Arabidopsis* transgenic plant line RV86-5 was further characterized. RV86-5 carries an inducible transgene that expresses a variant ubiquitin called UbK48R. In the UbK48R sequence, Lys 48 is changed to Arg. As a consequence, ubiquitin chain formation, which is

necessary to target proteins for degradation, is perturbed. Substrates accumulate in the cell which leads to cell death (Schlögelhofer *et al.*, 2006).

To gain further insight into UbK48R-induced cell death, two different strategies should be pursued: In a first approach, the physiological pathways in RV86-5 should be analysed to further characterize the type of cell death observed after UbK48R expression. A second approach should use EMS mutants, recently generated in the RV86-5 line, to isolate and to further investigate mutants that suppress the cell death phenotype. The EMS-induced alleles of the most promising mutant lines should be mapped to identify components involved in the UbK48R-induced cell death.

A second line of research was the analysis of the N-end rule pathway, a distinct protein degradation path of the ubiquitin-proteasome system that has been shown to be involved in senescence (Yoshida *et al.*, 2002), which is a plant specific type of programmed cell death. The N-end rule connects the fate of a protein with the nature of the amino acid at the amino terminus (Bachmair *et al.*, 1986). As only little is known about the N-end rule pathway in plants, a gene with a potential role in the N-end rule degradation pathway of proteins should be analysed with the help of model substrates generated by the ubiquitin protein fusion technique. This method permits to create test proteins with a defined amino acid at the amino terminus and to investigate the fate of this test protein in the cell. The first aim was to generate plant lines that carry both a test protein with a distinct amino acid at the amino terminus and a mutation either in an already known N-end rule ligase, in the predicted gene of interest, or in both. After identification of these mutant lines, histochemical and immunological analyses should reveal the potential participation of the gene of interest in the N-end rule pathway.

2 MATERIAL AND METHODS

2.1 Material

2.1.1 Chemicals and enzymes

Chemicals used in this work were ordered from the following companies: Duchefa (Haarlem, The Netherlands), Merck (Darmstadt), Roche (Penzberg), Roth (Karlsruhe), Serva (Heidelberg) and Sigma-Aldrich (Steinheim). Enzymes were purchased from Fermentas (St. Leon-Rot), Invitrogen (Karlsruhe), New England Biolabs (Frankfurt/M) and Roche (Penzberg).

2.1.2 Plant material

All plant lines that were used in this work were *Arabidopsis thaliana*.

Accession Columbia (Col-0)
Accession Landsberg *erecta* (L*er*)
RV86-5 in accession Col-0 (transgenic plant line as described in Schlögelhofer, 2002)
prt1 (from Potuschak *et al.*, 1998)

2.1.3 Media for Arabidopsis thaliana

Ara plates:

In general, seeds were put on plates with solid Ara medium (Ara plates):

Plates for *Arabidopsis* seedlings:

Ara medium:	MS salt	4.3 g
	Sucrose	10 g (1 %)
		30 g (3 %) for early experiments
	MES	0.5 g
	Plant agar (Merck)	8 g
	pH 5.7 with KOH	
	=> ad 1000 ml with dH_2O	
500 x vitamin-mix:	Myo-inositol	5 g
	Thiamine	1 g
	Nicotinic acid	50 mg
	Pyridoxine	50 mg
	Biotin	10 mg
	=> ad 100 ml with dH_2O	

After autoclaving and before use, 2 ml of the 500 x vitamin-mix were added to 1000 ml of liquid medium.

Plates that were used for the transgenic plant line RV86-5 additionally contained hygromycin (25 mg/ml) for transgene selection.

HD plates:

To induce the transgene, Dexamethasone (Dex) was added to the medium to a final concentration of 0.7 µM. Dex was dissolved in 96 % EtOH, the concentration of the stock solution was 7 mM.

HDM plates:

In addition to Dex, also Methotrexate (0.1 mg/l) was added to the medium to rule out silencing of the transgene. Methotrexate was dissolved in 0.1 M HCl, the concentration of the stock solution was 1 mg/ml.

Liquid growth medium:

For kinetic studies, plants were grown in ½ GAMBORG's B5 liquid medium and seeds were put directly into the flask.

½ GAMBORG's B5 medium:

GAMBORG's B5 Basal salt mixture	1.526 g
Sucrose	5 g
pH 5.7 with KOH	
=> ad 1000 ml with dH_2O	

After autoclaving and before use, 0.5 ml 1000 x GAMBORG B5 vitamin mix (Duchefa) was added.

Plates for sensitivity assay:

For the sensitivity assay, all substances listed in Table 2.1 were used. Hormones and salts were added to normal Ara medium; for the different sugars, sucrose was replaced in each particular medium.

Substance	Concentration in medium	Concentration of stock solution	Solvent
Sucrose	1 % [30 mM]		
Sucrose	6 % [175 mM]		
Glucose	0.3 % [15 mM]		
Glucose	3.2 % [175 mM]		
Mannitol	3.6 % [200 mM]		
NaCl	[150 mM]	[5 M]	dH_2O
LiCl	[100 mM]	[1 M]	dH_2O
ABA	[1 µM]	[10 mM]	DMSO
ACC	[1 mM]	[25 mM]	dH_2O
MeJA	[5 µM]	[5 mM]	EtOH
SA	[0.5 mM]	[0.5 M]	EtOH
2-iP	[0.5 mg/l]	[5 mg/ml]	DMSO
2,4-D	[0.4 mg/l]	[0.5 mg/ml]	EtOH

Table 2.1: List of substances that were added to Ara medium to assess the sensitivity of* Arabidopsis thaliana *plants to the diverse sugars, salts and hormones. *For the different salts and hormones, stock solutions were prepared and the adequate amount was added to the already prepared Ara medium, whereas the different sugars were directly replaced during the preparation of the Ara medium.*

2.1.4 Markers and antibodies

Markers for Agarose gels:

1 kb (GeneRuler 1 kb ladder Fermentas; 1kb ladder, NEB)

100 bp (GeneRuler 100 bp DNA ladder Fermentas; 100 bp ladder, NEB)

Markers for protein gels:

Protein marker: 7 µl of PageRuler^{TM} Prestained Protein Ladder (Fermentas)

Antibodies used for Western blot:

anti-HA antibody from rats (Roche)
IR Dye^{TM}800 Conjugated Affinity Purified anti-Rat IgG from goats (Rockland)

2.1.5 Buffers and solutions

2.1.5.1 General buffers and solutions

Calciumhypochlorite solution (CaOCl) for seed sterilization:

CaOCl	15 g
dH_2O	500 ml

The solution is stirred for 15 min and stored overnight in the fridge to let the insoluble rest of the CaOCl deposit. Before use, 0.1 % of Triton-X100 (20 %) is added to the solution.

Extraction buffer for isolation of genomic DNA for PCR:

Tris-Cl pH 7.5	200 mM
NaCl	250 mM
SDS	0.5 %
EDTA	25 mM

Ferguson solution for protein isolation:

Tris-Cl pH 6.8	50 mM
SDS	4 %
β-Mercaptoethanol	10 %

GUS-reaction solution:

Na-Phosphate, pH 7	100 mM
EDTA	10 mM
K-Ferricyanide	0.5 mM
K-Ferrocanide	0.5 mM
Triton X-100 20 %	0.1 %
X-Gluc	1 mM

TE solution:

Tris-Cl pH 7.5	10 mM
EDTA	1 mM

50 x TAE stock solution:

Tris base	242 g
Glacial acetic acid	57.1 ml
Na_2EDTA x 2 H_2O	37.2 g

6 x DNA loading buffer:

Glycerol	50 %
EDTA pH 8.0	0.2 M
Orange G	0.005 %

2.1.5.2 Buffers and solutions for Northern blot

MOPS:	MOPS	41.8 g
	=> ad 800 ml with DEPC-dH_2O	
	adjust pH 7.0 with NaOH	
	3 M DEPC-NaOAc	16.6 ml
	0.5 M DEPC-EDTA	20 ml
	=> ad 1000 ml with DEPC-dH_2O	
6 x RNA loading buffer:	Ficoll	150 mg
	0.25 mM EDTA	200 µl
	Orange G	0.15 %
	=> ad 1000 µl with DEPC-dH_2O	
MasterMix (per sample):	10 x MOPS	2.4 µl
	Formaldehyde	4.5 µl
	Formamide (deionised with mixed-bed resin, Biorad)	12 µl
	DEPC-dH_2O	4.5 µl
	6 x RNA loading buffer	0.75 µl
RNA marker:	0.24 – 9.5 kb RNA ladder (Invitrogen)	3 µl
	MasterMix	24 µl
	Ethidium bromide [10 mg/ml]	0.53 µl
20 x SSC:	3 M NaCl	
	0.3 M Na-Citrate x 2 H_2O	
	=> adjust pH 7.0 with 1 M NaOH	
Church-and-Gilbert buffer:	1 % BSA	
	1 mM EDTA	
	0.5 M Na-phosphate buffer	
	=> adjust pH 7.2	
	7 % SDS	

2.1.5.3 Buffers and solutions for Protein gels and Western blot

12 % polyacrylamide gel – separating and stacking gel:

Separating gel:	30 % acrylamide	2 ml
	1.5 M Tris (pH 8.8)	1.3 ml
	10 % SDS	50 µl
	10 % APS	50 µl
	TEMED	4 µl
	dH_2O	1.6 ml
Stacking gel:	30 % acrylamide	330 µl
	1M Tris (pH 6.8)	250 µl
	10 % SDS	20 µl
	10 % APS	20 µl
	Bromophenolblue	0.003 %
	TEMED	2 µl
2 x loading sample buffer (LSB):	Glycerol	50 %
	DTT	20 mM
	SDS	2 %
	Tris-Cl pH 6.8	125 mM
	Bromophenolblue	0.003 %
Electrophoresis buffer (5 x):	Tris	7.55 g
	Glycine	36 g
	SDS	2.5 g
	=> ad 500 ml with dH_2O	
Transfer buffer:	Glycine	190 mM
	Tris	25 mM
	Methanol	20 %
	SDS	0.05 %

10 x PBS:	Na_2HPO_4 (anhydrous)	10.9 g
	NaH_2PO_4 (anhydrous)	3.2 g
	NaCl	90 g
	=> ad 1000 ml with dH_2O, pH 7.2	

2.1.6 Primers

Primers for *sud* mapping:

nga63-F	5' - GCC TAA ACC AAG GCA CAG AAG - 3'
nga63-R	5' - TCA TCA GTA TTC GAC CCA AG - 3'
F3F19-F	5' - CCA CAA AAC AAT TTG GTT CAC TC - 3'
F3F19-R	5' - TCC CGT TGG GGA TAT TAA AG - 3'
F7P12-F	5' - TCG AGG ATA TGT TTC GTG TTT G - 3'
F7P12-R	5' - ACA GTT TTG ATG CAT TGT GTG AG - 3'
F2J6-F	5' - CAA GAT GGG CTG CCA TAT TAT C - 3'
F2J6-R	5' - TAA CAG GAA GTC CAC CGT CAG - 3'
ciw1-F	5' - ACA TTT TCT CAA TCC TTA CTC - 3'
ciw1-R	5' - GAG AGC TTC TTT ATT TGT GAT - 3'
F23H11-F	5' - GAT ATG GGA GTA AGT ATG AAA TCG G - 3'
F23H11-R	5' - TTC GTC CGG GTA AAA GTC AAG - 3'
NF5I14-F	5' - GTTGAGTCTTGGCATCACAGTTC-3'
NF5I14-R	5' - CTG CCT GAA ATT GTC GAA AC - 3'
AthATPASE-F	5' - CCT GGG AAC GGT TCG ATT CGA G - 3'
AthATPASE-R	5' - GTT CAC AGA GAG ACT CAT AAA CCA - 3'
F18P14-F	5' - ATT CCC GCA ATT TAT TTT GTT C - 3'
F18P14-R	5' - GTT TGA TGG CAG ATT TGT TTT C - 3'
T20K24-F	5' - CTT GCA CTT GTG GAT GAT GAG - 3'
T20K24-R	5' - CTT TGT TGA GCC CTT CGT TC - 3'
F2H17-F	5' - ATT GCA TAC CAC GCA GTT CAC - 3'
F2H17-R	5' - CCA TTT TGC CCT TTC CTT CTA C - 3'
nga162-F	5' - CAT GCA ATT TGC ATC TGA GG - 3'
nga162-R	5' - CTC TGT CAC TCT TTT CCT CTG G - 3'
ciw11a-F	5' - GTT TTT TCT AAT CCC CGA GTT GAG - 3'
ciw11a-R	5' - GAA GAA ATT CCT AAA GCA TTC - 3'

MZN14-F	5' - CAA TAC ACT TTA TCC AGA TGC TG - 3'
MZN14-R	5' - GGG ATT TGT TGA TTG AAA AAG GAC - 3'
MUO22-F	5' - ATT GAT CAT ATC GCC CAA CAC - 3'
MUO22-R	5' - ACA TTG CAG CAG GAT AGG TTG - 3'
T32N15-F	5' - ATC TGA AAA TCC TTG CGT GAG - 3'
T32N15-R	5' - TTG TGA CGA ATA GTG AAA GGA GAG - 3'
T6H20-F	5' - CGG CTG AAA CTT GGA AGG GAC - 3'
T6H20-R	5' - AGG AAG AAC GTG TGA TTG TG - 3'
ciw4-F	5' - GTT CAT TAA ACT TGC GTG TGT - 3'
ciw4-R	5' - TAC GGT CAG ATT GAG TGA TTC - 3'
F27K19-F	5' - TGC TTT TGA AGA GAT GGT TAT TAG G - 3'
F27K19-R	5' - CCC CAT TTC ACT TAT CAT TGG - 3'
nga6-F	5' - AGC GAA TCC GAA AAT AAT GGA G - 3'
nga6-R	5' - TGG ATT TCT TCC TCT CTT CAC - 3'
ciw5-F	5' - GGT TAA AAA TTA GGG TTA CGA - 3'
ciw5-R	5' - AGA TTT ACG TGG AAG CAA T - 3'
C6L9-F	5' - GTG CCT TTG TTC AAT TCT AGT G - 3'
C6L9-R	5' - AAA CAA CTA AAT CGC CGT CAC - 3'
T3H13-F	5' - TTT GGT GGG TCA AGA GTC AAG - 3'
T3H13-R	5' - GCA AAA GTC ATT ACG GAC AAT AC - 3'
T26M18-F	5' - CAA TTA GCG GAG GCC ACT TC - 3'
T26M18-R	5' - GGG CAA AAG CTT CCA GTA C - 3'
F28A21-F	5' - GCA TCA TCA TTC ATC ACC AAC - 3'
F28A21-R	5' - TGT GAA GTG TTT GTC TTT GTG - 3'
F6E21-F	5' - TTC TTT GTT CAA GTT CCA TGT CTC - 3'
F6E21-R	5' - CGG TGA TTG TCT CAA GTG TTT G - 3'
F23E13-F	5' - TGA CCG TTG AAA GTG TTG TTG - 3'
F23E13-R	5' - GCC CGA GAA GCC TGA TAG - 3'
MHF15-F	5' - CTC CTC CTT TAA TTT TCT CTC TGT G - 3'
MHF15-R	5' - AGT TCC AGC TTT GGA CTT CTT C - 3'
nga151a-F	5' - ATC TCA TAC TGA CCC ATA TGT TCC - 3'
nga151a-R	5' - ATT GTA CAG TCT AAA AGC GAG AG - 3'
nga139-F	5' - AGG GTT TCG TTT CAC TAT CCA G - 3'
nga139-R	5' - TGA GAG CTA CCA GAT CCG ATG - 3'
MPL12-F	5' - GTC CCC AAA ACC AAT CAT AAG - 3'

MPL12-R	5' - TCC GAG TGA GAA GAG AGT TTG - 3'
MNC17-F	5' - GTA CCG GAT CTG TGT TGT GAA G - 3'
MNC17-R	5' - GTG CTC AAG GAA ATG GGA TAG - 3'

Primers for N-end rule analysis:

GarlicLB1	5' - GCC TTT TCA GAA ATG GAT AAA TAG CCT TGC TTC C - 3'
PRT 6602-6582	5' - CCA CCT TCT GTT TAT CTA CAC - 3'
PRT 6423-6446	5' - CAG AGG AAG AGC AAG AAC GAG AAT - 3'
ubrdn3	5' - GTT TCT TGT TCT GGG GAG GAT GGT TT - 3'
ubrup2	5' - CAG GTC ACG GGG TAA AGA TTT GAC C - 3'
ubrup3	5' - AGG ACA ATA GGT ACA TAC TCA TTT GTT - 3'

Primers for RT-PCR and transgene sequence analysis:

35Sp	5' - CAC TGA CGT AAG GGA TGA CGC AC - 3'
DHFR end seq	5' - CTC TGA GGT CCA GGA GGA A - 3'
GUSdn	5' - CTG TGG GCA TTC AGT CTG GAT C - 3'
GUSup	5' - GGG ATA GTC TGC CAG TTC AGT TC - 3'

Primers for transgene detection:

DHFRup	5' - ATC CCC ATA TTT TGG GAC AC - 3'
pTAdown	5' - GAC CCT TCC TCT ATA TAA GGA AGT TC - 3'

2.2 Methods

2.2.1 Seed sterilization

All seeds that were put on solid or in liquid growth medium were sterilized to avoid bacterial or fungal growth. Therefore, a small amount of seeds (50 to 100) were put into a 1.5 ml Eppendorf tube and 0.5 ml Calciumhypochlorite solution was added. The seeds were shaken for 15 min at room temperature, centrifuged for 15 sec and washed with 0.5 ml sterile dH_2O for three times. After washing, the seeds were dried in the lamina flow for at least one day and then spread on plates.

2.2.2 Plant growth

In general, sterilized seeds of A*rabidopsis* were put on plates with solid Ara medium. To vernalize the seeds, the plates were incubated for 3 days at 4 °C before they were moved to the growth chamber. Seedlings with a minimum age of three weeks were put on soil and grown in the green house. All plants were grown under long day conditions (16 h light / 8 h dark).

For kinetic studies, RV86-5 was grown in liquid culture. Therefore, 50 to 75 sterilized seeds of RV86-5 were put into a flask with 100 ml liquid ½ GAMBORG's B5 medium supplemented with vitamins. The flasks were put in a growth chamber under long day conditions (16 h light / 8 h dark) on a rotating platform to assure constant shaking. 14 days after sowing, the transgene was induced with Dex (stock solution: [70 mM], dissolved in DMSO), when 143 µl of the stock solution was added to 100 ml of liquid media to obtain a final concentration of 100 µM. Control flasks were supplemented with the same amount of pure DMSO to exclude side effects of the solvent. Samples were harvested before transgene induction, and seven and 14 days after induction, respectively. For harvesting, the whole plant content of one flask was first dried on filter paper and then frozen in liquid nitrogen.

2.2.3 Isolation of DNA, RNA and proteins from Arabidopsis

2.2.3.1 Isolation of genomic DNA for PCR

Fresh plant material was harvested, put into a 1.5 ml Eppendorf tube and deep frozen in liquid nitrogen. For DNA preparation, a spoon full of sand and 200 µl extraction buffer were added to the frozen plant material and homogenized with an electric homogenizer until no further plant pieces were visible. After adding another 200 µl extraction buffer and vortexing, the sample was centrifuged (14000 rpm at RT for 5 min) and the supernatant was transferred into a new Eppendorf tube. One volume of isopropanol was added

and the sample was kept at room temperature for 5 min after inverting for three to four times. The DNA was precipitated by centrifugation (14000 rpm at RT for 5 min) and the pellet was washed with 500 µl 70 % EtOH. After drying the pellet in a speed vac the pellet was resuspended in 70 µl TE and heated for 10 min at 65 °C (stirring with a yellow tip after 5 min). The dissolved DNA was transferred into a fresh Eppendorf tube and kept at -20 °C for further use.

2.2.3.2 Isolation of genomic DNA for quantitative analysis

The plant material was ground to a fine powder in liquid nitrogen under constant cooling. Total DNA was isolated from 1 g of ground tissue powder by using the Qiagen DNeasy Plant Maxi Kit (68163, Qiagen) according to the manufacturers' protocol with the following alterations: No sample was vortexed but only inverted carefully and the lysate was filtrated after precipitation through Miracloth (Calbiochem) when poured on the Qiashredder. At the end, the DNA was eluted in two steps with a total of 1.8 ml AE buffer. The DNA was precipitated with 1/10 vol. of 3 M KOAc and 2.5 vol. 96 % EtOH at -20 °C overnight. After centrifugation (13500 rpm at 4 °C for 30 min), the DNA was washed with 70 % EtOH and recentrifuged (13500 rpm at 4 °C for 5 min). Finally, the dried DNA was resuspended four times with a total of 120 µl dH_2O.

2.2.3.3 Isolation of RNA

RNA was extracted from fresh plant material frozen in liquid nitrogen. Grinding was carried out in a pre-cooled mortar with liquid nitrogen, and the plant material was kept deep frozen during the whole grinding process. For RNA isolation, 100 mg of the ground plant powder was used; RNA was purified by using RNeasy Plant Mini Kit (Quiagen, 74104) according to the manufacturers' protocol and at the end, the RNA was eluted in 2 x 30 µl RNase-free dH_2O.

2.2.3.4 Isolation of proteins

Fresh plant material was harvested into a 1.5 ml Eppendorf tube and deep frozen in liquid nitrogen. For protein isolation, a spoon full of sand and 200 µl prewarmed (5 min at 37 °C) Ferguson solution were added to the frozen plant material and ground with an electric homogenizer until no further plant pieces were visible. The samples were then centrifuged (14000 rpm at RT for 1 min) and the supernatant was transferred into a fresh tube. Afterwards, the solution was incubated for 10 min at 95 °C and in-between the samples were vortexed briefly for two to three times. Finally, they were again centrifuged (14000 rpm at RT for 10 min) and the supernatant was mixed with 1 vol. of 2 x LSB in a fresh Eppendorf tube. The samples were stored at -20 °C until they were further analysed on a polyacrylamide gel.

2.2.4 Chemical genetics

For the chemical genetics assay, the Syngenta Chemical Compounds Library was screened. Therefore, 20 to 30 seeds of transgenic plant line RV86-5 were put on 1 ml of solid medium that was poured into a well of a 24-well tissue culture plate. The medium was supplemented with vitamins and, according to the assay, with Dex and/or with one of the different substances of small molecular weight with unknown identity. Dex was added to a final concentration of [0.6 µM] and the different substances to a final concentration of 6 ppm, 3 ppm, 1 ppm and 0.3 ppm. All chemicals were dissolved in DMSO and the same volume of DMSO (2 %) with or without additional substance was added to the Ara medium to exclude interference with DMSO. The seeds were grown in a light chamber under long day conditions. For control, RV86-5 seeds and Col-0 WT seeds were used.

Similar to the experimental conditions for the screening of the Syngenta Chemical Compounds Library, the proteasome inhibitor MG132 (Biomol) was tested. Here, no Dex was added to the medium, and the concentrations of MG132 varied between 20 µM and 400 µM. MG132 was dissolved in DMSO, the concentration of the stock solution was 20 mM.

2.2.5 Northern blot

For Northern blot, RNA was isolated (cf. 2.2.3.3) and 1/10 of the eluted RNA was loaded on a 1 % Agarose gel to estimate its concentration. 7 µg RNA of each sample was used for the Northern blot and precipitated with 1/10 vol. 3 M NaOAc, pH 5.2 (treated with DEPC-dH_2O) and 2.5 vol. of 96 % EtOH at -20 °C overnight. The samples were centrifuged (14000 rpm at 4 °C for 15 min), washed in 80 % DEPC-EtOH and dried in a speed-vac. The pellet was dissolved in 24 µl MasterMix on ice for 10 min.

The samples and the marker were heated up to 95 °C for 5 min and thereafter kept on ice. Just before loading, they were centrifuged briefly to collect any condensate. For the gel, 1.4 g Agarose was boiled in 100 ml DEPC-dH_2O. After cooling down, 11.16 ml 10 x MOPS and 3.5 ml formaldehyde were added and the gel was poured. As running buffer, 1 x MOPS was used at 100 Volts. After the gel run, all parts that did not contain any sample were removed, the upper left corner of the gel was cut off and a picture was taken with a ruler placed left to the gel. The gel was incubated for once in DEPC-dH_2O and twice in 10 x SSC to remove the formaldehyde, each time for 15 min. A nylon membrane (Hybond-N nylon membrane, Amersham) cut the same size as the gel was dipped in 50 % methanol (mixed with DEPC-dH_2O) and rinsed in DEPC-dH_2O and 20 x SSC. Finally, the RNA was blotted on the membrane in 20 x SSC overnight and cross-linked with a UV-Stratalinker (Stratagene) the next day.

Probes for Northern blot were produced by PCR with Brown-Taq and purified from the 1 % Agarose gel using NucleoSpin-Extract Kit II (Macherey-Nagel 740609.050), according to the manufacturers' protocol. Two different probes were used:

Probe against cDNA fragment of DHFR:
0.5 kb *Xho*I and *Xba*I fragment from pRTMR, provided by Andreas Bachmair

Probe against the Ch42 locus (=Chlorata 42, At4g18480):
1.7 kb *Xho*I and *Hin*dIII fragment from the Ch42 plasmid, provided by Andreas Bachmair (Koncz *et al.*, 1990)

For hybridization of the blot with the probes, the membrane was first pre-hybridized in 20 ml Church-and-Gilbert buffer at 65 °C for at least 30 min. The radioactive labelling of the probe was done using a hexa-nucleotide mix (Roche):

DNA	60 – 90 ng
=> ad 19 µl with dH_2O	
denatured for 5 min at 95 °C, then kept on ice	
10 x random hexa-nucleotide mix, on ice	3 µl
5mM dNTP without dCTP, on ice	3 µl
α32-P dCTP (~ 40 µCi / µl)	4 µl
Klenow-polymerase (2 U/ µl), Roche	1 µl
=> total volume: 30 µl; incubated for 1 h at 37 °C	

After exchanging the buffer with a fresh aliquot, the denatured probe (5 min at 90 °C) was added to the membrane and the blot was hybridized at 65 °C overnight. The next day, the blot was rinsed with 30 ml of the first washing solution (2 x SSC, 0.1 % SDS) and then incubated with 50 ml of the first washing solution at 65 °C. After 40 min it was replaced by 50 ml of the second washing solution (1 x SSC, 0.1 % SDS) for washing at 65 °C for 25 min, then the membrane was incubated with the third washing solution (0.5 x SSC, 0.1 % SDS) for 15 min at the same temperature. The last washing was done with the forth washing solution (0.1 x SSC, 0.1 % SDS) at 65 °C for 3 min. The membrane was sealed in a plastic bag and was exposed to X-OMAT AR films (Kodak) and to a phosphor-imager screen. The screen was scanned with a Typhoon scanner and the results analyzed with the software package ImageQuant (GE Healthcare/ Amersham).

To remove the radioactive probe from the membrane, the Northern blot was incubated with the stripping solution (60 % deionised formamide, 2 x SSC) at

75 °C. After 90 min, the blot was rinsed with 50 ml of the first washing solution and incubated with another 50 ml of the same solution for 5 min at 65 °C.

2.2.6 Western blot

For Western blot, the investigated proteins were first isolated (cf. 2.2.3.4) and then separated on a polyacrylamide gel. Therefore, 10 µl of protein extract from the investigated plant line was mixed with 2 x LSB and incubated for 5 min at 99 °C. Afterwards, the samples were separated on a 12 % polyacrylamide gel. The gels were run at 120 V, and after the electrophoresis, the stacking gel was removed. To compare the amount of protein extract used for the blotting, a gel with the same amount of proteins was stained with 20 ml of Imperial™ Protein Stain (Pierce) for 2 h and afterwards destained with dH_2O.

To determine the amount of the protein of interest, the protein extracts were transferred from the gel to a membrane via electro-blotting. Therefore, the polyacrylamide gel was rinsed after the gel electrophoresis for a few minutes with dH_2O and transfer buffer. In addition, the membrane (Whatman Protran BA85) was incubated for 10 min in transfer buffer. Then the blot was set up in the following order: sponge, 2 sheets of 3 MM gel blotting paper, polyacrylamide gel, membrane, 2 sheets of 3MM gel blotting paper, sponge. After one hour of blotting at 50 V and 4 °C, the gel was discarded and the membrane was treated according to the protocol recommended when working with the Odyssey Infrared Imaging System (LICOR Biosciences). The membrane was first washed for several minutes in 1 x PBS and then blocked in Odyssey Blocking Buffer (LICOR Biosciences) for one hour. Afterwards, it was incubated overnight at 4 °C with the primary anti-HA antibody from rats (SIGMA), which was diluted 1:1000 in Odyssey Blocking Buffer and 0.1 % Tween. The next day, the membrane was washed four times for 5 min with 1 x PBS + 0.1 % Tween before it was incubated for one hour at RT with the secondary antibody IR Dye™800 Conjugated Affinity Purified anti-Rat IgG

from goats (Rockland), which was diluted 1:5000 in Odyssey Blocking Buffer + 0.1 % Tween. After four times of washing in 1 x PBS + 0.1 % Tween for 5 min and one time in 1 x PBS without Tween, the membrane was dried and kept in darkness until the bands were detected with the Odyssey Infrared Imager (LICOR Biosciences).

2.2.7 Histochemical detection of GUS activity

Histochemical GUS-staining was carried out either with whole plant seedlings, aged two weeks, or with stem leaves of older plants that had already been put on soil after having grown for three weeks on Ara plates. The plant material was transferred into the GUS-reaction solution, and after vacuum infiltration for 10 min, the plant samples were incubated overnight at 37 °C for at least 18 h. Chlorophyll was removed with 70 % EtOH by incubating the plants for at least three hours. The plantlets were transferred into fresh 70 % EtOH, and destained samples were stored at 4 °C.

2.2.8 Mapping

For the first-pass mapping, a total of 32 primer combinations were used (Table 2.2). Each PCR was carried out according to the standard protocol for small fragments (cf. 2.2.9.1) with PCR mix #1, as each amplified fragment was smaller than 500 bp. The PCR product was separated on 3 % Agarose gels (cf. 2.2.10) and the ecotype at each marker position was determined. Two markers (F3F19 and F7P12, both on chromosome I) amplified a fragment with identical size in the two accessions Col-0 and L*er*, therefore the PCR product was subsequently digested with *Dra*I. Digestion with *Dra*I of the PCR product produced with marker F3F19 resulted in two smaller fragments in Col-0 accession, as only this ecotype possesses a cleavage site for *Dra*I. *Dra*I digestion with the PCR product generated with marker F7P12 resulted in two smaller fragments in L*er* accession.

Chr.	Marker	Position (Mbp)	Primer combination	Size fragment (bp)	
				Col-0	L*er*
I	nga63	3.2	nga63-F, nga63-R	128	99
I	F3F19	4.4	F3F19-F, F3F19-R	100, 138	243
I	F7P12	12.6	F7P12-F, F7P12-R	315	100, 215
I	F2J6	16.0	F2J6-F, F2J6-R	371	328
I	ciw1	18.0	ciw1-F, ciw1-R	159	135
I	F23H11	21.5	F23H11-F, F23H11-R	300	250
I	NF5I14a	24.0	NF5I14a-F, NF5I14a-R	221	240
I	AthATPASE	28.1	AthATPASE-F, AthATPASE-R	86	70
II	F18P14	2.4	F18P14-F, F18P14-R	123	144
II	T20K24	8.0	T20K24-F, T20K24-R	222	240
II	F2H17	15.2	F2H17-F, F2H17-R	250	280
III	nga162	4.6	nga162-F, nga162-R	107	89
III	ciw11a	9.8	ciw11a-F, ciw11a-R	192	242
III	MZN14	10.7	MZN14-F, MZN14-R	150	143
III	MUO22	11.4	MUO22-F, MUO22-R	248	185
III	T32N15	16.2	T32N15-F, T32N15-R	238	196
III	T6H20	16.0	T6H20-F, T6H20-R	273	293
III	ciw4	18.9	ciw4-F, ciw4-R	189	215
III	F27K19	21.0	F27K19-F, F27K19-R	237	281
III	nga6	23.0	nga6-F, nga6-R	159	137
IV	ciw5	0.7	ciw5-F, ciw5-R	164	144
IV	C6L9	3.7	C6L9-F, C6L9-R	364	287
IV	T3H13	4.7	T3H13-F, T3H13-R	275	150
IV	T26M18	6.1	T26M18-F, T26M18-R	330	271
IV	F28A21	9.2	F28A21-F, F28A21-R	198	169
IV	F6E21	14.1	F6E21-F, F6E21-R	199	225
IV	F23E13	16.0	F23E13-F, F23E13-R	264	246
V	MHF15	1.9	MHF15-F, MHF15-R	295	268
V	nga151a	4.7	nga151a-F, nga151a-R	198	170
V	nga139	8.4	nga139-F, nga139-R	174	132
V	MPL12	18.4	MPL12-F, MPL12-R	319	293
V	MNC17	23.6	MNC17-F, MNC17-R	168	187

Table 2.2: List of markers used to identify the* sud *mutation during the mapping process. *All markers are listed with their name and their exact position in the* Arabidopsis *genome, and the size of the amplified fragment in the two different accessions Col-0 and* L*er is specified. For the two markers F3F19 and F7P12, the PCR product had to be additionally digested with* DraI*.*

2.2.9 Polymerase chain reaction (PCR) amplification

For PCR, two different standard protocols were used, one for small fragments with a size between 100 bp and 500 bp, and another one for longer fragments (> 500 bp).

2.2.9.1 Standard protocol for small PCR fragments

For PCR products of the mapping population analysis with fragment length between 100 bp and 500 bp, the following standard protocol with PCR mix #1 was used. Each PCR reaction was carried out with a total volume of 40 µl and was executed in a 200 µl PCR-tube. The reaction was performed with a home-made Taq-polymerase (Brown-Taq), provided by Dr. Iain Searle:

PCR mix #1:	PCR buffer (10 x)	4 µl
	dNTP [2.5 mM]	3.2 µl
	$MgCl_2$ [25 mM]	1.2 µl
	Primer-F [10 mM]	1.5 µl
	Primer-R [10 mM]	1.5 µl
	Brown-Taq [5 U/µl]	0.3 µl
	DNA template (from genomic DNA isolation)	1 µl
	dH_2O	27.3 µl

For PCR analysis of the *PRT1* allele with a PCR fragment of 180 bp, a different protocol with PCR mix #2 was used. The final volume of each PCR reaction was 50 µl, and no additional $MgCl_2$ was added. The PCR program for both reactions was the same, and it started with a denaturation step at 94 °C for 45 sec, followed by 37 cycles. The first 10 cycles (94 °C for 7 sec, 56 °C for 30 sec and 72 °C for another 30 sec) used a higher annealing temperature than the last 27 cycles (94 °C for 7 sec, 53 °C for 30 sec and 72 °C for another 30 sec). The amplified fragments were separated on a 3 % Agarose gel (cf. 2.2.10), whereas the *PRT1* allele was additionally digested with *Mnl*I before loading (cf. 2.2.12).

PCR mix #2:	PCR buffer (10 x)	5 µl
	dNTP [2.5 mM]	5 µl
	Primer-F [100 pmol/µl]	0.5 µl
	Primer-R [100 pmol/µl]	0.5 µl
	Brown-Taq [5 U/µl]	0.5 µl
	DNA template	
	(from genomic DNA isolation)	2 µl
	dH_2O	36.5 µl

2.2.9.2 Standard protocol for longer PCR products (> 500 bp)

The following protocol was mainly used for PCR reactions to characterize the *PRT6* locus. However, also general PCR with fragments larger than 500 bp were performed with the following standard protocol with PCR mix #3. Each PCR reaction had a volume of 50 µl and was carried out in a 200 µl PCR-tube:

PCR mix #3:	LA-Taq buffer (10 x)	5 µl
	$MgCl_2$ [25 mM] for LA-Taq	5 µl
	dNTP [2.5 mM]	8 µl
	Primer-F [100 pmol/µl]	0.5 µl
	Primer-R [100 pmol/µl]	0.5 µl
	LA-Taq [5 U/µl]	0.5 µl
	DNA template	
	(from genomic DNA isolation)	2 µl
	dH_2O	28.5 µl

The PCR program started with a denaturation step at 95 °C for 5 min, followed by 32 cycles (95 °C for 30 sec, 55 °C for 30 sec and 68 °C for 8 min) and a final cycle of 68 °C for 10 min. The amplified fragments were separated on a 1 % Agarose gel (cf. 2.2.10).

2.2.10 Agarose gel electrophoresis

PCR products were separated by Agarose gel electrophoresis. Agarose concentration of the gel was depending on the size of the expected DNA fragments amplified with PCR. For DNA fragments with a length between 100 bp and 500 bp, gels with an Agarose concentration of 3 % were used, whereas for larger fragments Agarose gels with only 1 % Agarose were prepared. Agarose was dissolved in 1 x TAE buffer and boiled up in the microwave. After cooling down, 5 µl ethidium bromide [5 mg/ml] were added to 100 ml of liquid and the gel was poured into a plastic tray, sealed at the edges with tape and cast with appropriate combs. The gel was transferred into a gel tank filled with 1 x TAE buffer. DNA samples were mixed with 1/6 vol. of 6 x loading buffer and loaded into the gel slots. Small gels (50 ml) were run at 50 V, large gels (100 ml) at 80 V. Gel images were captured by a Kodak DC-120 ZOOM digital camera and processed with the Kodak Digital Science 1D V.3.0.2 software.

2.2.11 RT-PCR

Extracted RNA from 100 mg ground powder (cf. 2.2.3.3) was dissolved in 30 – 40 µl DEPC-dH_2O. 5 µl of this liquid were taken into a small Eppendorf tube and mixed with 6.4 µl DEPC-dH_2O. This sample was heated in a thermoblock at 65 °C for 5 min and put on ice immediately after for a quick chill. The following components were added to the Eppendorf tube:

RT-PCR mix:	RT buffer (5 x)	4 µl
	RNasin	0.6 µl
	dNTP (10 mM)	2 µl
	oligo dT (50 – 100 µM)	1 µl
	AMV reverse transcriptase	1 µl

The mixture was incubated for 1 hour at 42 °C; 1 µl of the RT-PCR reaction was used as template for a 50 µl PCR reaction. The PCR followed the protocol for large PCR fragments, except for the use of Brown-Taq instead of LA-Taq and the absence of $MgCl_2$ in the PCR mix that was replaced by dH_2O.

2.2.12 Digestions with restriction endonucleases

Digestions of DNA samples were carried out with restriction endonucleases according to the manual of the manufacturer. The reaction was normally performed in 1.5 ml Eppendorf tubes with a final volume of 20 µl and an enzyme concentration of 1-5 U enzyme / µg DNA.

2.2.13 Isolation of DNA for sequencing

To verify the correctness of a transgenic sequence, DNA of the examined plant line was first isolated (cf. 2.2.3.1) and amplified with PCR. The PCR product was loaded on a Agarose gel, the expected fragment was cut out under 70 % UV-light intensity to avoid damages of the DNA and was isolated with NucleoSpin Extract II (Macherey-Nagel) according to the manufacturers' protocol. 50 % of the eluted DNA was submitted for sequencing to ADIS (Automatic DNA Isolation and Sequencing) at the Max Planck Institute for Plant Breeding Research.

2.2.14 Analysis of DNA content

For analysis of the DNA content in plants, DNA was isolated (cf. 2.2.3.2) from 1 g of ground tissue powder, and 1/12 of the eluted DNA (10 µl) was diluted in 990 µl dH_2O. The absorbance of the solution was measured with the spectrophotometer at a wavelength of 260 nm.

3 RESULTS

3.1 Perturbation of the ubiquitin-proteasome pathway with UbK48R

3.1.1 Growth of the UbK48R expression line RV86-5 in induced and non-induced state

The transgenic plant line RV86-5 contains an inducible UbK48R transgene that is under control of a Dexamethasone (Dex) inducible promoter (cf. 1.3.2). To compare the growth of transgenic plants in induced and non-induced state, seeds of RV86-5 were put on plates with solid medium (Ara plates). On Ara plates that were only supplemented with hygromycin (Hyg), no expression of the variant ubiquitin UbK48R or dihydrofolate reductase (DHFR)

took place; however, the presence of the complete transgene could be checked with Hyg, as the transgene expressed hygromycin phosphotransferase (HPT). This expression has no influence on the phenotype. Therefore, RV86-5 plants grew normally on Ara plates with Hyg and were phenotypically identical to wild type (WT) plants (Fig. 3.1.A). However, seeds that were sown on HDM plates – Ara plates that were supplemented with Hyg, Dex (for inducible transgene expression) and Methotrexate (MTX; to rule out silencing of the transgene) – showed severe perturbation of growth from the beginning and the seedlings died a few days after germination (Fig. 3.1.B).

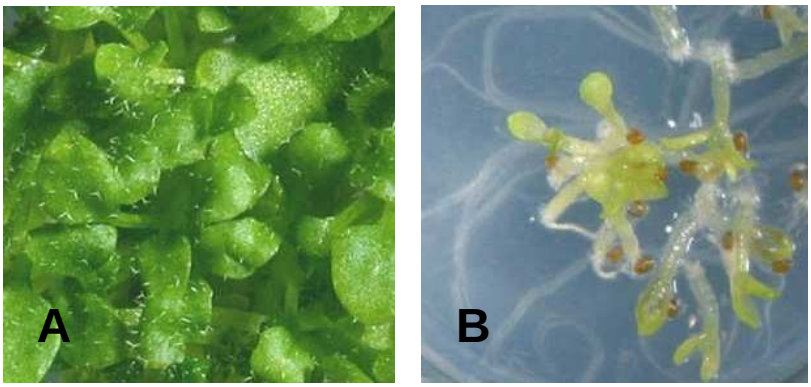

Fig. 3.1: Comparison of growth of plant line RV86-5 in induced and non-induced state. *14-day old seedlings of* Arabidopsis *plant line RV86-5 containing a Dex-inducible transgene showed normal growth on Ara plates in the absence of Dex (**A**). The Ara plates were supplemented with Hyg, but not with Dex. In contrast, the seedlings died soon after germination when grown on HDM plates that were supplemented with Hyg, Dex and MTX (**B**). Pictures taken from Schlögelhofer* et al.*, 2006.*

3.1.2 Kinetic studies with plants expressing UbK48R

To study kinetic aspects of cell death induction at later stages of development, seeds were germinated in a flask with liquid medium (half strength of GAMBORG's B5 medium, supplemented with vitamins). The flasks were put on a rotating platform under constant shaking to ensure an adequate supply of oxygen during growth. After 14 days, the plants had developed cotyledons and four primary leaves. At this time point, Dex was added to a final concentration of 100 µM to induce UbK48R expression.

Dex was dissolved in dimethyl sulfoxide (DMSO), and to avoid any hypothetical influence of the solvent to the plant growth, the same amount of DMSO (143 µl) that was added to the liquid culture samples was also added to the control flasks.

First visual differences in growth could be detected five to seven days after addition of Dex to the liquid medium. The differences in growth were clearly visible in the flask with 28-day old seedlings where the UbK48R transgene expression had been induced with Dex 14 days earlier (Fig. 3.2).

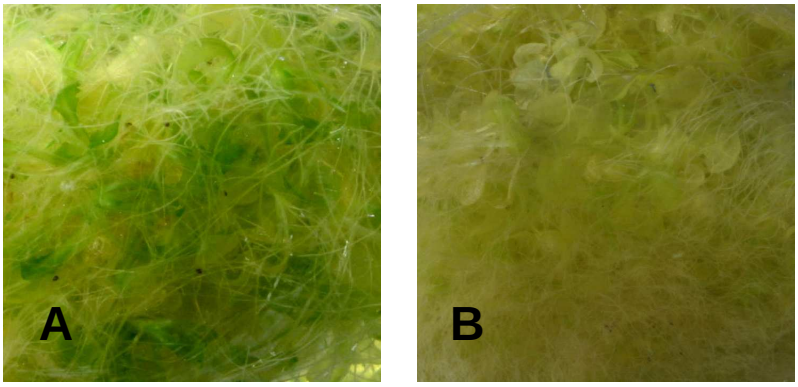

Fig. 3.2: Comparison of 28-day old RV86-5 plants, grown in liquid culture in the absence and in the presence of Dex. *Both pictures show roots and shoot material grown in liquid medium for 28 days, intermingled in the Erlenmeyer flask. Dex was only added to (**B**), 14 days after sowing. Non-induced control plants (**A**) do not express the inducible transgene and show significant better growth than the Dex-induced seedlings. Pictures taken from Schlögelhofer* et al*., 2006.*

The senescence of the plants where the UbK48R expression was induced with Dex was much more advanced than in the control flasks and could be observed by the high amount of yellowish and brownish leaves compared to the control plants that were not induced.

Experiments with plants grown on plates that were supplemented with solid Ara medium gave similar results. Analogous to the liquid culture experiment, seeds were sown on Ara plates and after three weeks of growth, the plantlets were induced with Dex, dissolved in DMSO. For activating the transgene, the DMSO – Dex solution was applied with a pipet directly and evenly on the solid

Ara medium; the control plants were only supplemented with the same amount of DMSO without Dex.

A difference in growth and the beginning of senescence could be detected four to six days after induction. Similar to the liquid culture experiment, the plant leaves started to become yellowish, whereas the control plants continued their growth normally. This can be seen in Fig. 3.3, where induced (A) and non-induced (B) plants are shown seven days after induction, grown under normal light conditions.

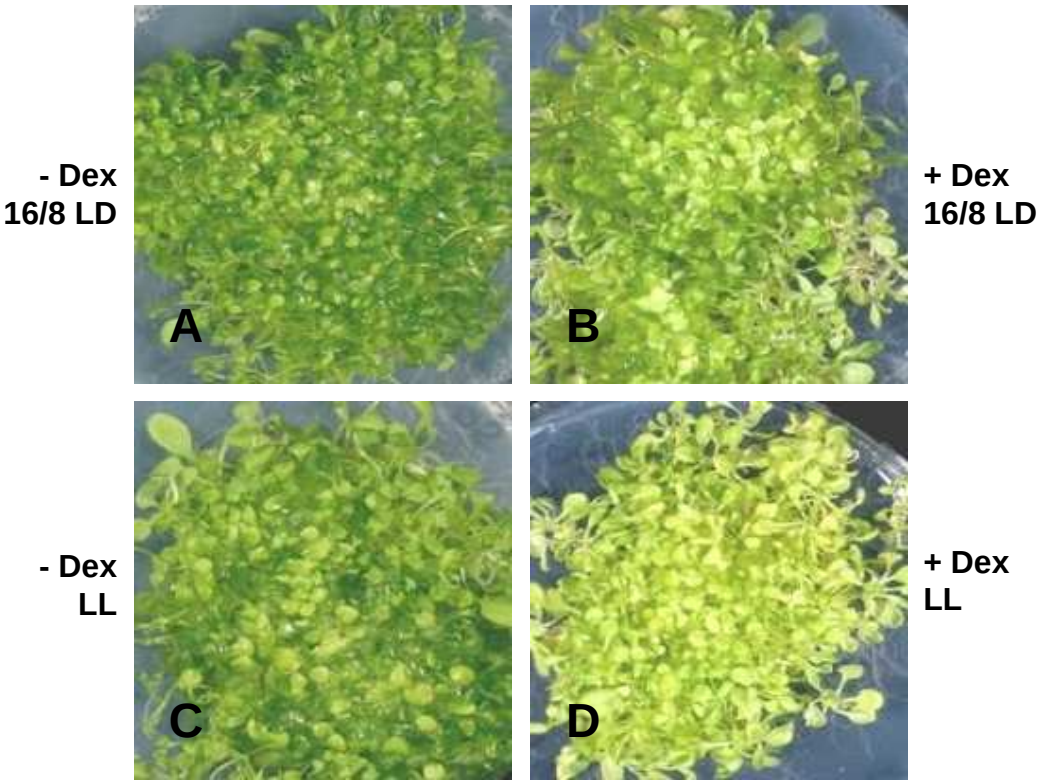

Fig. 3.3: Dex induction and light stress in the UbK48R expression line grown on plates with solid Ara medium: *Comparison of 28-day old RV86-5 plants grown on Ara plates under varying conditions. 21-day old plantlets were induced with Dex (**B**,**D**), whereas the control plants (**A**,**C**) were not supplemented with the glucocorticoid. Two plates – an induced and a non-induced one – were grown in a 16 h light / 8 h dark cycle (**A**,**B**) and the other two plates under stress light conditions in continuous light (**C**,**D**). The Dex-induced plants grown in continuous light (**D**) showed the most severe cell death symptoms. Plants that were exposed to only one stress factor like Dex-induction under normal light conditions (**B**) or continuous light in the absence of Dex (**C**) revealed also cell death symptoms when compared to the non-induced plate (**A**) grown under normal light conditions, but to a minor extent. Abbreviations: LD – light-dark cycle; LL – continuous light; -Dex – no Dex was added; +Dex – Dex was added. Pictures taken from Schlögelhofer* et al.*, 2006.*

These experiments demonstrated that the transgene expression clearly perturbs cellular processes and can lead to cell death. However, transgene induction in growing seedlings needed around one week before first visual symptoms could be detected, whereas the germination of RV86-5 seeds on plates already supplemented with Dex to induce the transgene from the beginning accelerated the appearance of cell death symptoms.

To investigate whether the observed senescence constitutes a fast or a slow form of programmed cell death (PCD), the DNA content of the plantlets was measured. The hypersensitive response (HR), which is a fast form of PCD, results in loss of DNA, and as one possible consequence, DNA laddering may be observed. Unlike the HR, senescence is a slow form of PCD, and one hallmark of this form is that DNA is degraded only at its latest stages.

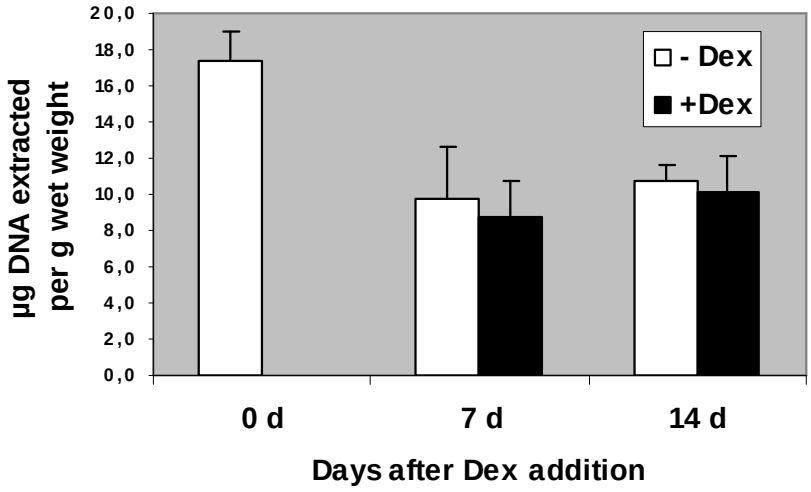

Fig. 3.4: Content of total DNA in RV86-5 plants expressing the variant ubiquitin UbK48R. *Dex was added to 14-day old seedlings grown in liquid culture and plants were harvested at the indicated time points. Each column shows the average amount of total DNA analysed in three independent experiments. Figure taken from Schlögelhofer* et al.*, 2006.*

Therefore the DNA content of plants of various ages, grown in liquid culture, was analysed. Samples of plant material were taken at three different time points: First at the beginning of the experiment, before Dex was added to the liquid culture plants, and then seven and 14 days after the variant ubiquitin UbK48R was initiated by Dex. The plants were induced 14 days after

germination so that the youngest plants analysed were aged 14 days and the oldest ones 28 days. DNA was isolated and the absorbance was quantified in a spectrophotometer. Fig. 3.4 summarizes the results of these measurements.

The DNA analyses were repeated three times for each time point and for both the control and the Dex-induced plants. Comparison of the DNA amount in Dex-induced plants with the one found in control plants, both seven and 14 days after UbK48R induction, did not show any significant differences in the DNA content between UbK48R-induced and non-UbK48R-induced plants, indicating that no fast DNA degradation in UbK48R-induced plants took place. However, two interesting observations could be made: First, the DNA amount decreased in both groups during the first week after transgene induction, which might be explained by the expansion growth of the plants during this time. Second, this decrease did not continue during the second week after Dex induction, as the same amount of DNA was measured in both groups seven and 14 days after initiation of UbK48R induction, respectively. Here, the constant DNA amount of both samples may be caused by a reduced expansion growth due to diminished nutrient availability in the liquid growth medium.

The kinetics of cell death induction caused by adding Dex to the plants is quite slow. To see whether an additional abiotic stress factor like heat or light could speed up these kinetics, seedlings were exposed to increased light intensity (continuous light) as a stress factor. Therefore 14-day old plants that were grown on plates were induced with Dex, dissolved in DMSO. For activating the transgene, the DMSO – Dex solution was again applied with a pipet directly and evenly on the solid Ara medium; the control plants were only supplemented with the same amount of DMSO without Dex. Subsequently the plantlets were grown both further on under normal light conditions (16 h light / 8 h dark cycle) and under stress light conditions (continuous light) just like control seedlings without Dex treatment. A comparison in Fig. 3.3, panels A and B vs. C and D, shows that increased light

intensity could indeed enhance the cell death effects of induced transgene expression.

The induced seedlings exposed to continuous light showed the first changes in leaf colour from green to yellow already four days after addition of Dex, whereas this effect could be detected only seven days after Dex-induction in plants that were grown in a 16 h light / 8 h dark cycle. The untreated plants could cope with the increased light intensity without any problems and even after seven days in continuous light no change in leaf colour could be seen. The UbK48R induced seedlings were clearly more affected in continuous light after seven days, indicating that additional stress factors like increased light intensity could indeed speed up cell death induction.

3.1.3 Chemical genetics as an alternative approach

In collaboration with other groups of the Max-Planck-Institute and Syngenta, some experiments were carried out with substances of small molecular weight to see whether these substances have any influence on the investigated plant line RV86-5. Therefore, a screen was performed and seeds of RV86-5 were sown on solid Ara medium supplemented with vitamins, Dex and different amounts of the test substance. The initial concentrations of the chemicals in the first screen were 0.3 ppm and 3 ppm. The seeds were put on tissue culture plates with 24 wells; each well was filled with 1 ml of Ara medium and each substance was tested in two wells. To rule out substances that modify the phenotype of RV86-5 plants without transgene expression, seeds were also sown on Ara medium supplemented only with vitamins and the test substance but not with Dex. On every plate, the four central wells served as a control where the Ara medium was only supplemented with vitamins but not with any test substance. Dex was only added to the two upper wells but not to the two lower ones. Altogether, 120 different substances were tested. Fig. 3.5 shows one plate with 10 different substances, 13 days after sowing.

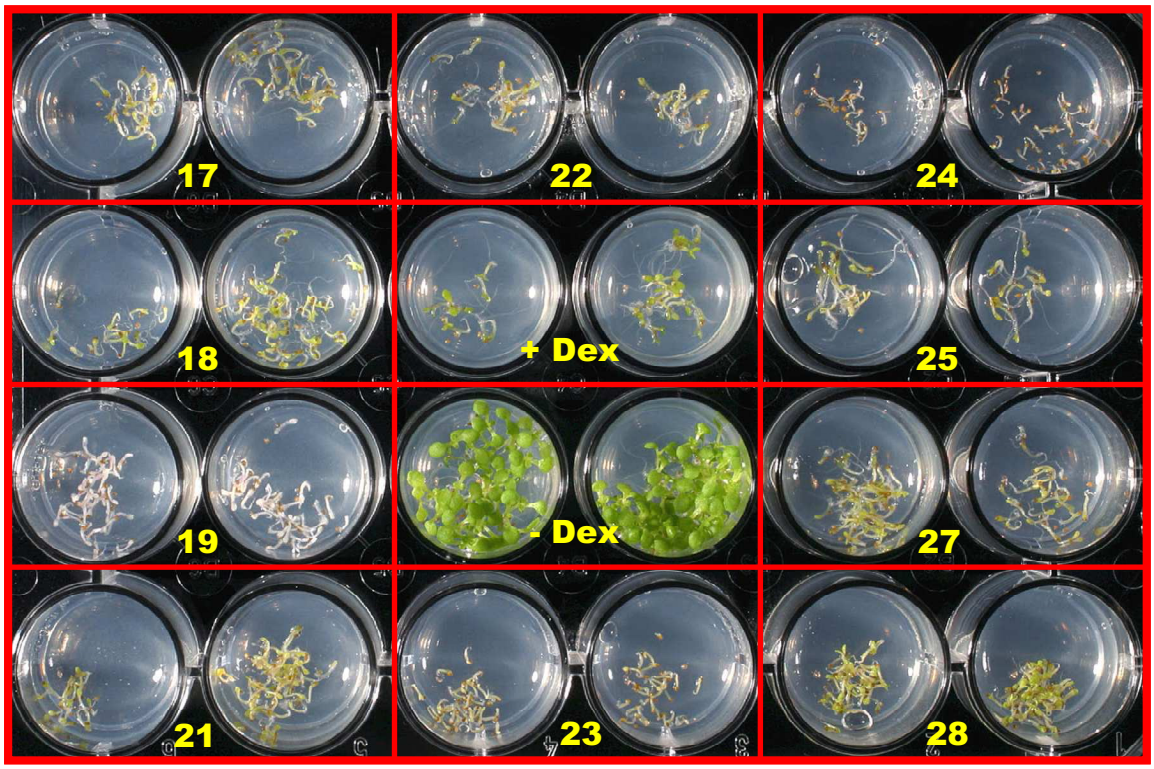

Fig. 3.5: Chemical genetics: Example for a 24-well plate with 13-day old Dex-induced seedlings of RV86-5 on Ara medium supplemented with 10 different chemicals (numbered 17 to 28). *Each well, except the four central ones, contained Ara medium, vitamins, Dex for transgene induction and one chemical at a concentration of 3 ppm. The four central wells served as control and contained Ara medium and vitamins but no additional chemical; the two upper wells (+ Dex) were supplemented with Dex to induce the UbK48R transgene, whereas the two lower ones (- Dex) did not contain the glucocorticoid.*

Several chemicals had an influence on the growth of RV86-5, but most of the test substances showed only negative effects and inhibited germination or slowed down growth. Only one substance was identified in the first screen that influenced RV86-5 plants in a positive way, i.e. RV86-5 plants with the induced transgene showed better growth in the presence of the test substance than the induced control plantlets without it. This chemical, Cantharidin (CAN), was tested as compound #28 and is a monoterpene anhydride. When CAN was supplemented to the Ara medium in a concentration of 3 ppm, UbK48R induced plants grew better and showed a stronger phenotype than the induced control plants. Therefore further growth experiments with different concentrations of this substance were carried out in a second screen, this time varying the CAN concentration between 0.3 ppm and 6 ppm (Fig. 3.6).

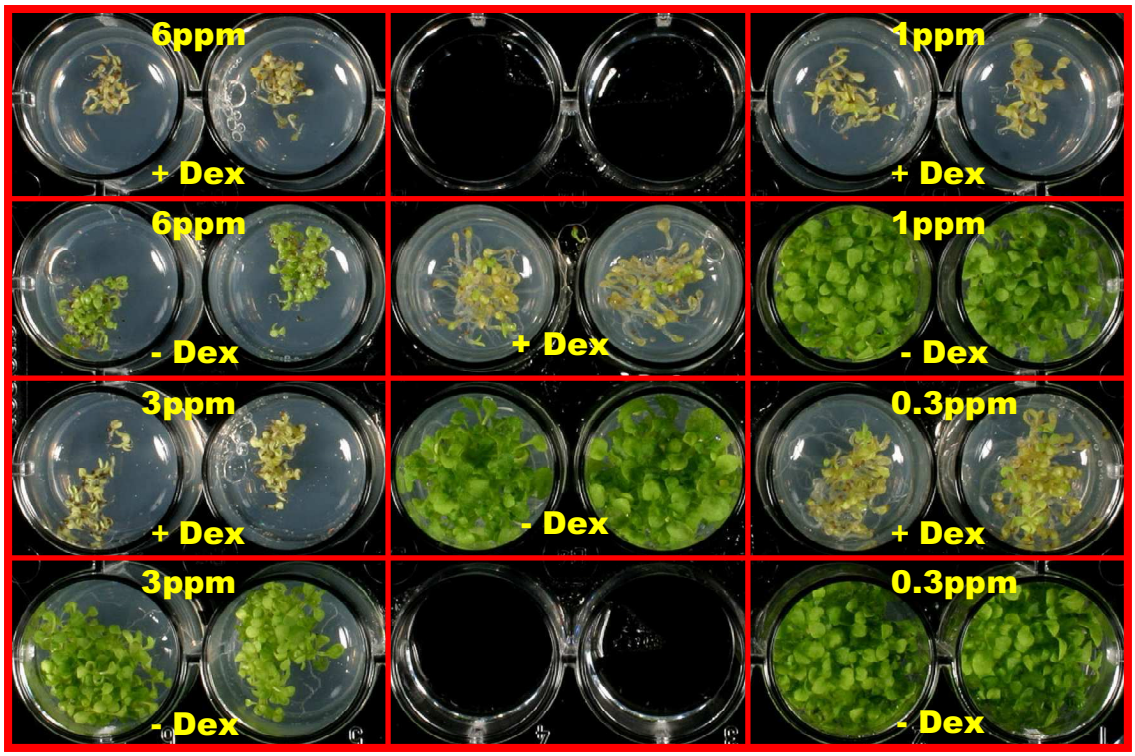

Fig. 3.6: Chemical genetics: Tissue culture plate with 21-day old plantlets of RV86-5 grown on Ara medium that contained different concentrations of CAN, varying between 0.3 ppm and 6 ppm. *CAN was added both to Ara medium supplemented with Dex (+ Dex) and to Ara medium without Dex (-Dex). The four central wells served as control and contained Ara medium and vitamins but no test substance. Only the two upper wells were supplemented with Dex for transgene induction, whereas the two lower ones (- Dex) did not contain the glucocorticoid. Stronger growth of Dex-induced plant line RV86-5 due to CAN compared to the control plants could only be shown in lower concentrations (0.3 ppm and 1 ppm), whereas higher concentrations (3 ppm and 6 ppm) affected the growth also of non-induced RV86-5 plants.*

The repetition with different concentrations of CAN however revealed that the positive results observed in the first screening could not be repeated. Dex-induced plants grown on Ara medium supplemented with 3 ppm CAN showed a weaker phenotype than the Dex-induced control plants without the test substance. Furthermore, CAN in concentrations of 3 ppm or higher influenced negatively also the growth of non-induced plants, as it could be demonstrated clearly in the assay with Ara medium supplemented with CAN in a concentration of 6 ppm. Only plantlets grown on Ara medium with 0.3 ppm CAN showed slightly better growth in the first days after germination, but also died three to four weeks later, similar to the Dex-induced control plants. Besides this, plants grown on Ara medium with a CAN concentration of 3 ppm

showed reduced root growth and the roots turned brown three to four weeks after germination.

To see whether structually related substances to CAN have any effect on UbK48R-induced plants, three different structural analogues were tested in the same way as in the second screen, carried out with different concentrations of CAN in the Ara medium. Similar to the experiment carried out with CAN, no positive effect on UbK48R-induced plants could be detected, and the growth on the different Ara media with varying concentrations of the three structural analogues from 0.3 ppm to 6 ppm did not reveal a better phenotype than the one of Dex-induced control plants not supplemented with additional chemicals.

3.2 Selection of UbK48R-induced cell death suppressor mutants from EMS mutagenesis

3.2.1 State of the art

After choosing the transgenic plant line RV86-5 for further investigations, approximately 210 000 seeds of this plant line were mutagenized with 0.1 % Ethyl methanesulfonate (EMS). The aim was to cause a mutation due to which the plant could cope better with the expression of the variant ubiquitin UbK48R than the non-mutagenized plant line RV86-5, i.e. to identify plants that survive the Dex-induced UbK48R expression in contrast to RV86-5. To generate an appropriate mutation, one third of the seeds – approximately 70 000 – was treated with EMS for 8 hours, one third for 12 hours and one third for 16 hours.

At first, the seeds mutagenized for 12 hours were further characterized. Therefore the seeds were put on soil and about 30 % of them germinated. The M2 generation was harvested in 36 batches. About 25 000 M2 seeds were

screened on HDM plates and the survivors of this screen were rescued, put on normal Ara plates and later on soil. These seeds of the M3 generation were again collected and re-screened to find surviving plants on HDM plates (cf. Schlögelhofer, 2002).

Among these screened mutant lines, 19 plant lines that survived considerably better than other plant lines were chosen for further investigations and tested for mRNA expression of the transgene. Because plant line EMS 12-2 showed good resistance to Dex and a superior level of mRNA when expressing the transgene, it was chosen for further characterization and renamed *sud1* (for *suppressor of ubiquitin UbK48R-induced cell death1*; Kerzendorfer, 2002).

Similar to the seeds mutagenized for 12 hours, the seeds of the 16-hour mutagenesis were also characterized and put on soil. Like in the 12-hour mutagenesis, about 30 % of the 70 000 seeds germinated. The M2 generation was harvested in 48 batches. About 100 000 seeds of all 48 batches were screened on HDM plates. Similar to the seeds of the 12-hour mutagenesis, the survivors were again rescued by putting them on normal Ara plates and later on soil. The seeds of each individual plant were collected separately and re-screened on HDM plates (cf. Kerzendorfer, 2002).

3.2.2 Screening for additional mutants

In order to obtain additional mutant lines besides *sud1*, the batches of the 16-hour mutagenesis were further investigated in this work. 75 plant lines of the M3 generation were re-screened on HDM plates containing 3 % of sucrose. Mutant seeds were sown on one half of the HDM plate and non-mutagenized seeds of RV86-5 on the other half. By growing mutant lines and RV86-5 plants on the same HDM plate, better growing mutant plants could easily be identified. In addition to these 75 plant lines of the 16-hour mutagenesis, three more plant lines of the 12-hour mutagenesis that showed a promising phenotype were re-screened. Table 3.1 summarizes some of the plant lines

Plant Line		Growth on HDM plates 17 days after sowing
EMS 38-4	++/-	Both mutant and control plants yellowish/white; many mutant plants with dark green eophyll and violet stained petioles
EMS 39-2	++/-	Mutant plants greener than control plants, further grown and with eophyll
EMS 42-2	+	Mutant plants strong and well developed; nearly all leaves dark green; control plants brownish/white and only some with eophyll
EMS 42-4	++/-	Mutant plants stronger than control plants and much more developed; 50 % of the mutant plants with eophyll, 50 % yellowish/white like control plants
EMS 50-1	++	Best growing mutant plants of this screening; strong mutant plants with dark green eophyll and violet stained petioles and apices; control plants yellowish/brown and only sporadically with green eophyll
EMS 50-4	++	Best growing mutant plants of this screening; strong mutant plants with dark green eophyll and violet stained petioles and apices; control plants yellowish/brown and only sporadically with green eophyll
EMS 53-2	++/-	Mutant plants stronger than control plants with eophyll; leaves reddish/brown; control plants yellowish/white
EMS 57-2	+	Mutant plants stronger and much better developed than control plants; all plants with dark green eophyll; some cotyledons yellowish/brown; violet stained petioles; control plants brownish/white
EMS 58-1	+	Mutant plants much stronger and further developed than control plants; all plants with dark green eophyll; control plants brownish/white and only single plants with eophyll
EMS 66-3	+	Mutant plants much stronger and further developed than control plants; nearly all plants with dark green eophyll; control plants brownish/white and only single plants with eophyll
EMS 67-1	++/-	Mutant plants stronger and leaves darker green than control plants; numerous plants with eophyll
EMS 67-4	++/-	Mutant plants stronger and leaves darker green than control plants; numerous plants with eophyll; 60 % germination in both groups
EMS 68-1	++/-	Mutant plants with light green cotyledons; numerous plants strong with green eophyll; control plants with brownish cotyledons and weak
EMS 73-3	+/-	Mutant plants greener than control plants; several mutant plants with eophyll; cotyledons of control plants light yellow
EMS 76-1	+/-	Mutant plants slightly stronger than control plants; mutant plants with green eophyll; control plants with brownish cotyledons and only some plants with eophyll
EMS 81-1	+	Mutant plants much stronger and further developed than control plants; nearly all plants with dark green eophyll; control plants brownish/white and only single plants with eophyll
EMS 82-3	+	Mutant plants stronger and greener than control plants; many plants with dark green eophyll
EMS 84-2	++/-	Mutant plants with yellowish cotyledons and bigger than control plants; those with brownish cotyledons; several mutant and control plants with dark green eophyll
EMS 84-6	++/-	Both mutant and control plants with yellowish cotyledons; some control plants with brownish cotyledons; several mutant plants with green eophyll and violet stained petioles; only some control plants with eophyll
EMS 28-1	+	Mutant plants without growth of cotyledons; many cotyledons stuck in their seed coat; strong growth of fungi
EMS 30-1	+	Mutant plants stronger than control plants; several plants with dark green eophyll

Table 3.1: (Previous page) - Description of growth development of 17-day old plantlets originating from the EMS mutagenesis in comparison with control plants of RV86-5, both grown on HDM plates. *21 EMS-mutagenized plant lines with an above average surviving phenotype are listed. Nine plants – marked in green - were selected for further investigations. Eophyll – first leaves of a seedling, other than cotyledons;* ***+/-*** *: Mutant line grows slightly better than control plants;* ***++/-*** *: Mutant line grows better than control plants;* ***+*** *: Mutant line grows considerably better than control plants;* ***++*** *: Mutant line grows much better than control plants.*

that grew better than plants of the non-mutagenized plant line RV86-5. Seven plant lines (EMS **42-2**, EMS **50-4**, EMS **57-2**, EMS **58-1**, EMS **66-3**, EMS **81-1** and EMS **82-3**) that showed better growth and a much higher survival ratio on HDM plates than RV86-5 plants were selected from these 75 plant lines for further investigations. Besides these seven lines of the 16-hour mutagenesis, two of the three tested lines of the 12-hour mutagenesis (EMS **28-1** and EMS **30-1**) were also chosen due to their clear phenotype.

3.2.3 Transgene expression levels

Plant lines EMS 50-1 and EMS 50-4, originating from the same batch, showed very strong growth, comparable to non-transgenic WT plants. Although both plant lines were still resistant to Hyg and MTX, which indicated that the transgene was still expressed, the unusually strong growth suggested that these plant lines may have a reduced number of UbK48R repeats in the transgene. Therefore plant line EMS 50-1 was tested with PCR for having lost one or more UbK48R repeats. The PCR was carried out with the two primers “DHFRup” and “pTAdn” to amplify a 2 kb fragment of the transgene containing all eight UbK48R repeats. As shown in Fig. 3.7, plant line EMS 50-1 had indeed lost six or seven of the eight repeats of the ubiquitin UbK48R present in the transgene.

The normal size of the fragment of 2 kb was amplified in the three non-mutagenized control plant lines, whereas EMS 50-1 only showed a fragment of 600 bp. Two of the control lines showed additional laddering of the ubiquitin transgene with less than eight repeats, which was probably a PCR

artefact, due to internal initiation by a partially extended primer. Compared to these lines, the EMS 50-1 mutant expressed only one or two instead of eight repeats of the variant ubiquitin. This result explained the unusually strong growth, as very little modified ubiquitin was expressed, so that the excess of native ubiquitin was sufficient to prevent a perturbation of the ubiquitylation process. Due to these results, this plant line was not further investigated.

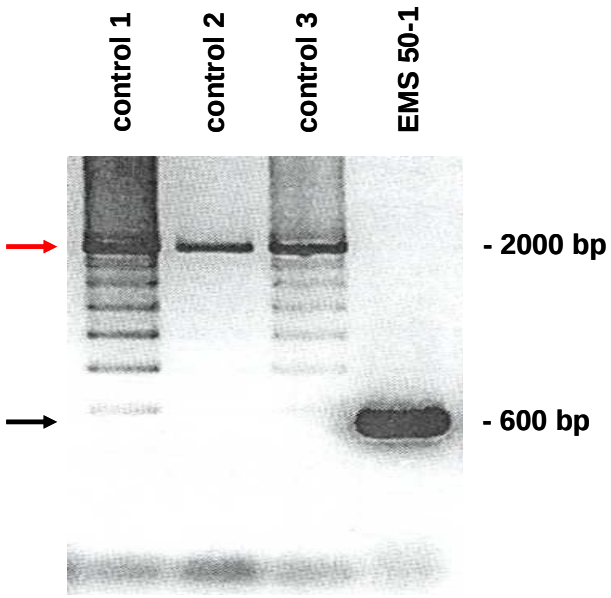

Fig. 3.7: PCR results of mutant line EMS 50-1 on an Agarose gel to check the correct expression of all UbK48R repeats in the transgene. *The expected fragment size including all eight repeats was 2 kb, as it could be detected in all three control samples (red arrow). In control 1 and in control 3, additional laddering could be observed, probably as a PCR artefact; here, transgene fragments with seven or less UbK48R moieties were amplified. The amplified fragment in mutant line EMS 50-1 however revealed a size of only 600 bp (black arrow), indicating a loss of at least six UbK48R moieties in EMS 50-1.*

The RNA levels of the remaining eight mutant lines were determined in a Northern blot experiment to verify the correct expression of the inducible transgene. Therefore, seeds of all eight mutant lines were put on Ara plates and cultivated for six weeks in a growth chamber. To activate transgene expression, Dex was dissolved in water in a concentration of 0.6 mM and 1 ml of this solution was applied with a pipet directly and evenly on the Ara medium of each plate. Eight hours after induction, all plants were harvested and frozen in liquid nitrogen. RNA was isolated from 100 mg of ground plant material, separated on an Agarose gel and blotted on a nylon membrane.

A 680 bp fragment of DHFR was used as probe and hybridized with the RNA. After stripping off the DHFR probe from the membrane, a second probe – Chlorata 42 - was applied and served as reference. The results of the phosphor-imager screen are shown in Fig. 3.8.

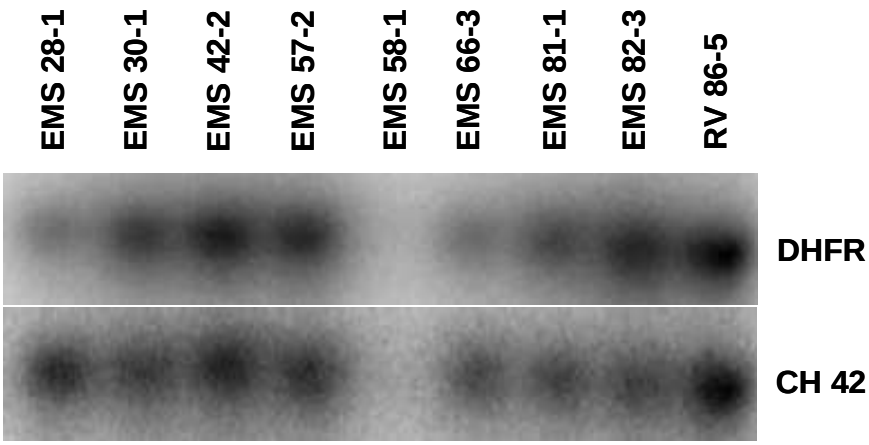

Fig. 3.8: Northern blot results of eight different EMS mutant lines and the non-mutated plant line RV86-5. *A 680 bp fragment of DHFR was used as a probe to detect mRNA levels of the transgene; Chlorata 42 (CH42) served as control.*

Except for mutant lines EMS 28-1 and EMS 66-3, all mutants showed transgene expression levels that were comparable to the level of control plant line RV86-5. The mRNA expression of the transgene was reduced in mutant line EMS 28-1 and EMS 66-3. All expression levels were also quantified with a Phosphor-Imager-Screen. The obtained data was analysed: First, the ratio of the values of the two probes was calculated (DHFR / CH42) and then this value of RV86-5 was normalized to 1. Fig. 3.9 shows the mRNA levels of the EMS mutants in comparison to the ratio of RV86-5.

Compared to the mRNA ratio of control line RV86-5, only two lines – EMS 28-1 and EMS 66-3 – differed markedly, as they did not express the same amount of transgene mRNA as plant line RV86-5 but less than 75 % (EMS 66-3) or 50 % (EMS 28-1). The other six investigated mutant lines revealed an expression level nearly identical with the one found in the non-mutated control line RV86-5.

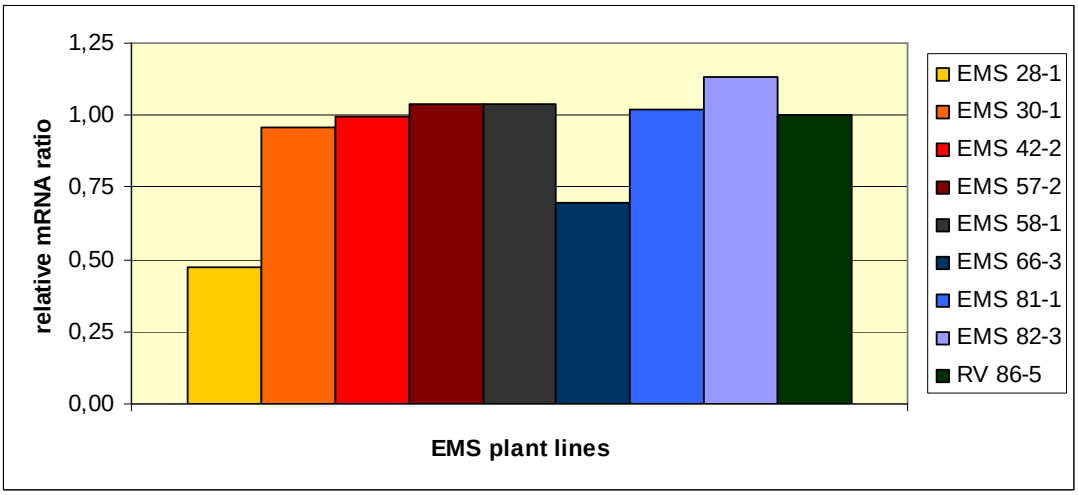

Fig. 3.9: Ratio of Dex-induced transgenic mRNA levels in comparison to control mRNA of non-mutated plant line RV86-5. *Except for mutant lines EMS 28-1 and EMS 66-3, all mutants could express the transgene to the same amount as the non-mutated control line RV86-5.*

3.2.4 Complementation analysis

Next, a complementation test was carried out with the eight mutant lines to see if the mutations were independent from each other. Therefore, all mutants were crossed to each other to define complementation groups. A complementation test can be used to see whether the mutation of a recessive mutant is allelic with the mutation of another recessive mutant line that shows the same phenotype. Both mutant lines are crossed with each other and the offspring of the F1 generation is tested for its phenotype. If the mutant phenotype still exists in the F1 generation, the two mutations must be in the same gene. If the progeny shows WT phenotype, the mutations are independent from each other and must be located in different genes.

The seeds of all crosses were put on HD plates (Ara plates supplemented with Hyg and Dex) and grown in the growth chamber. The plant growth was compared after four weeks. According to the complementation test theory, the offspring of two genetically independent EMS mutants should die in the F1 generation and crosses of EMS mutants with mutations in the same gene should survive on HD plates. The results are summarized in Table 3.2.

	EMS 28-1	EMS 30-1	EMS 42-2	EMS 57-2	EMS 58-1	EMS 66-3	EMS 81-1	EMS 82-3
EMS 28-1	-	surviving	surviving	surviving	surviving	surviving	surviving	surviving
EMS 30-1	surviving	-	dead	n.s.	surviving	dead	dead	dead
EMS 42-2	surviving	dead	-	dead	surviving	dead	dead	dead
EMS 57-2	surviving	n.s.	dead	-	dead	n.s.	dead	dead
EMS 58-1	surviving	surviving	surviving	dead	-	surviving	dead	dead
EMS 66-3	surviving	dead	dead	n.s.	surviving	-	dead	dead
EMS 81-1	surviving	dead	dead	dead	dead	dead	-	dead
EMS 82-3	surviving	dead	dead	dead	dead	dead	dead	-

Table 3.2: Results of the complementation assay with crosses of all eight selected mutants with each other, indicating the state of four-week old plantlets in the F1 generation grown on HD plants. *According to the rules of the complementation assay, F1 plants of a cross between two recessive suppressor mutants die on HD plates if the two mutations are independent from each other, and F1 plants survive if they are in the same gene. Except for mutant line EMS 28-1 and mutant line EMS 58-1, all mutants seemed to have independent recessive mutations, as the offspring of their crosses died on HD plates in the F1 generation. Therefore, as all plants of a cross survived when the mutant line EMS 28-1 was involved, it could be assumed that EMS 28-1 carried a dominant mutation. The results for mutant line EMS 58-1 are not clear, as plants of some crosses survived, whereas other plants died, indicating a partially dominant mutation. Abbreviations: surviving – all progenies of the cross were still alive after four weeks; dead – all progenies of the cross have died after four weeks; n.s. – not specified.*

The crosses revealed several remarkable results: First of all, plant line EMS 28-1 seemed to have a dominant mutation. Plants of all crosses, where this line was involved, survived. If EMS 28-1 were a recessive mutant, this mutation would be identical with all other mutations. As crosses among other mutant lines showed no surviving plants, e.g. between EMS 81-1 and EMS 82-3, the other mutant lines seemed to have independent mutations. Similar to these results, mutant line EMS 58-1 was also considered to be dominant or at least semi-dominant and not recessive, as crosses with EMS 30-1, EMS 42-2 and EMS 66-3 resulted in surviving plants in the F1 generation – which would have implied the same mutation – but crosses of these three lines among each other produced only non-surviving plantlets, which meant that all three mutations were independent from each other. Besides these two dominant

mutant lines, all mutations in the other tested plant lines seemed to be independent recessive mutations, as each cross resulted in dying plants in the F1 generation.

In consideration of all results achieved in the accomplished experiments for transgene expression and complementation, four independent recessive mutant lines were selected for further investigations and due to the expected mutation renamed ***sud*** (for "***s****uppressor of* ***u****biquitin UbK48R-induced cell* ***d****eath*").

- EMS 30-1 => ***sud2***
- EMS 42-2 => ***sud3***
- EMS 57-2 => ***sud4***
- EMS 82-3 => ***sud5***

3.3 Further characterization of *sud* mutants

3.3.1 Dominant mutation: *sud1*

The *sud1* mutant line was the most promising line from the 12-hour EMS mutagenesis. It showed a good *sud* phenotype on HDM plates (Fig. 3.10) and an above average level of transgene expression, as it was shown in Northern blot analyses (Kerzendorfer 2002); therefore it was selected for further characterisation. Backcrossing of *sud1* with RV86-5 indicated that *sud1* is a dominant mutation.

In previous experiments it was shown that plants carrying the *sud1* mutation showed higher resistance to salicylic acid (SA). To see if this phenotype is linked to the *sud* mutation, seeds of *sud1* were sown on Ara plates supplemented with different concentrations of SA, varying from 0.5 mM

to 2 mM. Seeds of the non-mutated plant line RV86-5 served as control. The *sud1* seeds that were used had been backcrossed with RV86-5 to decrease the possibility of unlinked mutations. The results obtained, however, revealed no difference in growth between *sud1* mutant plants and the control plants. Therefore it was assumed that the SA phenotype was not linked to the *sud1* mutation and the unlinked mutation was eliminated by backcrossing.

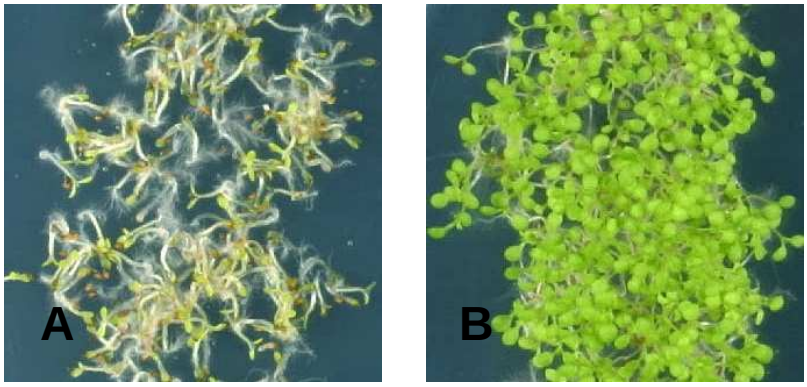

Fig. 3.10: Comparison of growth on HDM plates between plants of the non-mutated plant line RV86-5 and suppressor mutant line* sud1*: *Seedlings of* Arabidopsis *plant line RV86-5, containing only the Dex-inducible transgene, died on HDM plates (**A**) soon after germination, whereas the EMS-mutagenized plant line* sud1*, carrying both the Dex-inducible UbK48R-transgene and a UbK48R-induced cell death suppressor mutation, survived on HDM plates and showed strong growth (**B**).*

In another assay, the same backcrossed mutant line of *sud1* was used to test the growth of *sud1* mutants on Ara medium supplemented with different concentrations of MG132, a proteasome inhibitor. The aim of this experiment was to see if the EMS-induced mutation also increased the survival chances in the case that the proteasome was inhibited. The concentrations of MG132 varied between 20 µM and 400 µM. On Ara medium that contained MG132 in a concentration of 200 µM but no Dex for transgene induction, *sud1* mutant plants grew indeed much stronger than RV86-5 control plantlets (Fig. 3.11). It could be observed that seedlings of the *sud1* mutant line survived much better in the presence of the proteasome inhibitor than the RV86-5 control plants that did not carry the UbK48R-induced cell death suppressor mutation.

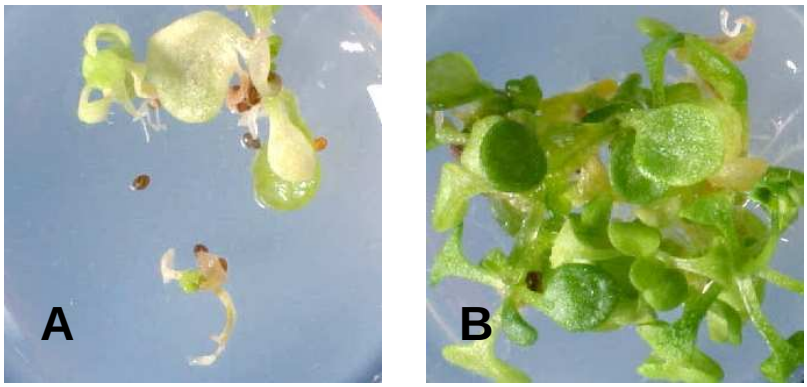

Fig. 3.11: Comparison of growth on Ara medium, supplemented with the proteasome inhibitor MG132, between plantlets of the non-mutated plant line RV86-5 and suppressor mutant line* sud1*: *Ara medium was only supplemented with vitamins and MG132 in a concentration of 200 µM, but not with Dex for transgene induction. Seedlings of* Arabidopsis *plant line RV86-5, containing only the non-induced transgene, died soon after germination (**A**), whereas the EMS-mutagenized plant line* sud1*, carrying both the Dex-inducible UbK48R-transgene and a suppressor mutation of UbK48R-induced cell death, showed a higher resistance to the proteasome inhibitor MG132 (**B**). Pictures taken from Schlögelhofer* et al.*, 2006.*

Due to these promising results, *sud1* was chosen for identifying the mutated allele and was therefore crossed with *Arabidopsis* ecotype Landsberg *erecta* (L*er*) to create a mapping population (cf. also mapping 3.4). Although this process was repeated twice with two different mapping populations, the mapping failed in both cases. In the first mapping population, no candidate region with homozygous ecotype could be identified. Therefore, a second mapping population was created and characterized. Unfortunately, this second mapping process also failed as all progenies in the F3 generation showed silencing effects and died on HDM plates after transgene induction. A classification of the plants in mutation containing plants and no mutation containing plants was not possible anymore. As experiments with *let* mutations (cf. 3.3.3) in the RV86-5 background indicated that at least two of three *let* mutations were dominant like the *sud1* mutation (Fig. 3.12), no third mapping population with *sud1* mutant plants was created and the focus was changed to another promising line with recessive character: *sud2*.

3.3.2 Recessive mutations: *sud2*, *sud3*, *sud4* and *sud5*

The four selected recessive mutant lines of the EMS treatment – *sud2*, *sud3*, *sud4* and *sud5* – were further investigated and characterized. To eliminate mutations not linked to the examined *sud* phenotype, all four lines were backcrossed with RV86-5. The seeds of those crosses were put on Ara plates and the best growing plant of each mutant line with the highest seed yield was selected for the F2 generation. 20 plants of each mutant line were grown in the F2 generation and the *sud* phenotype of each line was tested in the F3 generation on HD – and HDM plates. According to their growth, the two best growing lines of *sud3*, *sud4* and *sud5* with the strongest *sud* phenotype were selected for further investigations:

- ***sud3***: ***sud3* # 4-15** and ***sud3* # 4-16**
- ***sud4***: ***sud4* # 1-3** and ***sud4* # 1-4**
- ***sud5***: ***sud5* # 3-17** and ***sud5* # 3-18**

Before selecting two lines of *sud2* for further experiments, this mutant line was additionally tested in sensitivity assays to reveal a supplemental phenotype potentially linked to the *sud* mutation. In a first assay with non-backcrossed *sud2* seeds, several promising phenotypes had been found. In this first assay, *sud2* seeds were sown on plates with different types of sugar in varying concentrations and with different hormones. Mutant line *sud2* showed differences in growth compared to the RV86-5 control plants on various media, as e.g. plates with medium that was supplemented with 6 % of sucrose revealed a chlorotic phenotype with reduced root growth. Chlorotic leaves were also detected with medium supplemented with 3.2 % of glucose and on plates with medium containing 3.6 % mannitol. Besides this, *sud2* mutants showed differences in growth compared to RV86-5 on medium supplemented with NaCl [150 mM] and MeJA [5 µM]. NaCl generated little plants with a chlorotic phenotype, whereas MeJA also generated little plants, but with brownish-yellowish leaves.

Due to these results with the original *sud2* line, the sensitivity assays were repeated with the backcrossed *sud2* plant lines. Six out of 20 plant lines of the F2 generation, that showed a clear *sud* phenotype on HDM plates, were again tested on medium supplemented with 3.2 % glucose, MeJA [5 µM] and NaCl [150 mM]. The differences in phenotype observed in the first assay could only be detected with some but not with all mutant plant lines. At least one mutant line always showed no difference in growth compared to the RV86-5 control. Therefore no detected growth difference observed in the first assay was linked to the putative *sud* mutation. Out of the six F2 lines selected for the growth tests, two lines that showed the best growth in all assays were finally selected for further investigations on *sud2*:

- ***sud2***: ***sud2* # 4-16** and ***sud2* # 4-20**.

Seeds of all chosen recessive mutant lines from *sud2*, *sud3*, *sud4* and *sud5* were put on Ara plates and six plants of each line were grown on soil. The strongest plant, i.e. the one with the highest yield of seeds, was chosen for further investigations:

- ***sud2***: ***sud2* # 4-16-5**
- ***sud3***: ***sud3* # 4-16-2**
- ***sud4***: ***sud4* # 1-4-2**
- ***sud5***: ***sud5* # 3-17-5**

From each recessive mutant, seeds of the selected line were again screened in sensitivity assays. Experiments were performed with medium supplemented with 1 % or 6 % of sucrose, 0.3 % or 3.2 % of glucose and 3.6 % of mannitol. Besides these sugars, the chemicals NaCl [150 mM] and LiCl [100 mM] were tested as well as the hormones ABA [1 µM], ACC [1 mM], MeJA [5 µM], SA [0.5 mM], 2IP [0.5 mg/l] and 2,4-D [0.4 mg/l]. All chemicals and hormones were added to Ara medium containing 1 % sucrose. For direct growth comparison, seeds of the non-mutated plant line RV86-5 were also put on each plate. After 26 days, no differences in growth could be detected for the

mutant line *sud4*. Mutant lines *sud2* and *sud5* showed a difference with one substance, but whereas *sud2* plants grew better on mannitol (3.6 %), *sud5* plants did not support growth on SA [0.5 mM] as well as the control plants and only half of the plantlets survived on Ara medium supplemented with SA. The most sensitive mutant line however was *sud3*, as these plants showed different growth with four different substances. A chlorotic phenotype could be detected on plates supplemented with 3.2 % glucose and reduced growth speed on medium with 0.3 % glucose. In contrast to *sud2* plants, all *sud3* plantlets died on medium supplemented with 3.6 % mannitol, just as on medium where SA was added in a concentration of [0.5 mM].

In addition to these substances, all four recessive mutants were also screened on Ara medium supplemented with the proteasome inhibitor MG132, since sensitivity assays with *sud1* mutant plants had revealed a higher resistance of this mutant line to MG132 (Fig 3.11). Similar to the assay with *sud1*, seeds of all four mutant lines were grown on Ara medium supplemented with different concentrations of MG132, varying from [100 µM] up to [400 µM]. MG132 was dissolved in DMSO and added to the Ara medium before sowing, whereas the amount of DMSO was always constant and only the concentration of MG132 dissolved in DMSO varied.

Only *sud5* plants grew slightly better than the control plants of RV86-5 and Columbia (Col-0). The other three lines *sud2*, *sud3* and *sud4* showed no phenotypic differences in growth compared to the control plants.

All four recessive mutants were crossed with 86L*er* to create mapping populations. 86L*er* is an *Arabidopsis* plant line with L*er* ecotype where the UbK48R transgene had been introgressed (cf. 3.4.1). Mutant line *sud2* was finally selected for a more detailed mapping process (cf. 3.4.2), whereas the mapping populations that were carried out with the mutant lines *sud3*, *sud4* and *sud5* have not been further analysed yet. The crosses of *sud3*, *sud4* and *sud5* with 86L*er* are listed in Table 3.3.

Mutant line	♂	Cross	♀
sud3	86L*er* #16-3-2	**x**	*sud3* # 4-15-1
sud3	86L*er* #39-4-1	**x**	*sud3* # 4-15-1
sud3	86L*er* #48-2-2	**x**	*sud3* # 4-15-1
sud4	86L*er* #22-3-1	**x**	*sud4* # 1-4-2
sud4	86L*er* #39-4-3	**x**	*sud4* # 1-4-2
sud4	86L*er* #48-2-1	**x**	*sud4* # 1-4-2
sud5	86L*er* #22-3-3	**x**	*sud5* # 3-18-2-1
sud5	86L*er* #39-4-3	**x**	*sud5* # 3-18-2-1
sud5	86L*er* #48-2-3	**x**	*sud5* # 3-18-2-1

Table 3.3: Summary of the nine crosses carried out with three different* sud *mutants (*sud3*, s*ud4*,* sud5*) and 86L*er *(cf. 3.4.1). *For the crosses, the pollen of 86L*er *plant lines was used to pollinate the pistil of* sud *mutant lines. Each recessive* sud *mutant line was crossed with three different 86L*er *lines to create mapping populations for further analyses.*

The seeds of all nine crosses with 86L*er* were put on Ara plates and the seeds of eight plants from each cross were harvested and stored for later analysis.

3.3.3 *let* mutants

In a second approach, a sequence similarity-based search was initiated to identify *Arabidopsis* genes potentially involved in the ubiquitylation process. In collaboration with bioinformatics specialists, a group of *Arabidopsis* genes that contained a ubiquitin conjugating domain were identified. As a distant relative of the four *Arabidopsis* genes has been shown to play a role in mammalian PCD, a potential role of the *Arabidopsis* genes in cell death pathways of plants had been hypothesized. Single mutant plant lines were obtained as T-DNA insertion lines from diverse stock centers and designated *let1*, *let2*, *let3* and *let4*.

To see if the *let* mutants show any similarity to the potential *sud* mutant plants selected on HDM plates, all four different mutant lines were crossed with the transgenic plant line RV86-5 to get plant lines carrying both the *let* insertion and the inducible UbK48R-expressing transgene. Homozygous single

let mutant lines containing also the UbK48R transgene were selected in the F2 generation by the technicians Kerstin Luxa and Michaela Lehnen. It was possible to obtain homozygous mutants for *let1*, *let2* and *let4* but not for *let3*.

In a first experiment, four plant lines were tested on HDM plates: single *let1* and single *let2* plants, a double mutant line with homozygosity for *let1* and heterozygosity for *let2* and another double mutant line being homozygous for *let4* and heterozygous for *let2*. Seeds of all four mutants were put on HDM plates. RV86-5 seeds served as control and were put on the same plate to compare the plant growth under equal conditions. All four *let* mutants survived much better on HDM plates than the control plants. The *let* plantlets were stronger and the leaves greener compared to the control (Fig. 3.12).

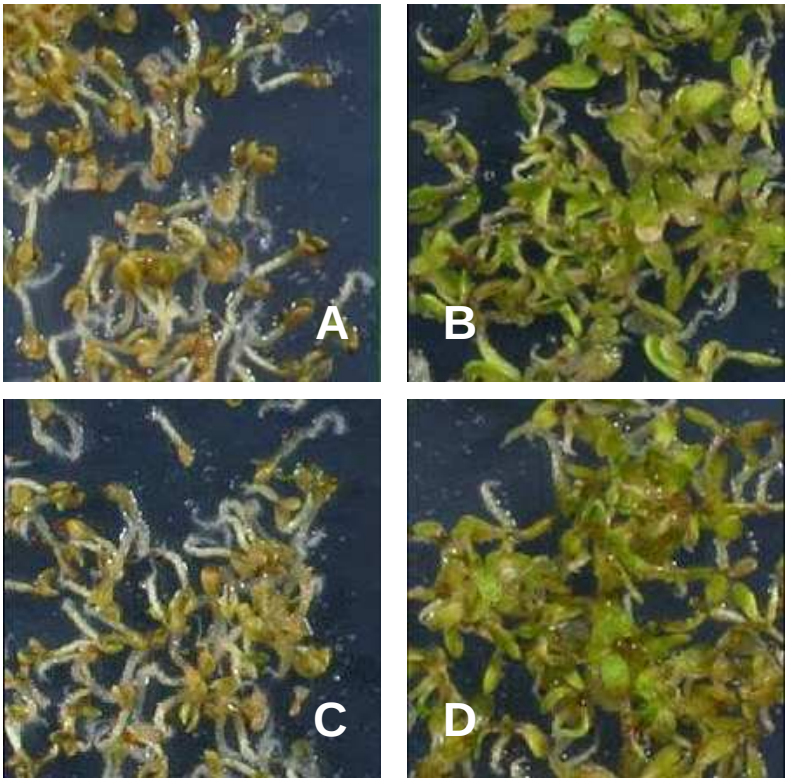

Fig. 3.12: Comparison of growth on HDM plates between RV86-5 plants that expressed only the UbK48R transgene and two mutant lines that expressed the UbK48R transgene and possessed a single* let *mutation: *Seedlings of* Arabidopsis *plant line RV86-5, containing only the Dex-inducible transgene, died on HDM plates soon after germination (**A,C**), whereas the* let *mutant lines that were both expressing the Dex-inducible UbK48R-transgene and possessing the* let1 *(**B**) or the* let2 *(**D**) mutation, survived on HDM plates and showed strong growth.*

The phenotype of the tested *let* mutants was similar to the one found in *sud* mutants from the EMS mutagenesis. To see if the *let* mutations were genetically related to one of the already identified *sud* mutations, plants carrying both mutations were generated for complementation analysis. The three homozygous *let* mutants *let1*, *let2* and *let4* were crossed with the four recessive *sud* mutants *sud2*, *sud3*, *sud4* and *sud5*, but not with *sud1*, as the complementation assay can only be applied to plant lines with recessive mutations. The seeds of each cross were put on HD – and HDM plates; seeds of the two parental lines served as control. According to the complementation test theory, the plants of these crosses should die on HDM plates if the *let* mutation is different from the *sud* mutation, and progeny should survive on HDM plates if the two mutations are related to each other and therefore located in the same gene – under the condition that both tested mutations are recessive. After 21 days, all crosses were still growing on HDM plates, some of them even better than the parental lines. No plantlets of any cross died and the phenotype was similar to the one of *let* mutants. According to the complementation test predictions that plants survive when the same gene is mutated, these results indicated that all four *sud* mutations were identical with the three *let* mutations. As it had been shown in former experiments that all four *sud* mutants were recessive lines and independent from each other, the obtained results suggested that the *let* mutations are dominant mutations.

To check the possibility of *let* being a dominant mutation, all three single *let* mutants were again crossed with the UbK48R-expressing plant line RV86-5 to observe the growth of the F1 generation. All plants of these crosses should die in the F1 generation in the case the *let* mutation is recessive. The seeds of all crosses were put on HD – and HDM plates with seeds of the *let* mutant line and RV86-5 as control, respectively. Two out of three *let* mutants (*let1* and *let2*) turned out to be dominant as their progeny showed a strong phenotype in the F1 generation. The offspring from the cross with *let4,* however, did not grow better than the control plants of the transgenic line RV86-5, indicating the recessive character of this *let* mutant.

Similar to the sensitivity assays done with the recessive *sud* mutants, all three *let* single mutant lines *let1*, *let2* and *let4* and the double mutants *let1 let2* and *let1 let4* were also tested on media supplemented with different sugars, hormones and chemicals. Plants carrying the *let* mutation showed no differences in growth with most of the added substances. Only Ara medium supplemented with SA [0.5 mM] and 2,4-D [0.4 mg/l] influenced the growth of some mutants compared to the control plantlets: The double mutant *let1 let2* produced significant smaller plants on Ara medium supplemented with 2,4-D compared to the control plants of RV86-5, whereas the other mutants showed normal growth. On Ara medium supplemented with SA, only two *let* mutants (*let1* and *let1 let2*) showed normal growth when SA was added to the Ara medium, whereas *let2*, *let4* and *let1 let4* presented reduced germination and decelerated growth.

To see if more phenotypical elements of *sud1* mutants could be found in *let* mutant plants, all *let* mutants were also screened on Ara medium supplemented with the proteasome inhibitor MG132 since sensitivity assays with *sud1* plants had revealed a higher resistance of this mutant line to MG132. Here however, all *let* mutants showed no higher resistance to MG132 unlike the *sud1* mutants and no differences in growth with the control plants of RV86-5 could be detected.

3.4 Mapping of a suppressor mutation

After having characterized dominant and recessive suppressor mutants, at least one unknown mutation should be identified in a mapping process. Final aim of the mapping process was to determine one or more genes that were thought to be involved in the cell death caused by expressing the variant ubiquitin UbK48R. For a mapping population, two different ecotypes are necessary. This approach permits to use markers that detect small fragments at the same position in the genome, but with different size depending on the ecotype. In this way, the ecotype at the investigated position can be

determined and conclusions about the presence of the mutation at the investigated position can be drawn due to the fact that the mutation can only be found in one ecotype. As the suppressor mutations were all generated in plants with Col-0 ecotype, L*er* was chosen as second ecotype to create a mapping population. Two mapping populations were made with the *sud1* mutant and normal L*er* ecotype (cf. 3.3.1), but both approaches could not be finished due to severe problems during the mapping process. After the failure of these two mapping populations, the mapping strategy was changed, and a L*er* plant line called 86L*er* that also expressed the UbK48R transgene was generated before the creation of a third mapping population with L*er* ecotype.

3.4.1 86L*er*

For obtaining plant lines with L*er* ecotype that also express the UbK48R transgene, the transgene of plant line RV86-5 (and later the progenies of the crosses) was introgressed all in all four times with L*er*, and the last cross was cultivated up to the F3 generation (Fig. 3.13). Introgression was supported by marker-based selection of a progeny with a high content of L*er* DNA. Finally, 48 plant lines were generated that were homozygous for the inducible transgene and largely made up of L*er* ecotype.

All seeds of a cross were sown on Ara plates supplemented with Hyg for transgene selection. The first cross resulted in four plants in the F1 generation. One plant line was selected to be further cultivated in the F2 generation. A PCR-based genotyping with 20 different plants out of this F2 generation was carried out with different mapping primers (cf. 3.4.3) to identify plant lines with an above average amount of L*er* DNA. One plant line that showed these characteristics (RV86-5 x L*er* # 3-11) was selected for a second cross with L*er*. Two more crosses were carried out with L*er*, always using plants from the F1 generation of a previous cross that showed a strong L*er* phenotype. To avoid new silencing effects of the transgene, each cross was also tested on HD – and HDM plates to see whether the transgene was

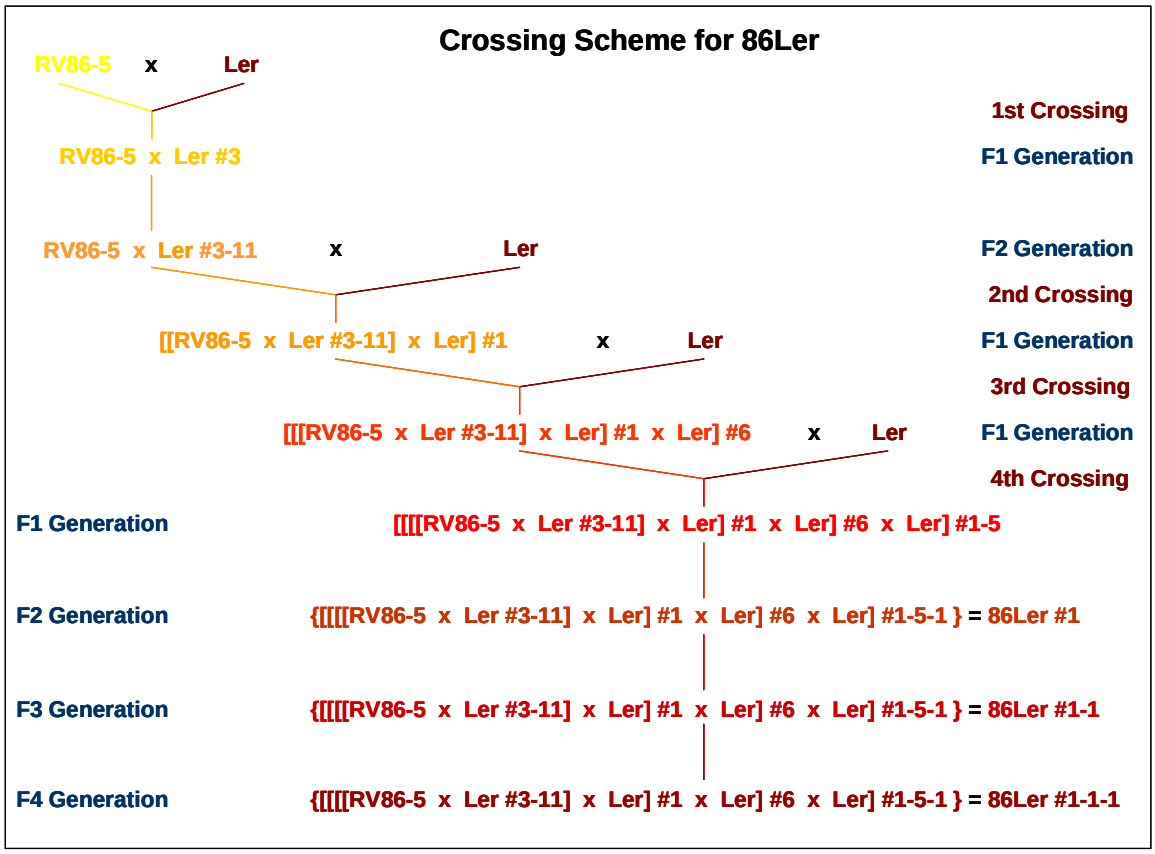

Fig. 3.13: Crossing scheme for 86Ler to generate plant lines with Ler ecotype expressing the UbK48R transgene: *To introduce the UbK48R transgene into Ler background, transgenic plant line RV86-5 was first crossed with Ler. In the F2 offspring of this cross, one plant line (RV86-5 x Ler #3), that had been genotyped by PCR to carry more Ler DNA than other lines, was again interbred with Ler. One F1 offspring line of this cross was crossed a third time with Ler, and one F1 offspring line of that cross was interbred a fourth time with Ler. Both F1 offspring lines were selected due to their strong Ler phenotype. The progenies of the last cross were grown up to the fourth generation, and in the F2 generation, the plant line was renamed 86Ler. Plants of the F3 and F4 generation were later used to create new mapping populations. The colour code from yellow (only Col-0 ecotype) via orange to dark brown (only Ler ecotype) symbolises the changes in ecotype from Col-0 to Ler during the crosses.*

expressed correctly. The proper expression of the transgene was proven when plants that survived on Ara plates supplemented only with Hyg died on HDM plates, as in this case the transgene was activated and the modified ubiquitin UbK48R caused plant cell death.

After the fourth cross with L*er*, the progenies were grown up to the fourth generation (F4) and renamed 86L*er* in the F2 generation to make clear that

86L*er* plant line #	Transgene	86L*er* plant line #	Transgene	86L*er* plant line #	Transgene
1	homozygous	17	heterozygous	33	homozygous
2	homozygous	18	heterozygous	34	heterozygous
3	homozygous	19	heterozygous	35	homozygous
4	heterozygous	20	heterozygous	36	homozygous
5	heterozygous	21	homozygous	37	homozygous
6	heterozygous	22	homozygous	38	heterozygous
7	homozygous	23	homozygous	**39**	homozygous
8	homozygous	24	homozygous	40	heterozygous
9	heterozygous	25	heterozygous	41	homozygous
10	heterozygous	26	homozygous	42	homozygous
11	homozygous	27	heterozygous	43	heterozygous
12	homozygous	**28**	homozygous	44	homozygous
13	heterozygous	29	homozygous	45	homozygous
14	heterozygous	30	heterozygous	46	heterozygous
15	homozygous	31	homozygous	47	homozygous
16	homozygous	32	heterozygous	**48**	homozygous

Table 3.4: Results of a homozygosity test for the UbK48R transgene with 48 plant lines of the new generated plant line 86Ler. *The distribution of the UbK48R transgene in the different 86Ler plant lines was tested with seeds of the F3 generation that were grown on Ara plates supplemented with Hyg for transgene selection. The whole offspring of plant lines with a homozygous distribution of the UbK48R transgene survived on the selective plates, whereas plant lines with only one UbK48R allele gave rise to both surviving and dying progeny on the selective medium. Plant lines written in red (and their offspring) were used for crosses to create mapping populations.*

this L*er* line contained the inducible UbK48R transgene of plant line RV86-5. All 48 plant lines of 86L*er* were tested on Ara plates supplemented with Hyg to control the distribution of the transgene. 28 out of 48 plants possessed two alleles of the inducible transgene and were therefore homozygous for it. The results of the homozygosity test for the UbK48R transgene are listed in Table 3.4.

Five different lines were finally selected for further experiments (86L*er* # **3**, 86L*er* # **16**, 86L*er* # **28**, 86L*er* # **39** and 86L*er* #**48**) and plants of the next generation (F3) were chosen to create new mapping populations.

3.4.2 Generation of and selection for a mapping population

After the failure with the two mapping populations created with the dominant suppressor mutation *sud1* (cf. 3.3.1), a new mapping population with a recessive suppressor mutant was generated. Due to the promising results in the previous analyses, the *sud2* mutation was selected for positional cloning. This mutant line showed good survival rates on HDM plates, complementation group analysis had revealed the recessive character of the mutation and the mRNA level of the UbK48R transgene indicated normal expression after Dex induction. Therefore this plant line was chosen to create a mapping population with L*er* ecotype to further characterize the mutated allele(s).

In a first approach, *sud2* was crossed with L*er* and the strongest plant of the F1 generation was selected for further investigations. 20 plants were cultivated in the F2 generation and their seeds were tested on HD – and HDM plates to make sure that they had not lost the *sud2* mutation or the transgene. Six plant lines that showed good growth on these selective plates were chosen for sensitivity assays to find additional phenotypes linked to the *sud2* mutation. Seeds were sown on medium supplemented with 3.2 % glucose, MeJA [5µM] and NaCl [150mM] and the growth of the plant lines was compared. Like the *sud2* plants that were backcrossed with RV86-5, none of these plants showed any differences to RV86-5, only the speed and the intensity of growth were slightly different.

Therefore the six plant lines were classified according to their growth abilities and two lines that showed the best growth in all assays were selected for crossing with the new 86L*er* plant lines:

- *sud2* x L*er* #2-1
- *sud2* x L*er* #2-2

A total of six crosses were carried out with different 86L*er* lines to create a mapping population (Table 3.5).

New name	♂	Cross	♀
Mapping population #1 (**MP #1**)	[*sud2* x L*er* #2-1-1]	**x**	86L*er* #3-2
Mapping population #2 (**MP #2**)	[*sud2* x L*er* #2-1-1]	**x**	86L*er* #16-2
Mapping population #3 (**MP #3**)	[*sud2* x L*er* #2-1-2]	**x**	86L*er* #16-1
Mapping population #4 (**MP #4**)	[*sud2* x L*er* #2-1-4]	**x**	86L*er* #39-3
Mapping population #5 (**MP #5**)	[*sud2* x L*er* #2-2-3]	**x**	86L*er* #28-1
Mapping population #6 (**MP #6**)	[*sud2* x L*er* #2-2-5]	**x**	86L*er* #48-1

Table 3.5: Summary of the six crosses carried out with five different* sud2 *mutant lines and six different 86L*er *plant lines to create mapping populations. *For the crosses, the pollen of* sud2 *mutant lines was used to pollinate the pistil of 86L*er *plant lines. The offspring of the crosses was used to identify the suppressor mutation of* sud2*. To simplify further investigations with the resulting plant lines, all crosses were renamed (MP #1 to MP #6).*

These six crosses formed the starting base for six different mapping populations with the recessive *sud2* mutation. The F1 generation of all six crosses was grown on Ara plates and later on soil and the strongest plant with the highest amount of seeds of each cross was selected for further cultivation. To accelerate the mapping process, seeds of the F2 generation were already tested on HD – and HDM plates to look at their growth behaviour and to exclude silencing. The results are summarized in Table 3.6.

Plant line	Growth on HD plates	Growth on HDM plates
MP #1-5	¼ surviving plants	**3** surviving plants
MP #2-6	¼ surviving plants	**1** surviving plant
MP #3-1	¼ surviving plants	**5** surviving plants
MP #4-5	¾ surviving plants	**1** surviving plant
MP #5-1	¼ surviving plants, slightly better growth than on HDM plates	¼ surviving plants
MP #6-1	¼ surviving plants,slightly better growth than on HDM plates	¼ surviving plants

Table 3.6: Number of surviving plants in the F2 generation growing on HD – and HDM plates six weeks after sowing. *The growth of six different mapping population, all generated with* sud2 *mutant lines, was analysed on selective plates. Only MP #5-1 and MP #6-1 showed the expected distribution of living and dead plantlets (ratio 1 : 4) on HD – and HDM plates. Plants of MP #4-5 survived unexpectedly well only on HD plates, whereas more plantlets than average died on HDM plates of MP #1-5, MP # 2-6 and MP #3-1.*

The expected distribution of surviving and dying plants on HD – and HDM plates in the F2 generation was 1 : 4, as one in four plants was supposed to carry both mutated *sud2* alleles, whereas three in four plants contained only one mutated allele (two in four) or no mutated allele at all (one in four). Whereas the offspring of MP #5-1 and MP #6-1 showed this distribution both on HD – as well as on HDM plates, the progenies of the mapping populations MP #1-5, MP #2-6, MP #3-1 and MP #4-5 revealed bigger differences between the growth on HD – and HDM plates. Especially MP #4-5 showed increased survival rates on plates supplemented with HD whereas only one plant on the HDM plate survived. Due to these results, the two mapping populations with the expected ratio, MP #5-1 and MP #6-1, were finally chosen to continue the mapping.

3.4.3 First-pass mapping with MP #5 and MP #6

50 plants of each population were put on soil to raise the F2 generation, as well as the surviving plants taken from HDM plates. The seeds of these plants - the F3 generation - were re-screened on HD – and HDM plates to divide plants into different groups according to their growth on HDM plates due to their genotype. Three possible states were expected:

- **Group 1: All plants die.** This would be the case if the plant has lost the mutation and cannot survive anymore the Dex treatment that starts the translation of the modified ubiquitin UbK48R. The ecotype at the position of the mutation should be L*er*.

- **Group 2: All plants survive.** In this case, the mutation is still present in the plant and prevents the plant from dying, even though the variant ubiquitin UbK48R is expressed. The ecotype at the position of the mutation must be Col-0, as both mutated alleles are necessary to express the recessive *sud2* mutation.

- **Group 3: Some plants die and some plants survive.** This distribution appears if the F2 mother plant is heterozygous concerning the mutation. In this case, plantlets in the F3 generation can contain various combinations (as in the F2 generation): The surviving plants feature two mutant alleles, whereas plants that die on HDM plates contain at least one WT allele at the SUD2 locus.

28 plant lines of MP #5-1 and 26 plant lines of MP #6-1 were sown on HD – and HDM plates. According to the predictions, all three different survival types could be found. To exclude misinterpretations of the mapping results, only the clear cut plant lines with defined genotype, i.e. those plant lines where all plants died or where all plants survived on HDM plates were selected for PCR-based genotyping.

In a first approach, 14 plant lines – seven of each mapping population – that did not survive the Dex treatment and 12 plant lines – six of each mapping population – that survived on HDM plates were selected for testing by PCR with selected markers.

For each plant line, leaves from at least six different plantlets were harvested to extract genomic DNA. The plants were aged 24 days and pooling of individual plants ensured a representative cross section of the genotype. A set of 29 different markers was used to get a rough estimation of the distribution of L*er* and Col-0 ecotype DNA on each of the five chromosomes in *Arabidopsis*. The markers for the mapping were chosen in a way that they covered all five chromosomes equally in a regular distance of around 20 cM (Fig. 3.14). The detection of the individual genotype of each plant line was possible due to the two different ecotypes L*er* and Col-0 present in the mapping population: All used markers detected in both ecotypes a DNA fragment at the same position of the chromosome, but according to the ecotype, the size of the fragment differed. Amplification with PCR and subsequent separation with gel electrophoresis on a 3 % Agarose gel revealed bands of different sizes that could be assigned to one of the two ecotypes.

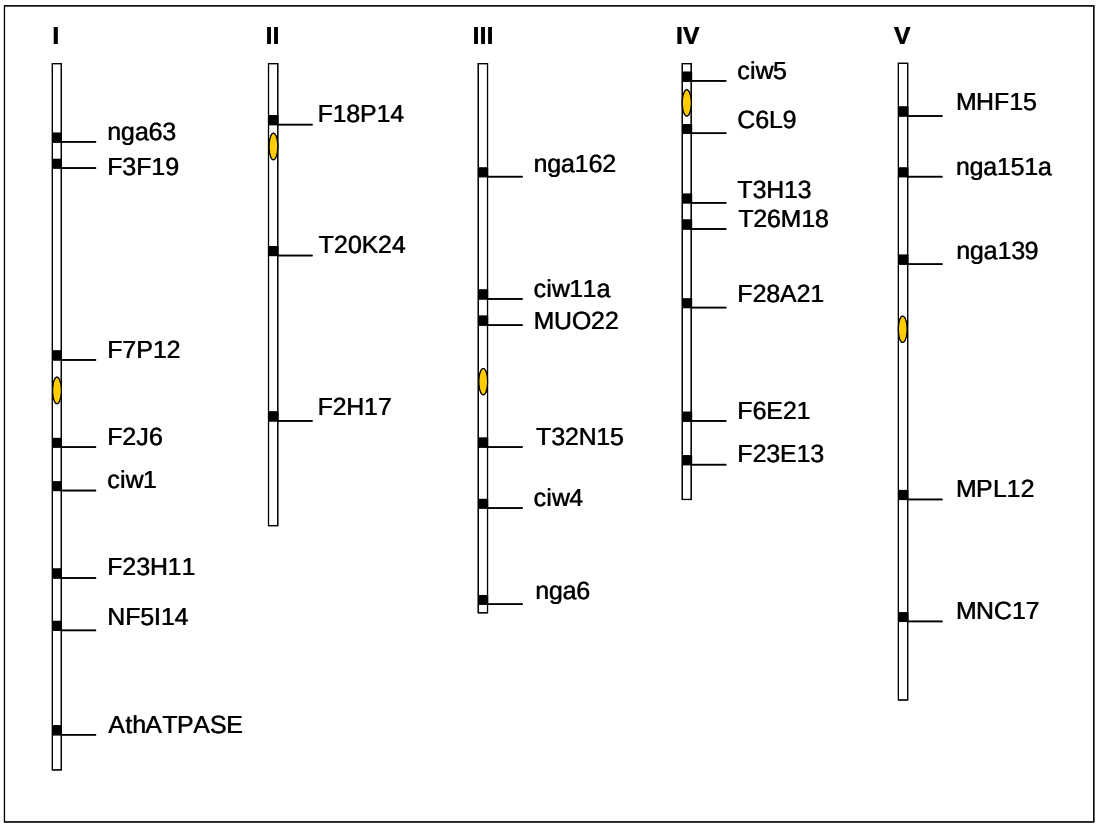

Fig. 3.14: Distribution of 29 mapping markers selected for mapping of the* sud2 *mutation. *The chosen markers are equally distributed on all five chromosomes (I to V) of* Arabidopsis thaliana *to ensure a general overview of the two genotypes* Ler *and Col-0 present in the genome at the investigated position. Yellow: centromer.*

In a first screening of the mapping population, the distribution of the ecotype of each plant line was analysed: Plants were homozygous with respect to one ecotype at one marker position if only one band was detectable. If the plant line was heterozygous at the investigated position because of the presence of two different alleles from two different ecotypes in the genome, the Agarose gel showed two bands. The marker system showed high accuracy: A test assay with different ratios of the two ecotypes demonstrated that two bands were detectable also with uneven distribution of the ecotype, e.g. 70 % of one ecotype and only 30 % of the other.

The size of an amplified fragment varied in the two ecotypes between 16 and 125 bp and could be separated on Agarose gels due to the high resolution. An example of an Agarose gel can be seen in Fig. 3.15, where the results of a

PCR with marker “MPL12”, located at the bottom arm of chromosome V, were separated on a 3 % Agarose gel.

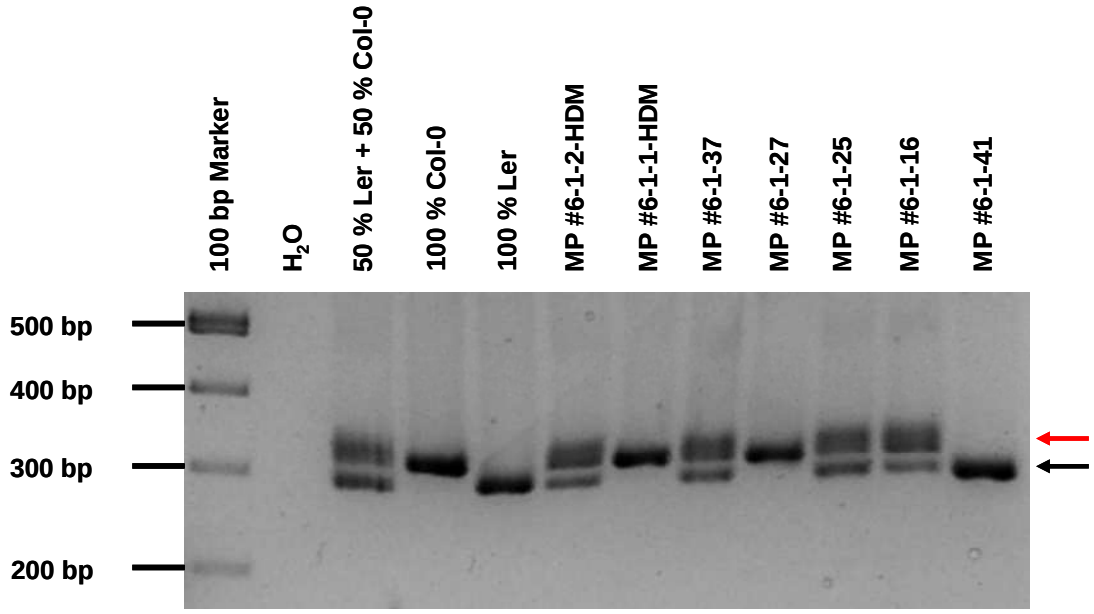

Fig. 3.15: Gel electrophoresis of PCR fragments of marker “MLP12” to analyse the genotype of different plant lines on the investigated position. *Seven samples with unknown genotype were examined. Samples that contained only Ler DNA showed a band at 293 bp (black arrow; MP #6-1-41), samples with only Col-0 DNA revealed a band at 319 bp (red arrow; MP #6-1-1-HDM and MP #6-1-27). If the sample contained both Ler and Col-0 DNA, both bands could be detected (MP #6-1-2-HDM, MP #6-1-37, MP #6-1-25 and MP #6-1-16).*

In this example, the fragment size of L*er* and Col-0 DNA differed by 26 bp. The high percentage of Agarose (3 %) used in the gel allowed detecting clearly this small difference in the size of the PCR fragments. The positive controls of L*er* (one band at 293 bp) and Col-0 (one band at 319 bp) as well as the mixed control with 50 % genomic DNA of each ecotype (two bands: one at 319 bp and one at 293 bp) showed clear-cut fragments. According to these control bands, the different samples could be assigned correctly to their individual genotype.

All five chromosomes of *Arabidopsis thaliana* were screened for their genotype at the position of the different markers (cf. Fig. 3.14). The results of this first rough mapping were summarized in a list and the different plant

lines were compared. The sought-after region was supposed to show two main characteristics in the genotype distribution:

- All plants of group 1 whose progeny died on medium supplemented with Dex should be largely homozygous for L*er* DNA.

- All plants of group 2 whose progeny were still in possession of the mutation and therefore survived on medium supplemented with Dex should be largely homozygous for Col-0 DNA.

In both mapping populations, exactly this genotype distribution could be detected in one region on chromosome III (Fig. 3.16 and Fig. 3.17).

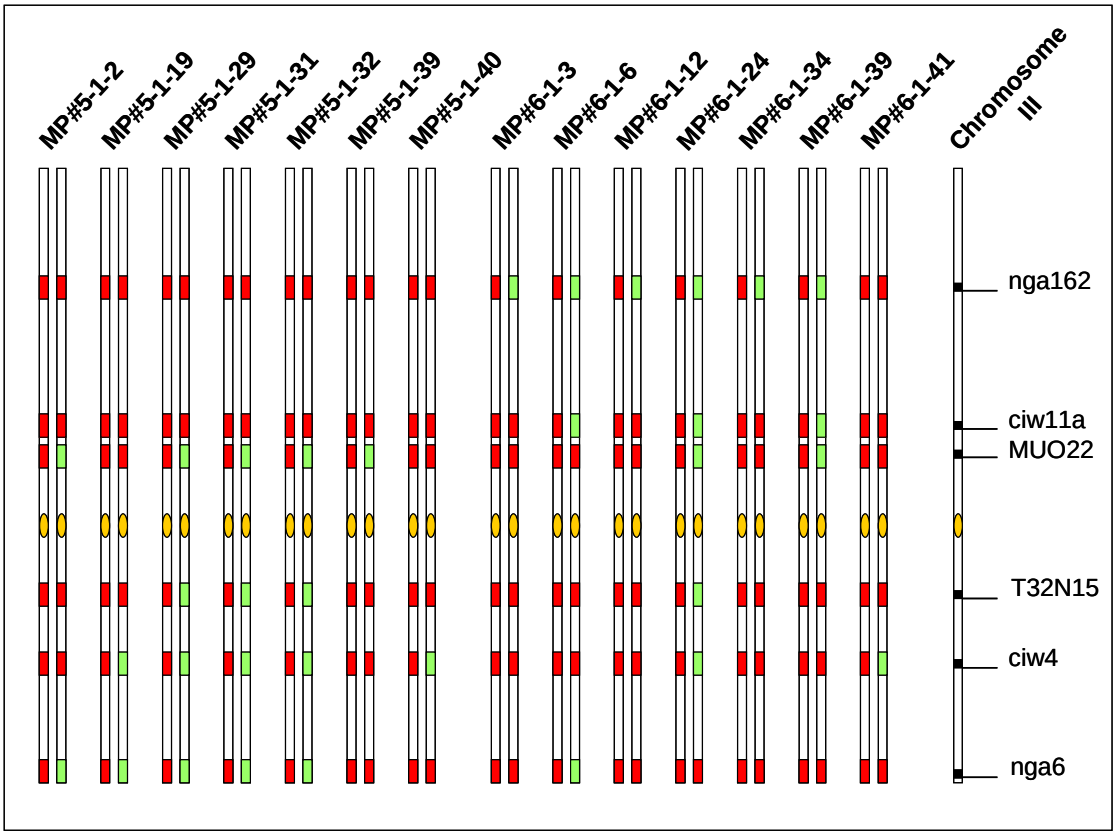

Fig. 3.16: Distribution of* Ler *(red) and Col-0 (green) genotype at six investigated positions of chromosome III. *All shown plant lines belong to group 1 (all plants die after Dex treatment due to a loss of the* sud *mutation), and the expected genotype was* Ler*. All genotype characterizations are based on the results of the PCR genotyping. Colour coding: Red – Ler genotype at the investigated position; green – Col-0 genotype at the investigated position; yellow – centromer.*

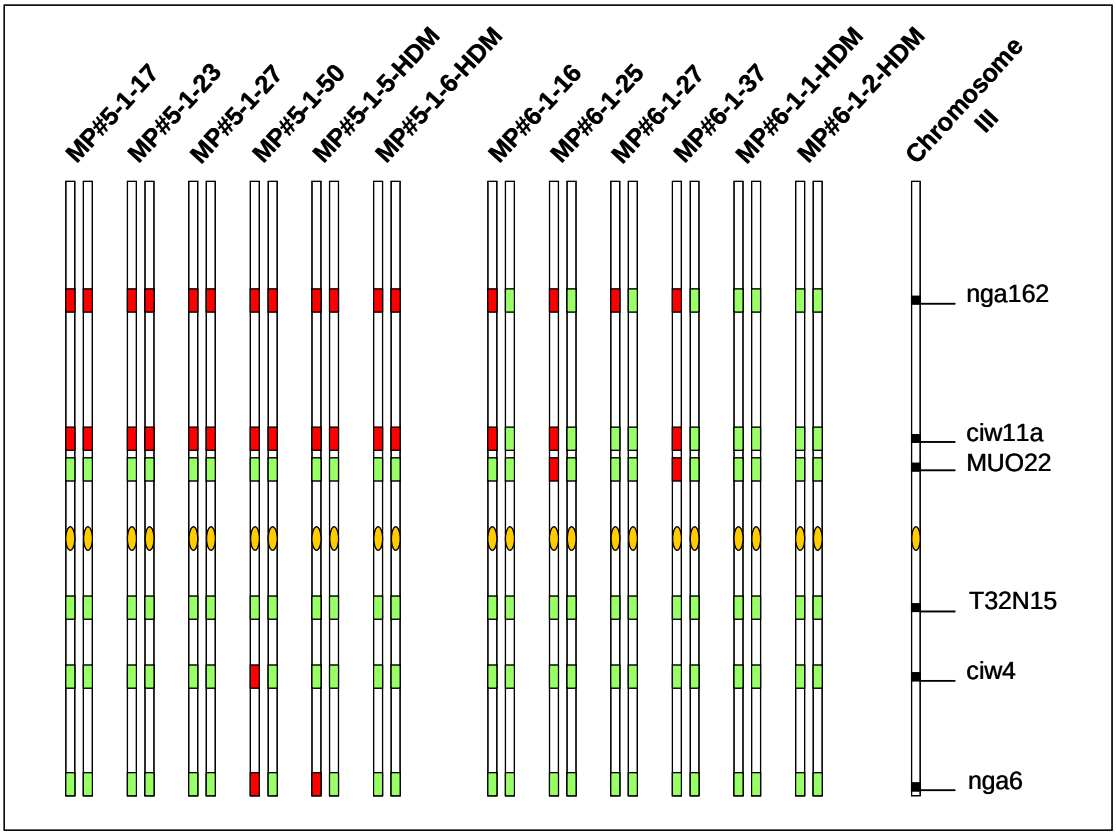

Fig. 3.17: Distribution of Ler (red) and Col-0 (green) genotype at six investigated positions of chromosome III. *All shown plant lines belonged to group 2 (all plants survive after Dex treatment due to the* sud *mutation), and the expected genotype was Col-0. All genotype characterizations are based on the results of the PCR genotyping. All investigated plant lines showed a region between marker "MUO22" and "ciw4" that contained only Col-0 DNA. Colour coding: Red – Ler genotype at the investigated position; green – Col-0 genotype at the investigated position; yellow – centromer.*

A closer look at the surviving plant group in comparison with the results obtained for plants of group 1 narrowed down the possible area of the sought-after mutation due to the PCR results: As the mutation is located in Col-0 genotype, the area north of marker "ciw11a" can be excluded. Genotyping of MP #5 plant lines with marker "ciw11a" at 9.77 Mbp and with marker "nga162" at 4.61 Mbp revealed only L*er* genotype in this part of chromosome III. This excludes the presence of the *sud* mutation in this area. Therefore the focus of interest was narrowed down to the region south of marker "ciw11a". Between marker "ciw11a" at 9.77 Mbp and marker "nga6" at 23.04 Mbp, only Col-0 DNA could be detected in group 2 plants (all plants survive). The only exception could be found with marker "ciw4" at 18.9 Mbp, where one plant

line out of 12 (MP #5-1-50) showed both Col-0 and L*er* genotype. As the *sud2* mutation is a recessive mutation, the plants have to be homozygous for Col-0 at the position of the mutated allele; therefore the region south of marker "ciw4" was excluded from the sought-after region. Due to these results, the main focus of the investigations was put on the region between marker "ciw11a" at 9.77 Mbp and marker "ciw4" at 18.9 Mbp. For further examination of this area on chromosome III, two more markers – "MZN14" at 10.7 Mbp and "T6H20" 17.2 Mbp – were used to narrow down the focused area. The results are summarized in Fig. 3.18 for plants of group 1 and in Fig. 3.19 for plants of group 2.

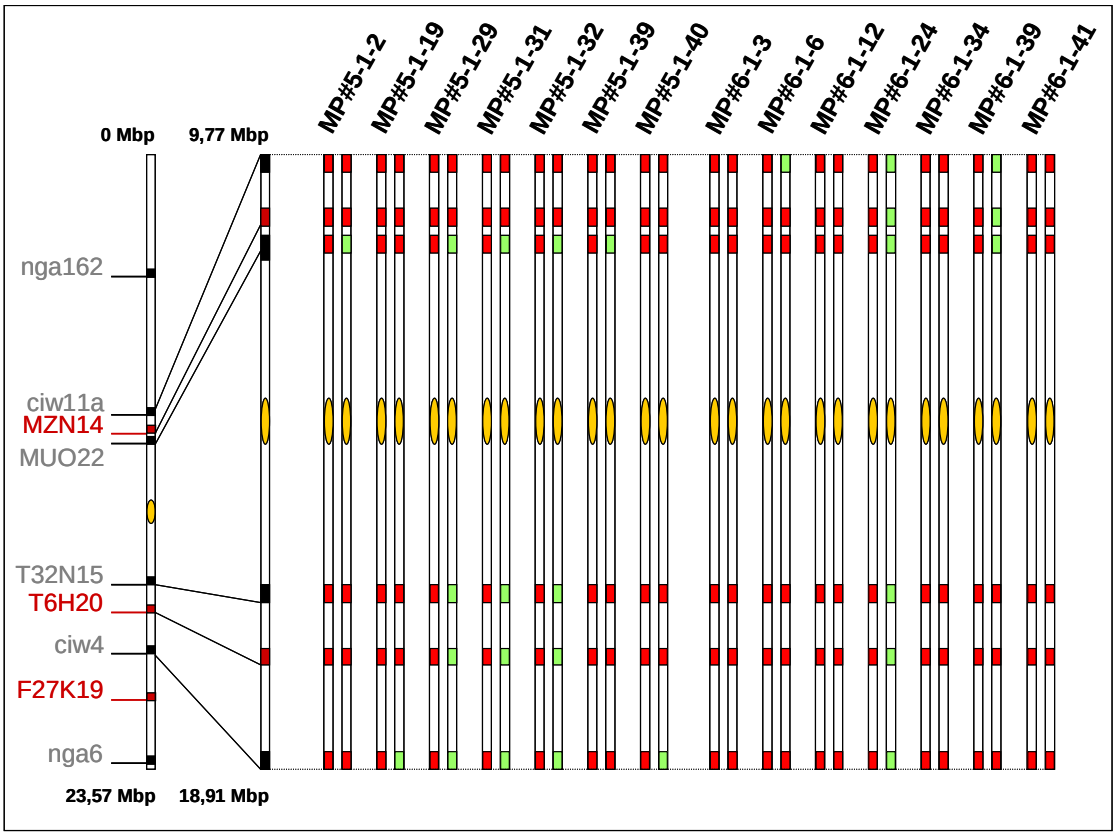

Fig. 3.18: Detailed view on the distribution of Ler (red) and Col-0 (green) genotype at six investigated positions of chromosome III, located between marker "ciw11a" at 9.77 Mbp and marker "ciw4" at 18.9 Mbp. *The markers written in red were additionally used for genotyping. All shown plant lines belonged to group 1 (all plants die after Dex treatment due to a loss of the* sud *mutation), and the expected genotype was Ler. All genotype characterizations are based on the results of the PCR genotyping. Colour coding: Red – Ler genotype at the investigated position; green – Col-0 genotype at the investigated position; yellow – centromer.*

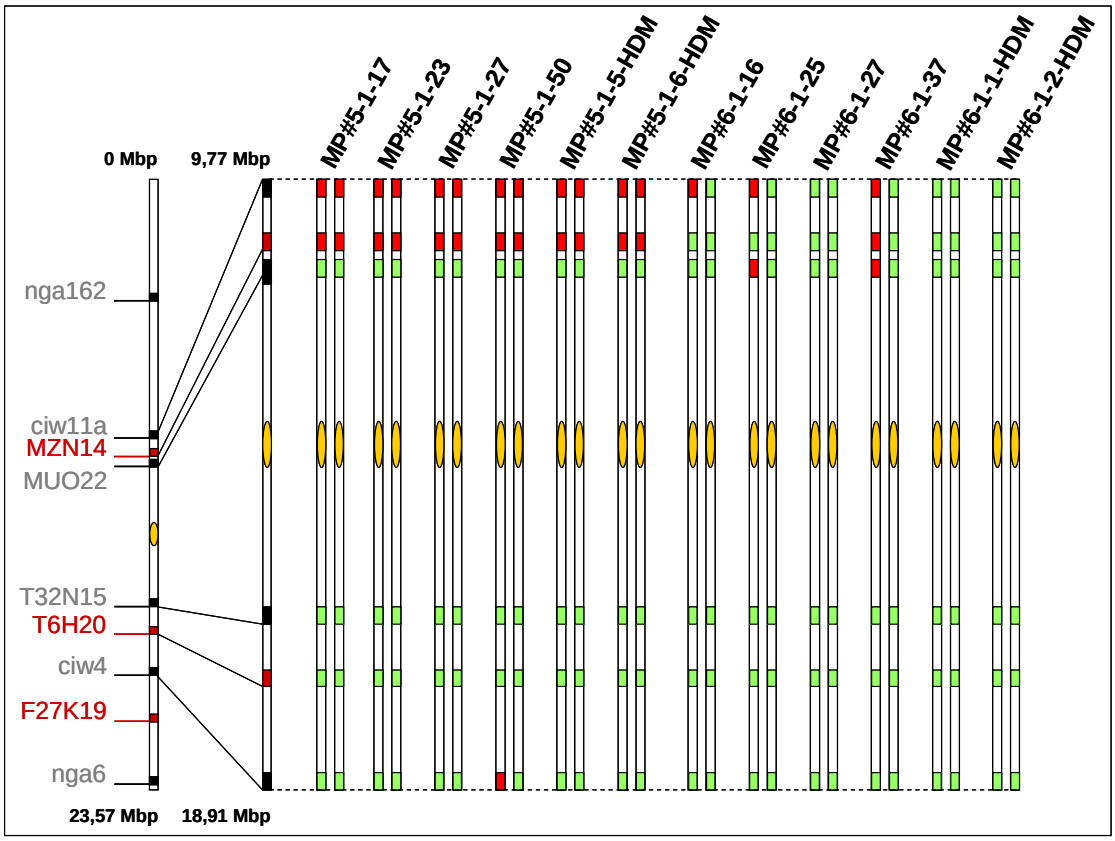

Fig. 3.19: Detailed view on the distribution of* Ler *(red) and Col-0 (green) genotype at six investigated positions of chromosome III, located between marker "ciw11a" at 9.77 Mbp and marker "ciw4" at 18.9 Mbp. *The markers written in red were additionally used for genotyping. All shown plant lines belonged to group 2 (all plants survive after Dex treatment due to the* sud *mutation), and the expected genotype was Col-0. The additional tested marker "MZN14" at 10.7 Mbp showed only* Ler *DNA in plants of MP #5 whereas the second marker "T6H20" at 17.2 Mbp revealed only Col-0 DNA in all tested samples. Therefore, the potential region of the* sud *mutation could be narrowed down from the top (top limit now marker "MZN14" instead of "ciw11a"), but not from the bottom. All genotype characterizations are based on the results of the PCR genotyping. Colour coding: Red – Ler genotype at the investigated position; green – Col-0 genotype at the investigated position; yellow – centromer.*

The additional markers partly narrowed down the investigated area: Marker "MZN14" at 10.7 Mbp revealed L*er* DNA in all plants of MP #5 that belonged to group 2 (all plants survive). Therefore the region north of "MZN14" could be excluded from the investigated area. The second additional marker "T6H20" at 17.2 Mbp gave the same results as marker T32N15 at 16.27 Mbp and did not narrow down the investigated area from south.

To narrow down the area of the potential *sud* mutation, three plant lines of group 2 with recombination events in the investigated region between marker

"MZN14" at 10.7 Mbp and marker "ciw4" at 18.9 Mbp were chosen to create a fine mapping population. The three selected plant lines (MP #5-1-50, MP #6-1-25 and MP #6-1-37) were crossed with 86L*er* #28-1 and 86L*er* #48-1 to create a new mapping population with additional recombination events in the interesting region, which are now investigated by Prabhavathi Talloji, another graduate student.

3.5 Analysis of the At5g02310 gene as a potential ligase of the N-end rule pathway

3.5.1 Selection of appropriate transgenic fusion protein lines

In this work, investigations of the N-end rule pathway were performed with the *Arabidopsis* gene At5g02310 to analyse its potential role in N-end rule protein degradation. A sequence search had revealed significant similarity of At5g02310 to the yeast ubiquitin ligase gene *Ubr1*, which is involved in the N-end rule pathway; therefore At5g02310 was chosen for further investigations. The *Arabidopsis* gene shows homologous sequence segments all along the entire open reading frame (ORF), indicating functional similarity between the two proteins. Due to the expected role in protein degradation and according to the already identified N-recognin in plants, PRT1, the gene was named *PRT6* for ***PR****O****T****EOLYSIS* ***6***.

To test a potential function for *PRT6* in the plant N-end rule pathway, the ubiquitin fusion degradation technique was used in which a fusion protein – consisting of a potentially unstable test protein and a metabolically stable reference protein – was introduced into the plant and cleaved into two independent proteins during or immediately after translation (cf. 1.3.3). As a GUS domain was included in the test protein and HA tags had been introduced in both the test and the reference protein, conclusions could be

drawn from the presence or absence of the test protein and its abundance compared to the reference protein.

As PRT6 was expected to be a potential ligase of the plant N-end rule pathway, another previously characterized ubiquitin ligase, PRT1, was used in the experiments as a control. PRT1 has been shown to use proteins with aromatic amino acid residues like Phe at the amino terminus as substrates (Stary et *al.*, 2003). The PRT6 sequelog in yeast, Ubr1, has two distinct binding sites for destabilizing amino acids at the amino terminus. Therefore, substrates both with basic residues and with (aliphatic and aromatic) hydrophobic ones can be bound. As PRT6 differed from PRT1, it was expected that if PRT6 functions also as a ubiquitin ligase, the substrates that are recognized and degraded by PRT6 should have either a basic or an (aliphatic) hydrophobic residue at the amino terminus. Therefore, two fusion proteins were used for further investigation of the potential PRT6 ligase: One with Arg as a basic amino acid at the amino terminus of the test protein and another one with Leu as an aliphatic hydrophobic residue. Besides those two lines, fusion proteins with Met were used as a stable control and Phe as a potential substrate protein for PRT1.

According to the first amino-terminal amino acid of the test protein after cleavage, these four fusion proteins were named as follows:

- **UPR-F-GUS** with **Phe** at the amino terminus of the cleaved test protein
- **UPR-L-GUS** with **Leu** at the amino terminus of the cleaved test protein
- **UPR-M-GUS** with **Met** at the amino terminus of the cleaved test protein
- **UPR-R-GUS** with **Arg** at the amino terminus of the cleaved test protein

These fusion proteins were transformed into *Arabidopsis* Col-0 WT plants and the two best expressing plant lines of each fusion protein were selected for

further experiments. To identify those lines, GUS staining was first carried out with plant leaves of the F2 generation of UPR-M-GUS plants. Met is a stabilising N-end rule amino acid, and therefore the test protein was stable and not degraded, a situation confirmed by the GUS staining. As the test protein was degraded normally in the other three plant lines, those plants had to be tested with RT-PCR to check the protein expression level. Thus, plants of the F2 generation were tested with reverse transcriptase PCR (RT-PCR) to select those plant lines with the highest expression level. For this purpose, RNA was isolated with the RNeasy Plant Mini Kit and the RT-PCR was carried out with one specific primer ("GUSup") and AMV reverse transcriptase. The two primers "GUSup" and "35Sp" were used for the subsequent PCR with the cDNA. The size of the expected band was at around 580 bp and could be detected with Agarose gel electrophoresis (Fig. 3.20).

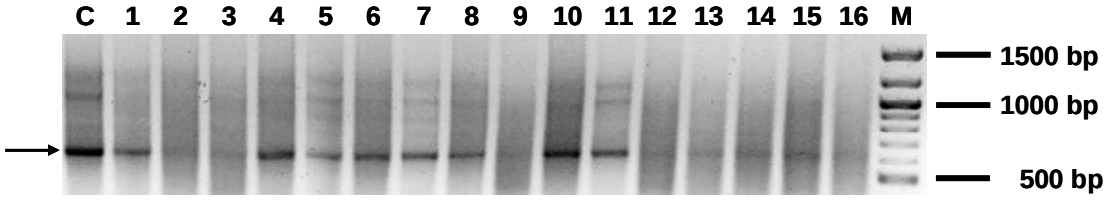

Fig. 3.20: Test for transgene expression – PCR results with cDNA on a 1 % Agarose gel. *Plants were tested on correct expression of the transgene with RT-PCR. The subsequent carried out PCR revealed the 580 bp fragment expected for the tested transgene (black arrow). 16 different plant lines were tested and in 50 % of the samples significant expression of the transgene could be detected. C: positive control; M: 100 bp marker.*

From all different UPR-X-GUS plant lines, at least five plants were examined, and the two plant lines with the highest expression level of the introduced transgene were chosen for further experiments:

UPR-**F**-GUS **#3**	UPR-**L**-GUS **#8**	UPR-**M**-GUS **#1**	UPR-**R**-GUS **#3**
UPR-**F**-GUS **#4**	UPR-**L**-GUS **#9**	UPR-**M**-GUS **#6**	UPR-**R**-GUS **#6**

3.5.2 Identification of *prt1* and *prt6* single and double mutants

After having selected the plant lines with strong transgene expression, these lines were crossed with a *prt1 prt6* double mutant line to generate plants that carry the UPR-X-GUS fusion protein transgene and a mutated *PRT1* and/or *PRT6* locus to study the different degradation profiles of the test protein in single and double mutant lines.

Before the crosses were carried out, both crossing partners were further characterized: As the *prt1 prt6* double mutant line growing in the F2 generation was from a cross between two single mutant plants, the potential double mutants were selected by testing for both the *prt1* and the *prt6* mutation.

To check the correct presence of the UPR-X-GUS fusion protein in the selected plant lines, all four lines were tested by sequencing to see whether the correct amino acid at the cleavage site was present. A PCR reaction was carried out with the two primers “GUSup” and “DHFR end seq” and the PCR product was applied to a 1 % Agarose gel. The expected fragment – a band at 1.1 kb – was cut out, and the amplified DNA was isolated with NucleoSpin Extract II. 50 % of the eluted DNA was used for sequencing, and the results, shown in Table 3.7, revealed the expected sequence encoding the amino-terminal amino acid after cleavage.

Fusion protein line	Sequence analysed
UPR-**F**-GUS #4-1	… CTT AGA GGT GGT **TTT AGA TCT** GT GGT …
UPR-**L**-GUS #8-1	… CTT AGA GGT GGT **CTT AGA TCT** GT GGT …
UPR-**M**-GUS #6-1	… CTT AGA GGT GGT **ATG AGA TCT** GT GGT …
UPR-**R**-GUS #6-1	… CTT AGA GGT GGT **AGA AGA TCT** GT GGT …

Table 3.7: Results of the sequencing carried out with each transgene of the four different UPR-X-GUS plant lines. *The* BglII *cleavage site at the border of reference and test protein (**AGA TCT**) is written in bold, the amino-terminal amino acid of the test protein is written in red. All four UBR-X-GUS fusion protein lines encoded the correct amino acid at the cleavage site: TTT encodes the amino acid Phe, CTT encodes Leu, ATG encodes Met and AGA encodes Arg.*

The potential *prt1 prt6* double mutant lines for the crosses were chosen out of 48 plants, progenies of five different F1 progeny plants from a *prt1* x *prt6* cross. All plants were tested with PCR: Genomic DNA was isolated from two to four fresh leaves of each plant line and PCR was carried out subsequently. At first, all plants were tested for the *prt1* mutation. This mutation can be detected by PCR with the two primers "PRT 6423 – 6446" and "PRT 6602 – 6582" and a subsequent digestion with *Mnl*I, as only the WT allele and not the mutated one possesses an *Mnl*I cleavage site. Due to this *Mnl*I site, the PRT1-fragment of the WT allele is shorter than the mutated one and can therefore be easily distinguished from the mutation after Agarose gel electrophoresis (Fig. 3.21).

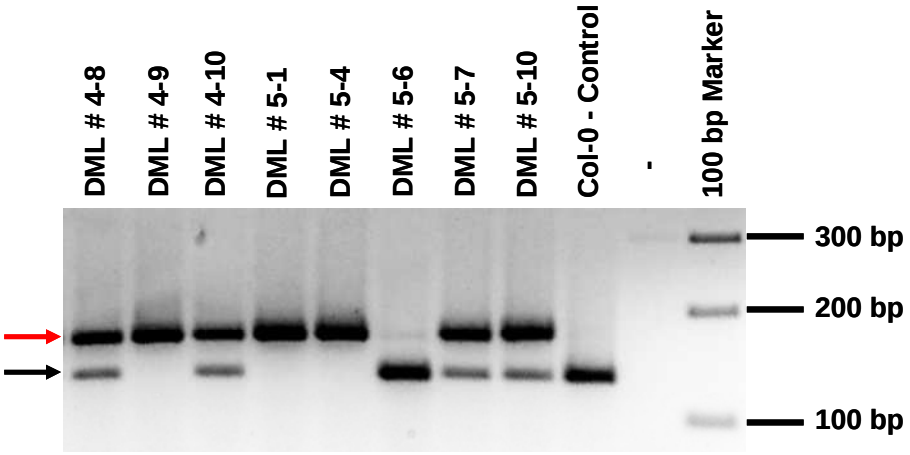

Fig. 3.21: Test for the* prt1 *mutation - PCR results after* MnlI *digestion on a 3 % Agarose gel. *Plants with the* prt1 *mutation show a larger fragment (179 bp, red arrow) compared to non-mutated WT plants (140 bp, black arrow). Three lines (DML # 4-9, DML # 5-1, DML # 5-4) are homozygous for the* prt1 *mutation, one line (DML # 5-6) has no mutated allele and is similar to Col-0 WT, four lines (DML # 4-8, DML # 4-10 , DML # 5-7, DML # 5-10) are heterozygous and carry one WT allele and one mutant allele) – DML – double mutant line.*

All plants homozygous for the *prt1* mutation were then tested for the *prt6* mutation. To this end, two different PCR tests were carried out: First one test to assess the presence of the WT allele and then a second one for testing the presence of the mutated allele. For the first test, the two primers "ubrdn3" and "ubrup3" were used to amplify a 1.7 kb fragment indicating the presence of at least one WT allele (Fig. 3.22).

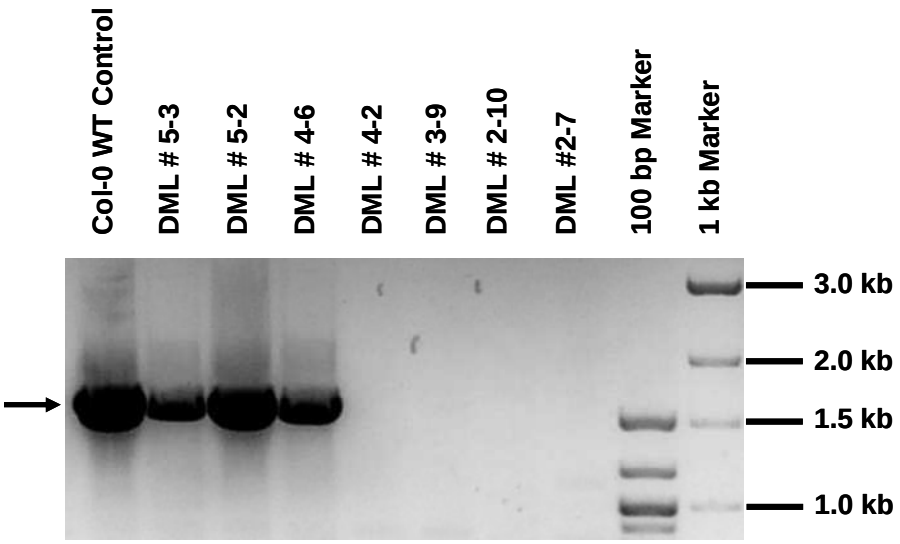

Fig. 3.22: Test for the presence of the* PRT6 *WT allele - PCR results on a 1 % Agarose gel. *Plants with at least one WT allele for* PRT6 *(DML # 4-6, DML # 5-2, DML # 5-3, Col-0 WT control) show a PCR fragment at 1.7 kb (black arrow). Plants with a homozygous* prt6 *mutation (DML # 2-7, DML # 2-10, DML # 3-9 and DML # 4-2) do not show any band; however, the potential* prt6 *mutant lines need to be analysed again with a positive* prt6 *mutant allele test as a failure of the DNA reaction cannot be excluded – DML – double mutant line.*

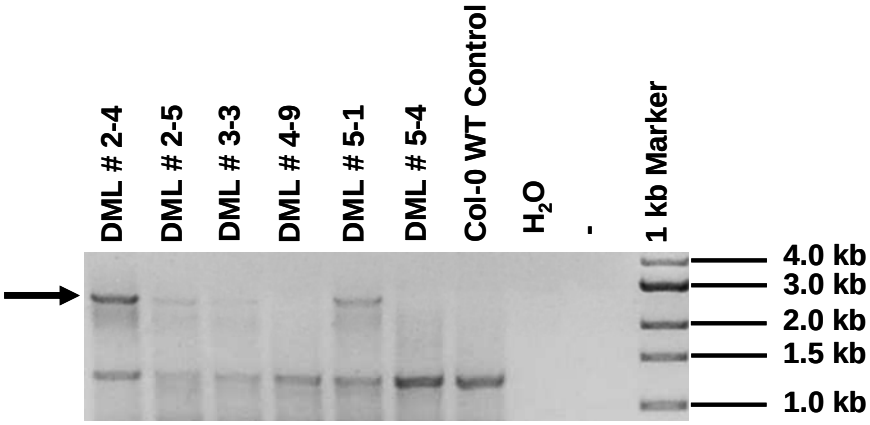

Fig. 3.23: Test for the presence of the* prt6 *mutant allele - PCR results on a 1 % Agarose gel. *Plants with at least one* prt6 *mutant allele (DML # 2-4, DML # 5-1 and (with weaker signal) DML # 2-5, DML # 3-3) show a PCR fragment of 2.7 kb (black arrow). WT plants with no mutated* prt6 *allele (DML # 4-9, DML # 5-4 and Col-0 WT control) do not show a band at 2.7 kb. Similar to the test results in Fig. 3.22, a failure of the DNA reaction cannot be excluded; therefore, the results of both tests (for the presence of the* PRT6 *WT allele and the* prt6 *mutant allele) have to be considered. – DML – double mutant line.*

Only the plant lines with no amplified fragment were used for the second test to check the presence of the mutated allele. As a failure in the first test during the amplification of the WT allele could not be excluded, and only a positive test result of the mutant allele would confirm the presence of the mutated allele, this additional PCR was necessary. The PCR was carried out with the primer combination “ubrdn3” and “GarlicLB1” and the expected fragment had a size of 2.7 kb (Fig. 3.23).

Double mutant line *prt1 prt6*	**Test 1**	**Test 2a**	**Test 2b**
	***prt1* mutant** allele homozygous?	***PRT6* WT** allele present?	***prt6* mutant** allele present?
((*prt6* #3-14 x Col-0) #5-3-8 x *prt1*) # **2-4**	**yes**	**yes**	**yes**
((*prt6* #3-14 x Col-0) #5-3-8 x *prt1*) # **2-5**	**yes**	**yes**	**yes**
((*prt6* #3-14 x Col-0) #5-3-8 x *prt1*) # **2-7**	**yes**	**no**	**no**
((*prt6* #3-14 x Col-0) #5-3-8 x *prt1*) # **2-10**	**yes**	**no**	**yes**
((*prt6* #3-14 x Col-0) #5-3-8 x *prt1*) # **3-3**	**yes**	**yes**	**yes**
((*prt6* #3-14 x Col-0) #5-3-8 x *prt1*) # **3-9**	**yes**	**no**	**yes**
((*prt6* #3-14 x Col-0) #5-3-8 x *prt1*) # **4-2**	**yes**	**no**	**yes**
((*prt6* #3-14 x Col-0) #5-3-8 x *prt1*) # **4-6**	**yes**	**yes**	**no**
((*prt6* #3-14 x Col-0) #5-3-8 x *prt1*) # **4-9**	**yes**	**yes**	**no**
((*prt6* #3-14 x Col-0) #5-3-8 x *prt1*) # **5-1**	**yes**	**yes**	**yes**
((*prt6* #3-14 x Col-0) #5-3-8 x *prt1*) # **5-2**	**yes**	**yes**	**no**
((*prt6* #3-14 x Col-0) #5-3-8 x *prt1*) # **5-3**	**yes**	**yes**	**no**
((*prt6* #3-14 x Col-0) #5-3-8 x *prt1*) # **5-4**	**yes**	**yes**	**no**

Table 3.8: Results of the PCR tests carried out with potential* prt1 prt6 *double mutant plants. *All plant lines shown above were at least homozygous for the* prt1 *mutant allele (“yes” in the first column). Only three lines out of 48 tested plant lines (# 2-10, # 3-9 and # 4-2) were homozygous for the* prt1 *mutant allele and for the* prt6 *mutant allele (“no” in the second column and “yes” in the third one). Four lines (# 2-4, # 2-5, # 3-3, # 5-1) were homozygous for the* prt1 *mutant allele and heterozygous for the* prt6 *mutant allele (“yes” in the second column and “yes” in the third one). Five lines (# 4-6, # 4-9, # 5-2, # 5-3, # 5-4) were only homozygous for the* prt1 *mutation and were carrying the* PRT6 *WT allele (“yes” in the second column and “no” in the third one). The plant lines written in green (# 2-4, # 2-5 and # 2-10) were chosen for carrying out further crosses.*

Table 3.8 shows the final results of the PCR-based double mutant selection. Only plant lines with at least the homozygous *prt1* mutation were listed. Among 48 potential double mutants that were tested, only three lines (# 2-10, # 3-9 and # 4-2) were homozygous in both mutant alleles *prt1* and *prt6*. All other mutant lines that were homozygous for *prt1* were only heterozygous for *prt6* (# 2-4, # 2-5, # 3-3, # 5-1), or did not contain the *prt6* mutation at all (# 4-6, # 4-9, # 5-2, # 5-3, # 5-4).

As some double mutant lines were growing slower than others, three lines (# 2-4, # 2-5 and # 2-10) that were flowering at the right time point for the crosses were finally chosen, although the two plant lines # 2-4 and # 2-5 were only heterozygous for *prt6*. The crosses were performed with the four different fusion protein lines UPR-**F**-GUS, UPR-**L**-GUS, UPR-**M**-GUS and UPR-**R**-GUS to obtain plant lines that carry both the different fusion protein and a *prt1 prt6* single or double mutation (Table 3.9).

New name	♂	**Cross**	♀
F7	UPR-**F**-GUS #4-1	**x**	((prt6 #3-14 x Col-0) #5-3-8 x prt1) # **2-10**
L8	UPR-**L**-GUS #8-1	**x**	((prt6 #3-14 x Col-0) #5-3-8 x prt1) # **2-4**
M11	UPR-**M**-GUS #6-1	**x**	((prt6 #3-14 x Col-0) #5-3-8 x prt1) # **2-5**
R15	UPR-**R**-GUS #6-1	**x**	((prt6 #3-14 x Col-0) #5-3-8 x prt1) # **2-5**

T*able 3.9: Summary of the four crosses carried out with the different fusion protein lines and* prt1 prt6 *double mutants.* *For the crosses, the pollen of UPR-X-GUS plant lines was used to pollinate the pistil of* prt1 prt6 *double mutant lines. To simplify further investigations with the resulting plant lines, all crosses were renamed.*

The seeds of the four crosses, shown in Table 3.9, were first put on Ara plates, supplemented with Hyg for transgene selection, and later on soil. Four different lines from the F1 generation of each cross were selected for further investigations and between 42 and 56 plants of each cross were grown to the F2 generation. As the fusion protein lines were crossed with a *prt1 prt6* double mutant line, the progenies in the F2 generation of this cross contained the transgene and different states of the *prt1* and *prt6* alleles.

Among these plant lines, *prt1* single mutants, *prt6* single mutants and *prt1 prt6* double mutant lines should be isolated. Therefore, the genotype was examined by harvesting two to four leaves of each plant. Genomic DNA was isolated with the quick genomic DNA isolation method, and PCR was carried out as described above.

First, all plants were tested for being homozygous for the *prt1* mutation or the WT allele. Only those plants with two identical alleles were subsequently analysed for the state of the PRT6 locus. Table 3.10, Table 3.11 and Table 3.12 summarize the different single and double mutants that could be characterized in the F2 generation. Whereas Table 3.10 shows the *prt1* single mutants, Table 3.11 lists the *prt6* single mutants and Table 3.12 specifies the potential *prt1 prt6* double mutant lines.

Plant line	***prt1* mutation**	***prt6* mutation**
F7 # 1-10	homozygous	WT allele
F7 # 4-2	homozygous	WT allele
F7 # 4-12	homozygous	WT allele
F7 # 6-1	homozygous	WT allele
L8 # 4-5	homozygous	WT allele
L8 # 4-11	homozygous	WT allele
L8 # 4-12	homozygous	WT allele
L8 # 6-3	homozygous	WT allele
L8 # 6-6	homozygous	WT allele
L8 # 6-11	homozygous	WT allele
M11 # 3-7	homozygous	WT allele
M11 # 3-14	homozygous	WT allele
R15 # 2-2	homozygous	WT allele
R15 # 2-4	homozygous	WT allele
R15 # 2-5	homozygous	WT allele

Table 3.10: List of 15* prt1 *single mutant lines, identified from plants expressing a test protein with Phe, Leu, Met or Arg as amino-terminal residue. *The* PRT1 *locus and the* PRT6 *locus of plant lines originating from the four crosses carried out between different fusion protein lines (**F**,**L**,**M** and **R**) and the* prt1 prt6 *double mutants (Table 3.9) were characterized by PCR in the F2 generation. All listed plant lines carry a homozygous* prt1 *mutation and contain the non-mutated* PRT6 *WT allele.*

Plant line	*prt1* mutation	*prt6* mutation
F7 # 6-13	WT allele	homozygous
L8 # 3-5	WT allele	homozygous
L8 # 6-8	WT allele	homozygous
M11 # 1-10	WT allele	homozygous
R15 # 2-6	WT allele	homozygous
R15 # 6-1	WT allele	homozygous

Table 3.11: List of six* prt6 *single mutant lines, identified from plants expressing a test protein with Phe, Leu, Met or Arg as amino-terminal residue. *The* PRT1 *locus and the* PRT6 *locus of plant lines originating from the four crosses carried out between different fusion protein lines (**F**,**L**,**M** and **R**) and the* prt1 prt6 *double mutants (Table 3.9) were characterized by PCR in the F2 generation. All listed plant lines carry a homozygous* prt6 *mutation and contain the non-mutated* PRT1 *WT allele.*

Plant line	*prt1* mutation	*prt6* mutation
M11 # 3-11	homozygous	homozygous
M11 # 7-7	homozygous	homozygous
R15 # 3-6	homozygous	homozygous
F7 # 1-4	homozygous	heterozygous
L8 # 1-5	homozygous	heterozygous
L8 # 1-13	homozygous	heterozygous
L8 # 6-12	homozygous	heterozygous
L8 # 1-9	heterozygous	homozygous
L8 # 3-9	heterozygous	homozygous

Table 3.12: List of nine mutant lines with at least one homozygous mutation of the* prt1 *or* prt6 *allele, identified from plants expressing a test protein with Phe, Leu, Met or Arg as amino-terminal residue. *The* PRT1 *locus and the* PRT6 *locus of plant lines originating from the four crosses carried out between different fusion protein lines (**F**,**L**,**M** and **R**) and the* prt1 prt6 *double mutants (Table 3.9) were characterized by PCR in the F2 generation. Three of the listed plant lines are* prt1 prt6 *double mutants (M11 # 3-11, M11 # 7-7 and R15 # 3-6), whereas four lines are homozygous for the* prt1 *mutation but only heterozygous for the* prt6 *allele (F7 # 1-4, L8 # 1-5, L8 # 1-13, L8 # 6-12) and two lines are heterozygous for the* prt1 *allele but homozygous for the* prt6 *mutation (L8 # 1-9, L8 # 3-9).*

Single mutant lines of *prt1* and *prt6* could be found in crosses with all four different UPR-X-GUS fusion proteins, *prt1 prt6* double mutant lines however only in plant lines with UPR-M-GUS – and UPR-R-GUS fusion proteins. Plant lines with UPR-F-GUS – and UPR-L-GUS fusion protein showed heterozygosity

in at least one locus. Therefore, two lines of this group (Table 3.12 – plant line F7 # 1-4 and L8 # 1-9) were chosen for supplementary PCR-based examinations in the F3 generation. 35 plant lines of each mutant were grown on soil and genotypic analyses were carried out as shown with plants in the F2 generation. Four *prt1 prt6* double mutant lines could finally be selected in the F3 generation (Table 3.13).

Plant line	***prt1* mutation**	***prt6* mutation**
F7 # 1-4-15	homozygous	homozygous
F7 # 1-4-20	homozygous	homozygous
F7 # 1-4-21	homozygous	homozygous
L8 # 1-9-1	homozygous	homozygous

Table 3.13: List of four* prt1 prt6 *double mutant lines, identified in plants expressing a test protein with Phe or Leu as amino-terminal residue. *The* PRT1 *locus and the* PRT6 *locus of plant lines originating from the two crosses carried out between different fusion protein lines (**F** and **L**) and the* prt1 prt6 *double mutants (Table 3.9) were characterized by PCR in the F2 generation. All listed plant lines carry both the homozygous* prt1 *mutation and the homozygous* prt6 *mutation.*

Plant line	***prt1* mutation**	***prt6* mutation**
F7 # 6-1-1	homozygous	WT allele
F7 # 6-13-1	WT allele	homozygous
F7 # 1-4-21	homozygous	homozygous
L8 # 6-6-1	homozygous	WT allele
L8 # 3-5-1	WT allele	homozygous
L8 # 1-9-1	homozygous	homozygous
M11 # 3-7-1	homozygous	WT allele
M11 # 1-10-1	WT allele	homozygous
M11 # 7-7-1	homozygous	homozygous
R15 # 2-4-2	homozygous	WT allele
R15 # 6-1-1	WT allele	homozygous
R15 # 3-6-2	homozygous	homozygous

Table 3.14: Selected plant lines for further investigations. *Originating from the four crosses carried out between different fusion protein lines (**F** ,**L** ,**M** and **R**) and the* prt1 prt6 *double mutants (Table 3.9), each possible combination has been identified. For each different fusion protein line, a* prt1 *single mutant line, a* prt6 *single mutant line and a* prt1 prt6 *double mutant line has been identified with PCR in the F3 generation.*

To make sure that all identified single and double mutant lines were correctly characterized, one plant line of each possible combination was selected for re-analyses in plants of the F3 generation. Similar to the plants of the F2 generation, DNA of two to four leaves was isolated and subsequently tested by PCR amplification of the regions of interest. Together with the double mutants in plant lines with UPR-F-GUS and UPR-L-GUS fusion proteins, one plant line of each combination was finally chosen for carrying out GUS staining and Western Blot analyses. Table 3.14 summarizes all selected plant lines of the F3 generation.

3.5.3 GUS staining

The GUS staining should reveal accumulation or degradation of the different test proteins in different mutant backgrounds according to the character of the amino-terminal residue. Therefore all plant lines of Table 3.14 were sown on Ara plates supplemented with Hyg for transgene selection, and in addition to these mutant lines also the non-mutated WT plants with the different fusion protein lines that served as controls. For the histochemical GUS staining assay, little plantlets aged 14 days were used. Fig. 3.24 shows the GUS staining results of all 16 different plant lines.

As expected, the stable M-GUS test protein accumulated in WT-plants, whereas the other N-end rule test substrates F-GUS, L-GUS and R-GUS were degraded in WT plants by the ubiquitin-proteasome pathway.

The M-GUS test protein was shown to be stable in all different single and double mutant lines. All *prt6* mutant plants expressing the F-GUS test protein could degrade the test protein like the F-GUS WT plants without any mutation. In *prt1* mutant plants, however, the test protein accumulated (strong GUS staining), and it could not be degraded by PRT6. Similar to the *prt1* single mutant, the test protein accumulated also in the *prt1 prt6* double mutant line, as the PRT1 ligase was non-functional, too.

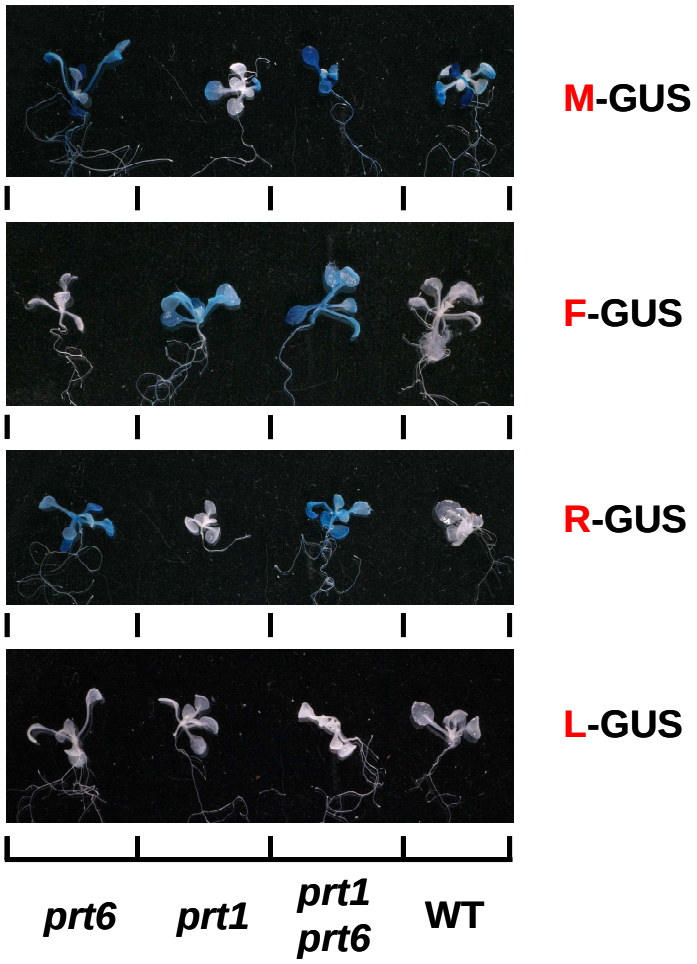

Fig. 3.24: GUS staining results of 14-day old seedlings expressing a GUS reporter protein with defined amino acid (M, F, R or L) at the amino terminus, determined as M-GUS, F-GUS, R-GUS and L-GUS. *GUS protein was expressed in different genetic backgrounds (WT,* prt1 *single mutant,* prt6 *single mutant and* prt1 prt6 *double mutant); according to the character of the amino-terminal residue, the GUS protein was stabilized or degraded in the different plant lines. Plant lines expressing the M-GUS construct served as stabilizing control lines and were shown to be stable in every examined background. The F-GUS test protein was stabilized only in plants with a* prt1 *mutation, whereas the R-GUS test protein was only stabilized in the presence of a* prt6 *mutation. No GUS staining could be detected in plants encoding an L-GUS test protein, indicating that this construct was degraded in all tested plant lines. Figure modified from Garzón* et al.*, 2007.*

Plants expressing the R-GUS test protein showed the complete opposite. Here, the degradation of the test protein was not affected in *prt1* mutant plants, but it was disturbed in *prt6* mutants, where the GUS enzyme substrate accumulated so that the plantlet was stained blue. Similar to plants expressing the F-GUS test protein, the *prt1 prt6* double mutant line was affected, as here the PRT6 ligase was non-functional, too. This suggested that PRT6 recognizes basic amino-terminal amino acid residues such as Arg.

The plants expressing the aliphatic hydrophobic test protein L-GUS showed no accumulation in any plant line. The test protein could be degraded in *prt1* mutant plants and in *prt6* mutant plants, just as in plants carrying a *prt1 prt6* double mutation. This suggested the existence of a third ubiquitin ligase that degrades proteins with aliphatic hydrophobic amino acids at the amino terminus.

3.5.4 Western blotting

To check whether these visual results of the GUS staining could be confirmed also by quantitative analyses, Western blots were performed in collaboration with Karolin Eifler to examine the proportional amount of HA tag containing proteins in protein extracts. As the fusion protein contained HA tags in both protein parts – a 1 x HA tag in the reference protein DHFR/Ub and a 3 x HA tag in the GUS test protein – the ratio of the two proteins could be determined quantitatively.

Proteins in seven-week-old plantlets were examined. Therefore, plants were first grown on Ara plates supplemented with Hyg for three weeks, and then plantlets were put on soil for another four weeks. One cauline leaf was used for preparing protein extracts that were first separated by SDS-PAGE and then transferred via Western blotting on a Nitrocellulose membrane. After incubation with an anti-HA antibody, a fluorescent dye-coupled secondary antibody was applied. The dye conjugate that was used in these experiments permitted an exact quantification of the HA-epitope containing proteins (Fig. 3.25).

Transgenic Col-0 plants expressing only the M-GUS fusion protein (lane 5) served as positive control, whereas WT plants with no transgene were used as negative control (lane 4). Although these WT plants did not contain any HA tags, they showed a background of bands on the blot, an effect that might be caused by non-specific binding of the secondary antibody, or by auto-fluorescence of some plant proteins, as fluorescence detection was used.

As the intensity of these background bands was low in the regions containing the reference protein (at around 30 kDa) or the test protein (at around 95 kDa), the results of the HA-tag containing proteins were not influenced.

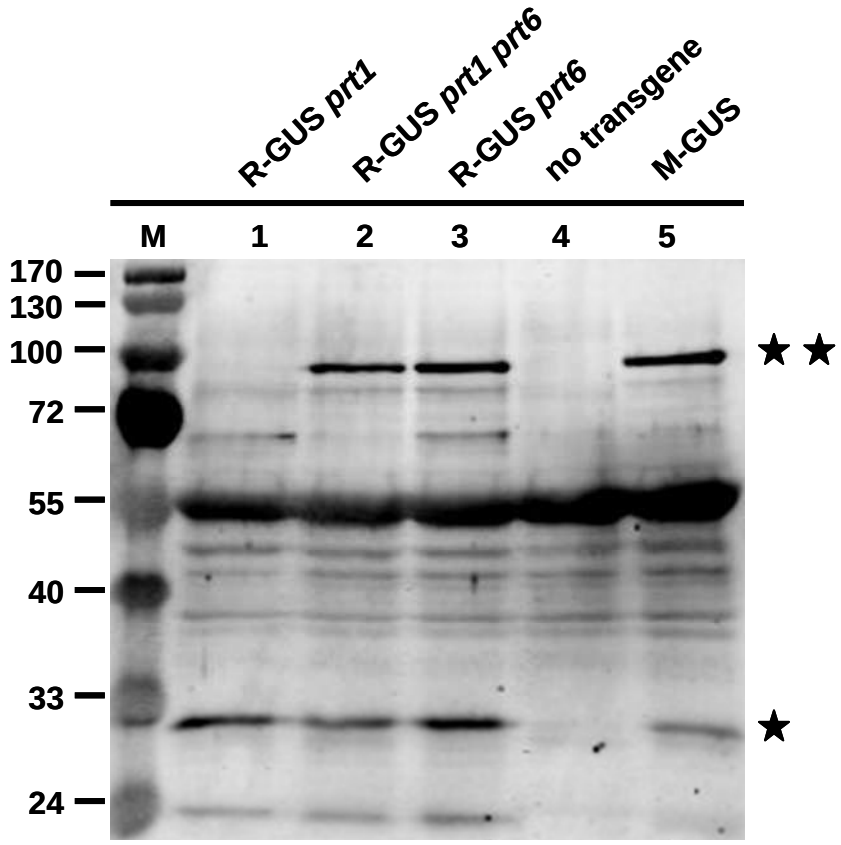

Fig. 3.25: Detection and quantitative measurement of N-end rule substrates by protein blotting using fluorescent secondary antibody excitation. *Protein extracts from 7-week old plants were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was incubated with two different antibodies, a primary antibody (anti-HA) and a secondary antibody that was coupled to infrared fluorescent dye IR800. Protein bands were detected with Odyssey Infrared Imager. A reference protein with one HA tag could be detected in all four transgenic plant lines at 30 kDa (one asterisk) but not in the WT control plant; a test protein with three HA tags and a defined amino acid at the amino terminus had a defined size of 95 kDa (two asterisks) and was stable in plants expressing M-GUS constructs (5) and in* prt6 *mutant plants expressing R-GUS constructs. Figure taken from Garzón* et al.*, 2007.*

The WT plants showed no bands at the expected positions of the reference and the test protein. The transgenic Col-0 plants with the M-GUS fusion protein however, revealed the presence of both the three HA epitope containing test protein (two asterisks) and the one HA tag containing reference protein (one asterisk).

The Western blot of the mutant plants confirmed the previously carried out qualitative GUS staining assay, as both tests showed corresponding results. All mutants expressing the R-GUS fusion protein showed a band at around 30 kDa, the size of the reference protein which indicated that the fusion protein was expressed correctly. No band at 95 kDa, the size of the test protein, could be detected in the R-GUS expressing *prt1* mutant plant line. This single mutant degraded the test protein normally and therefore no band was detectable. In both genetic backgrounds where *prt6* was mutated (either as a single mutation or as a double mutation together with *prt1*), however, the test protein accumulated and little degradation took place. Therefore a protein band at 95 kDa was detectable in both plant lines containing the *prt6* T-DNA insertion mutation. The band was at the same size as the one in the stable M-GUS plant line that served as positive control. These results confirmed the GUS staining assay (Fig. 3.24), where no blue staining could be detected in plant lines containing exclusively the *prt1* mutation and only plant lines with a mutated *prt6* locus accumulated the GUS containing test protein and showed therefore blue staining.

To see whether the expression levels varied according to the age of the investigated plant tissue, another western blot was carried out with 14-day-old plantlets. The proteins were extracted from one to two single plantlets and the blot was carried out in the same way as before. The results were similar to the protein extracts taken from 7-week old seedlings: All R-GUS mutant lines expressed the reference protein band at 30 kDa, like the M-GUS line without any *prt* mutation. The R-GUS *prt1* mutant line degraded the R-GUS test protein normally and so no test protein band at 95 kDa could be detected, whereas the test protein accumulated in the *prt6* single mutant and the *prt1 prt6* double mutant line (Fig.3.26).

Additional extracted plant lines showed varying results: Whereas the *prt1* mutant line carrying the F-GUS fusion protein transgene showed the expected results – accumulation of the reference and the test protein due to the absence of the PRT1 ligase – the *prt6* mutants carrying the L-GUS fusion

protein did not even express a detectable reference protein band. Both examined L-GUS mutant lines, the *prt6* single mutant and the *prt1 prt6* double mutant, gave the same results as Col-0 WT plants. Therefore no conclusion could be drawn from these experiments. However, a Western blot carried out later with 7-week old leaf material showed the correct expression of the reference protein and the absence of a test protein band (data not shown), so that the conclusion drawn from the GUS-staining assay could be confirmed.

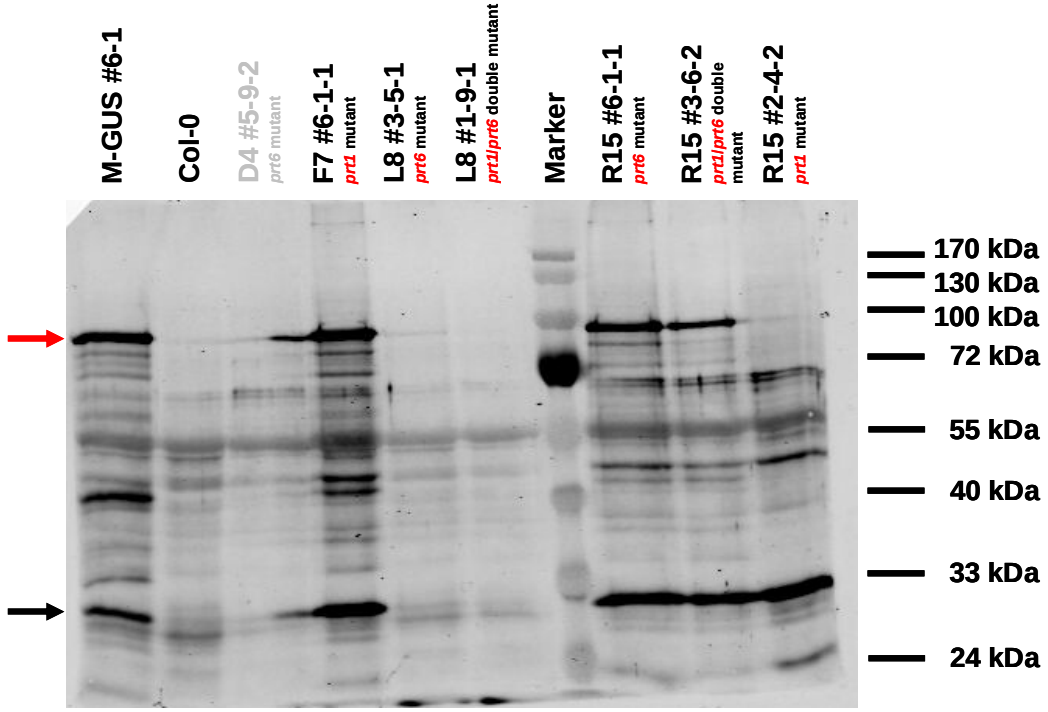

Fig. 3.26: Western blot carried out with extracted proteins of 14-day old seedlings that were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. *The membrane was incubated with a primary antibody (anti-HA) and a secondary antibody that was coupled to infrared fluorescent dye IR800. Protein bands were detected with Odyssey Infrared Imager. A reference protein with one HA tag could be detected in all plants carrying an R-GUS construct, an F-GUS construct and an M-GUS construct at 30 kDa (black arrow) but not in the WT control plant Col-0 or in plants expressing the L-GUS construct. A test protein with three HA tags and a defined amino acid at the amino terminus had a size of 95 kDa (red arrow) and was stable in plants expressing the M-GUS construct as well as in* prt6 *mutant plants expressing R-GUS constructs and in* prt1 *mutant plants expressing the F-GUS construct.*

After these qualitative assays of the accumulated or degraded test proteins, the quantitative values were analysed from pixel values detected by the Odyssey Infrared Imager. As the reference protein carried one and the test protein three HA tags, the quantitative ratio of the N-end rule substrates could be calculated. The results of the analysed plant lines are summarized in Table 3.15.

Plant line	**Plant genotype**	**Ratio of band intensity [test protein / reference protein]**
M-GUS # 6-1	**WT** plant	**3.4**
F7 # 6-1-1 (F-GUS)	***prt1*** single mutant	**2.0**
L-GUS # 8-1-7	**WT** plant	**0.3**
L8 # 3-5-1 (L-GUS)	***prt6*** single mutant	**0.3**
L8 # 1-9-1 (L-GUS)	***prt1 prt6*** double mutant	**0.2**
R-GUS # 6-1-3	**WT** plant	**<0.1**
R15 # 2-4-2 (R-GUS)	***prt1*** single mutant	**<0.1**
R15 # 6-1-1 (R-GUS)	***prt6*** single mutant	**1.2**
R15 # 3-6-2 (R-GUS)	***prt1 prt6*** double mutant	**1.0**

***Table 3.15**: **Ratio of fluorescence intensity that was calculated of the ratio for test vs. reference protein and measured with Odyssey Infrared Imager from different plant lines expressing various test proteins with defined amino acid at the amino terminus (M, F, L or R); values taken from gels as shown in Fig. 3.25.** The expected ratio of test versus reference protein was 3, as the test protein contained three HA tags and the reference protein one HA tag. This value was reached by plants expressing M-GUS constructs where the test protein was stabilized. The test protein was also stabilized in* prt1 *mutant plants expressing the F-GUS test protein and, to a lesser extent, also in* prt6 *mutant plants expressing the R-GUS protein. No stabilization could be observed in mutant plants expressing the L-GUS test protein. These calculated values corresponded to the results obtained with the histochemical GUS staining. Figure modified from Garzón* et al.*, 2007.*

The ratio for a completely stable test protein would be 3 as the reference protein carries one and the test protein three HA tags. This value was nearly achieved in the stable M-GUS fusion protein plant line. The second highest ratio could be measured in the F-GUS fusion protein line carrying the *prt1* mutation, where the ratio was 2.0. Concerning the levels of protein in the R-GUS fusion protein plant lines, the ratio in the *prt6* single and the *prt1 prt6* double mutant was at least 10-fold higher than in the *prt1* single mutant or

the non-mutated WT plants, although the absolute ratio reached only one third of the M-GUS level in non-mutated plants. The increased accumulation of test protein in *prt6* mutant lines showed the requirement of the PRT6 ligase for degrading proteins with basic terminal amino acids, but as the ratio stayed below the expected value, the possibility of another yet unknown ligase could not be excluded. The L-GUS fusion protein plant lines did not show any accumulation in the *prt6* mutant lines. The ratio did not differ between the WT plants and the mutant plant lines, which indicated that PRT6 was not the ligase required for degrading proteins with hydrophobic aliphatic amino termini.

4 DISCUSSION

4.1 Perturbation studies performed in the ubiquitin-proteasome system

The investigations of the ubiquitin-proteasome system via an inducible transgene expressing a modified ubiquitin that are presented in this work revealed an effective method to induce cell death in plants. Experiments to define more clearly the type of cell death gave no clear results, however it seems possible that the observed cell death is more related to leaf senescence. To gain more insight, suppressor mutants were selected from a population of EMS-mutagenized plants, revealing both dominant and recessive candidate genes. These mutant lines were further characterized and one recessive mutant, *sud2*, was selected and crossed with Landsberg *erecta* (L*er*) to create a mapping population. In the mapping process, the region of the mutation could be localized to chromosome III between 10.7 Mbp and 18.9 Mbp.

4.1.1 Inhibition of the ubiquitin-proteasome system

The ubiquitin-proteasome pathway is the dominant proteolytic system in plants. In this highly conserved pathway, ubiquitin serves as a reusable

recognition signal for selective protein turnover (Smalle and Vierstra, 2004). In an enzymatic cascade consisting of three different enzymes, ubiquitin is covalently attached to the target substrate. After the attachment of at least four ubiquitin moieties as a chain, the ubiquitylated protein is recognized by the 26S proteasome for degradation. One possibility to analyse the complex structure of this essential pathway is to disturb the normal process by expressing dominant negative variants of pathway components. In this work, a modified ubiquitin called UbK48R was expressed in *Arabidopsis*. The Lys to Arg change in ubiquitin prevents ubiquitin linkage via Lys 48 (Ling *et al.*, 2000), and can lead to programmed cell death (PCD) after induction of the transgene.

The transgenic plant line RV86-5 that was used in this work carries an inducible transgene with eight repeats of UbK48R. As proteins are marked for degradation by ubiquitin moieties covalently linked to each other via Lys 48 in a multiubiquitin chain that is attached to the target protein, the chain formation is perturbed by the variant ubiquitin. The variant ubiquitin interrupts the chain formation in such a way that UbK48R moieties can still be linked to an already existing chain, or directly to the substrate, but no further ubiquitin moieties can be added to the ubiquitin chain via position 48, as the Lys is replaced by Arg (cf. Fig. 1.8) – the chain elongation is discontinued. As an ubiquitin chain with at least four moieties is essential for a proteolytic substrate to be recognized by the proteasome for degradation, the protein-ubiquitin-conjugates accumulate in the cell and cause perturbations of the cell equilibrium.

4.1.2 Perturbation of the ubiquitin-dependent proteolysis pathway

Manipulations of the ubiquitin-proteasome pathway have been shown to be surprisingly specific. Experiments with the proteasome inhibitor PS-341 in *S. cerevisiae* (Fleming *et al.*, 2002) and human multiple myeloma cells (Mitsiades *et al.*, 2002) revealed well defined effects on the expression of genes involved in the ubiquitin-proteasome pathway. It could be shown in

mammalian cells that the proteasome inhibitor treatment induced both the intrinsic “mitochondrial” cell death pathway and the extrinsic one. Experiments in plants with virus-induced silencing of parts of the 26S proteasome demonstrated the activation of the PCD program including nuclear condensation and DNA fragmentation, accompanied by reduced proteasome activity and the accumulation of polyubiquitylated proteins (Kim *et al.*, 2003).

The modified ubiquitin UbK48R used in this work has already been effectively applied to study the role of ubiquitin in the ubiquitin-proteasome system. It was successfully used to inhibit proteolysis and cell cycle progression in yeast (Finley *et al.*, 1994) and to express substrates with premature termination of polyubiquitin chains in mammalian neurons (Patrick *et al.*, 2003). In plants, a transgene expressing the variant ubiquitin UbK48R has already been successfully introduced into tobacco, where it could be shown that the plants displayed necrotic lesions, similar to the ones developed as defence reactions after pathogen attacks (Bachmair *et al.*, 1990; Becker *et al.*, 1993; Conrath *et al.*, 1998). Experiments in plants with HisUbK48R, where the variant ubiquitin is additionally tagged with histidine at the amino terminus, demonstrated that the modified ubiquitin is indeed conjugated to proteins and expression of this variant led to a significant accumulation of ubiquitylated proteins (Ling *et al.*, 2000). In recently carried out experiments with UbK48R in *Arabidopsis*, where the transgene was expressed continuously, a number of changes in gene expression and in phenotype could be observed (Schlögelhofer *et al.*, 2006). Besides these changes, experiments with a cyclin-GUS fusion protein could demonstrate an increased half-life of the fusion protein, supporting the assumption that UbK48R expression interferes with the ubiquitin-dependent protein degradation (Schlögelhofer *et al.*, 2006), especially as cyclins are known substrates of the ubiquitin-proteasome pathway (Zachariae and Nasmyth, 1999).

To extend the results achieved in experiments with plants continuously expressing the ubiquitin variant, an inducible transgene expressing UbK48R

was used in this work. In the former experiments, the early expression of UbK48R led to severe damages of the plant and therefore prevented the analysis of effects that occur later in development (Schlögelhofer *et al.*, 2006). With the inducible transgene, it was possible to choose the initiating time point of the UbK48R expression independently and to study effects in gene and phenotype expression also later in mature plants.

4.1.3 Initiation of cell death pathways in UbK48R-induced plants

The most remarkable phenotypic change in *Arabidopsis* plants expressing UbK48R that could be observed was the tendency to initiate cell death. Necrotic lesions appeared soon after transgene induction and were followed by complete yellowing of the whole plant, resulting in the death of the entire plant. These two observed cell death symptoms can both be found in plant PCD: Whereas the observed necrotic lesion phenotype resembled to the one of lesion mimic mutants, the leaf yellowing seemed to be more related to leaf senescence. Lesion mimic mutants are characterized by the spontaneous formation of lesions that resemble hypersensitive response (HR) lesions, but which appear in the absence of pathogens; the transgene-induced lesion mimic phenotype is supposed to result from the activation of a PCD pathway (Mittler and Rizhsky, 2000). The HR is one of the most efficient and fastest reactions of plants to pathogen attacks and it is characterized by the rapid death of the affected plant cells (Lorrain *et al.*, 2003). Leaf senescence represents the final stage of leaf development and ensures the relocation of nutrients out of the leaf, generally to reproducing seeds (Lim *et al.*, 2007). Leaf yellowing reflects mainly chloroplast senescence of mesophyll cells which is the first step in senescence-associated PCD (Lim *et al.*, 2007), but by the time yellowing of the leaf can be seen, the majority of the senescence process has already occurred (Buchanan-Wollaston *et al.*, 2003). Leaf senescence is accompanied by increased expression of senescence-associated genes (Lim *et al.*, 2007) which is characteristic for cell death processes. Both HR and leaf senescence are therefore PCD programs of plants (Lim *et al.*, 2007).

Among the different forms of PCD that can be found in plants, cell death in pathogen-induced HR is the best characterized one (Lim *et al.*, 2007). Comparisons on the molecular level between the HR-associated PCD and senescence-associated cell death showed similarities in some, but not all involved genes. Crosstalks between these two forms exist. However, a few molecular markers are specific for each of them (Lim *et al.*, 2007). The two main differences between the HR and leaf senescence are the size of the tissue that is affected by the cell death, and the speed. Leaf senescence is wide spread and can affect one entire leaf, whereas the HR is a rather localized cell death that occurs in limited tissue areas (Lim *et al.*, 2007). Also the speed varies: Whereas the HR is considered to be a fast form of PCD which results in an early loss of DNA and possibly DNA laddering, leaf senescence is a much slower form of PCD that starts with chloroplast senescence in the mesophyll cells, and DNA loss can only be observed in its latest stages (Buchanan-Wollaston *et al.*, 2003).

The analysis of the DNA content in Dex-induced plants (Fig. 3.4) supports therefore the assumption that the observed cell death is more related to leaf senescence, as no loss of DNA could be observed in UbK48R induced plants. 14-day old plants were incubated with Dex to induce the transgene, and the DNA content was measured at the beginning of the experiment, seven days later and 14 days later, respectively. The DNA amount was decreased in the first week by nearly 50 % compared to the DNA content at the beginning of the experiment, but this change could be found both in Dex-induced and in control plants. Comparison of the DNA amount that was measured at the end of the first week with the amount quantified at the end of the second week did not show any changes, indicating that the DNA content is not affected in the second week after UbK48R induction. This would be in line with leaf senescence as observed cell death type because nuclear DNA is maintained until late in the senescence process to ensure gene expression (Buchanan-Wollaston *et al.*, 2003). One explanation for the considerable decrease of absolute DNA content in both groups within the first week after transgene induction might be the still ongoing expansion growth. This would be

consistent with a constant DNA content within the second week, when the diminished nutrient availability in the liquid growth media impedes further expansion growth.

On the other side, it could be demonstrated in this work that an additional stress factor accelerated the development of cell death symptoms (Fig. 3.3). This in turn would indicate a strong relation of the cell death phenotype to HR as a fast form of PCD. Light was chosen as additional stress factor as it had been shown that some *Arabidopsis* lesion mimic mutants – *lsd1* and *lsd3* – do not differ from WT under short day conditions, whereas cell death symptoms are developed under long light exposure (Dietrich *et al.*, 1994). Dex-induced and non-induced plants were grown both under a 16 h light / 8 h dark cycle, and under continuous higher light intensity as the additional stress factor. Whereas plants with one stress factor showed only slight differences to their control plants, the Dex-induced plants grown in continuous light revealed severe aggravation of the cell death phenotype with enhanced leaf yellowing. Both the intensity and the speed of the developed cell death symptoms were much stronger compared to the plants that were exposed to only one single stress factor. Due to these results, it can be hypothesized that the lesion mimic phenotypes, observed in mature plants which were grown under greenhouse conditions, result from the transgene expression in combination with one or more additional stress factors like light, drought or heat that are harmless when they occur alone (Schlögelhofer *et al.*, 2006).

4.1.4 Investigations of plant line RV86-5 with chemical genetics

To further characterize the UbK48R-induced cell death, a chemical genetics approach with 120 different substances was accomplished in this work. Chemical genetics has been shown to be a powerful tool to dissect essential cellular pathways like mitosis (Haggarty *et al.*, 2000). In a chemical genetics approach, small molecules are used as probes to perturb signalling pathways at the molecular level with the intention to identify novel proteins that are responsible for a particular phenotype (Schneekloth and Crews, 2005).

Analyses with chemical genetics are performed to detect the relationship between the structures of small molecules and their phenotypic effects in intact living systems (Haggarty *et al.*, 2004). Like in classical genetics, a phenotype-based screening is estabilished, but instead of mutations, small molecules are used (Haggarty *et al.*, 2004). In this work, a screen was executed to identify one or more small molecular substances that affected the UbK48R-induced cell death phenotype. In first experiments, the monoterpene anhydride Cantharidin (CAN) was shown to cause a stronger phenotype in RV86-5 plantlets grown in the presence of the substance compared to the phenotype of the control plants. The plants grew faster and seemed to deal better with the expression of the UbK48R ubiquitin variant. CAN is produced by the blister beetle, *Mylabris*, and is highly toxic to most animals (Dettner, 1997). It has been known in China as a medical agent for 2000 years where it has been used for treatment of cancer (Wang, 1989). Interestingly, it was recently demonstrated that CAN induced apoptosis in leukaemia cells by a p53-dependent mechanism and it was shown that resistance to oxidative stress caused cross-resistance to CAN (Efferth *et al.*, 2005). Furthermore, a demethylated analogue of CAN, Norcantharidin (NCTD) was also found to be involved in inducing apoptosis in human colon carcinoma (Peng *et al.*, 2002), hepatoma cells (Chen *et al.*, 2002) as well as in human oral cancer cell lines (Kok *et al.*, 2005). Therefore, it is possible that CAN also impinges on UbK48R-induced cell death. However, a repetition with varying substrate concentrations did not confirm the previous results, and three structural analogues of CAN that were tested in the same way did not show any changes in the cell death phenotype. As other approaches revealed more promising results and due to time constraints, the labour-intense approach with chemical genetics was not further pursued.

4.2 Suppressor mutants of UbK48R-induced cell death

To gain more insight and to investigate a possible connection between the ubiquitin-proteasome system and the observed PCD pathways from an

additional perspective, suppressor mutants were isolated and further characterized. These mutants survived the Dex-induced expression of the UbK48R transgene, whereas non-mutated plants died in the presence of Dex. One approach that was pursued in this work to identify *sud* (*suppressor of ubiquitin UbK48R-induced cell death*) mutants was the selection of suppressor mutants from a population of EMS-mutagenized plants. In addition, a candidate gene approach was accomplished and it could be shown that these mutants were also expressing the *sud* phenotype.

For the first approach, initiated by Peter Schlögelhofer in 2002, EMS-mutagenized seeds of the transgenic plant line RV86-5 were screened on HDM plates to select for mutant plants that survive despite the expression of the variant ubiquitin UbK48R. In this work, nine potential candidates were isolated and selected for further investigations. Analyses of the transgene expression levels revealed in six mutant lines that the mRNA expression of the transgene was similar to the one of the non-mutated transgenic plant line RV86-5. The complementation analysis identified one dominant mutation (EMS 28-1), one mutation with partly dominant character (EMS 58-1) and several recessive mutations. The different observations made with crosses of EMS 58-1 concerning the dominance of the mutation might indicate that the dominant *sud* mutation phenotype is possibly caused by haploinsufficency. This phenomenon can appear if the protein expression of the non-mutated WT allele is insufficient to rescue the defect function caused by the mutation (Greenberg, 2000). Interestingly, it has been shown in mice that a haploinsufficiency of the 40S ribosomal protein S6 is associated with activation of a p53-dependent checkpoint during gastrulation (Panic *et al.*, 2006) and that the regulation of the p53 tumor suppressor depends on the correct expression level of the S6 protein (Panic *et al.*, 2007).

Finally, four recessive independent mutants were selected for further investigations and renamed *sud2* (EMS 30-1), *sud3* (EMS 42-2), *sud4* (EMS 57-2) and *sud5* (EMS 82-3), in line with the already identified *sud1* mutant that was isolated by Claudia Kerzendorfer in 2002. All five *sud* mutants were

further characterized. It could be demonstrated that, similar to the transgenic plant line RV86-5 when the transgene was not expressed, the suppressor mutants grew normal under standard greenhouse conditions in the absence of Dex. The growth habit was similar to the one found in non-induced RV86-5 plants or Col-0 WT plants, indicating that the EMS-generated mutation(s) are in gene(s) that operate in distinct, rather specialized pathways. The results of the sensitivity assays support this assumption, as in growth experiments on media supplemented with varying concentrations of different sugars, hormones and salts no additional phenotype to the *sud* mutation could be identified. Both findings therefore approve a special role for ubiquitin and ubiquitylation in the specialized processes of PCD.

In two *sud* mutant lines, however, two remarkable findings could be made. It could be demonstrated in experiments with the dominant mutant line of *sud1* carried out in this work that this mutant showed also more resistance to the proteasome inhibitor MG132, indicating that the EMS-generated mutation, which has already been shown to suppress the UbK48R induced cell death, was also able to prevent cell death processes that occurred during perturbation of another essential component of the ubiquitin-proteasome system, the proteasome. These results imply that a decreased efficiency of the ubiquitin-proteasome pathway caused both by UbK48R expression or by proteasome inhibition, can be suppressed by the same mutation. However, the same experiments carried out with the four recessive *sud* mutations revealed no improved growth in comparison with the control plants of non-induced RV86-5 and Col-0 WT plants, indicating that the suppression of UbK48R-induced phenotypes can be found not only on one but on several levels.

The second line that revealed an anomalous reaction in an experimental assay was the recessive mutant line *sud2*. In the experiments, carried out in collaboration with and by Dr. Nikolaus Schlaich, it could be shown that *sud2* mutants were sensitive to the fungal pathogen *Hyaloperonospora parasitica* isolate WELA, an oomycete that causes downy mildew in *Arabidopsis*, but only

in certain accessions. Although the accession of *sud2*, Col-0, had been shown to express a nonhost resistance response when inoculated with the pathogen (Hermanns *et al.*, 2003), the inoculated *sud2* mutant plants displayed leaf yellowing (Schlaich, personal communications). As resistant host genotypes often recognize the pathogen in a gene-for-gene dependent manner and execute a response with first the production of H_2O_2 and then the initiation of a genetically programmed HR cell death (Hermanns *et al.*, 2003), it can be speculated that the *sud2* mutation might be located in one gene involved in one of these two processes.

A second approach to identify mutants carrying a suppressor mutation was made with reverse genetics. Therefore, mutant lines that were deficient in certain ubiquitin conjugation enzymes were chosen for analysis. In a reverse genetics approach, a gene of interest is selected, and in order to identify its function(s), plant lines with a mutation in this gene are investigated, as these mutants are assumed to have also a different phenotype (Peters *et al.*, 2003). Four selected mutant lines (*let1*, *let2*, *let3* and *let4*) were crossed with the transgenic plant line RV86-5 and homozygous single mutants (Fig. 3.12) as well as double mutant lines were tested in this work on HDM plates. Interestingly, no *let3* homozygous line could be identified, suggesting that a mutation of both *let3* alleles is lethal, whereas homozygous mutants in one of the other *let* genes seem viable. All tested mutants survived on HDM plates, in contrast to RV86-5, and showed a strong *sud* phenotype that was similar to the one of the EMS-generated mutant line *sud1*. Further analyses of the three single mutant lines *let1*, *let2* and *let4* by crossing them with RV86-5 revealed the dominant character in two out of three single *let* mutant lines (*let1* and *let2*), similar to *sud1*, whereas *let4* turned out to be recessive. The sensitivity assay, which was carried out on medium supplemented with the proteasome inhibitor MG132, however, did not demonstrate any increased resistance to MG132 as it was found in *sud1* mutants. Here, the perturbation of the proteasome-depending protein degradation could not be substituted by one of the *let* mutations, indicating that the dominant *sud* mutations can interact with cell death pathways on different levels. On the other side, the sensitivity

assays carried out with varying concentrations of different sugars, hormones and salts could generate some additional unique phenotypes. Besides the double mutant line *let1 let2* that expressed significant smaller plants when grown in the presence of 2,4-D, three tested *let* lines (*let2*, *let4* and *let1 let4*) revealed a higher sensitivity to SA with reduced germination and slowed growth. Interestingly, it has been shown that some cell death events are accompanied by high levels of SA (Schlögelhofer *et al.*, 2006), and it could be demonstrated in earlier experiments with tobacco plants that the level of SA was increased in UbK48R-expressing tobacco plants (Conrath *et al.*, 1998). Besides this, it was revealed that the induction of HR-related cell death, which is triggered by some *Arabidopsis* disease resistance genes, depends strongly on the independent generation of SA (Xiao *et al.*, 2003). However, a reduction of SA by a transgenic salicylate hydrolase that was co-expressed with UbK48R in *Arabidopsis* could not reduce the extent of observed cell death (Schlögelhofer *et al.*, 2006), indicating that SA might not be necessary for cell death events triggered by UbK48R expression.

4.3 Mapping of suppressor mutants

To identify EMS-mutated genes that generate the *sud* phenotype, mapping populations with two different *sud* mutants, *sud1* and *sud2*, were created. The first-pass mapping of the *sud2* mutant was successful and the region of interest could be narrowed down on chromosome III between the two flanking markers MZN14 at 10.7 Mbp and T6H20 at 17.2 Mbp. The first two mapping approaches however, carried out with the dominant *sud1* mutant, failed although two different mapping populations were created. In the first mapping population, three regions with homozygous distribution of the mutant accession (Col-0) could be detected in both groups of classification (group 1: all plants dying when grown on HDM plates and group 2: all plants surviving on HDM plates), but no region with homozygous distribution of L*er* accession was found, although this distribution was necessary for group 2 plants as *sud1* had been shown to be a dominant mutation. It was assumed

that the L*er* plant, which had been used for creating the mapping population, could have been contaminated with some Col-0 ecotype, resulting in Col-0 accession in alleles originating from the L*er* crossing partner, as it had been shown that contamination with other ecotypes can appear surprisingly frequent (Jander *et al.*, 2002). Therefore, the cross of *sud1* with L*er* was repeated with L*er* plants from the Max Planck Institute, where the risk of contaminations with other accessions was reduced to a minimum. This mapping population grew well up to the F3 generation where it was tested by selection on HDM plates; here, all plantlets died and no one survived. Hence, no separation into surviving plants (due to the mutated allele) and dying plants (due to a loss of mutation) could be made, and the mapping process was abandoned.

A possible explanation for the difficulties that occurred during these two mapping processes might be that the sought after *sud* mutation is not only one gene but a quantitative trait locus (QTL) as the determination of the map position is connected with the same problems that can be found in QTL mapping. In this case, the intensity of the *sud* phenotype would vary in the different generations, as the phenotype is not a clear-cut one but variable in different genetic backgrounds. The mapping of a QTL, however, is much more complicated and involves selfing of up to six further generations calculated from the F2 generation (Mauricio, 2001).

As the two mapping approaches with the dominant *sud1* mutation failed, a new mapping process was initiated with the recessive mutant line *sud2*. A consequence from the previously carried out experiences however was the reduction of potential influences of the genetic background to a minimum in future experiments. The variability in the genetic background has been shown to influence the characteristic traits in a way that the expression of one trait can be stronger or weaker. Therefore it was important to decrease the number of variability factors and the L*er* accession line was modified: To exclude a varying UbK48R expression in the plantlets, the inducible transgene was introduced in the L*er* accession line (86L*er*). In this way, the UbK48R

expression levels were always constant as the transgene was at all times present with two alleles in every progeny of the cross and a varying UbK48R expression could be excluded as a variable. Besides this, six mapping populations were created with *sud2* and different 86L*er* lines, and only the two mapping populations segregating on HDM plates in a mendelian fashion in the F2 generation were used for the mapping with polymorphic markers.

The mapping was based on a new set of primers, developed by Berendzen and co-workers (2005) to facilitate the map-based cloning approach. The first-pass mapping revealed an area on chromosome III around the centromere between the two flanking markers MZN14 at 10.7 Mbp and T6H20 at 17.2 Mbp that was composed of Col-0 accession in all tested plants of group 2 (surviving on HDM plates). In parallel, the plants of group 1 (dying on HDM plates) revealed for the most part L*er* ecotype, but never homozyguously Col-0, indicating that they had lost at least one allele of the recessive mutation. So far, the predicted area of the *sud* mutation is still too big to make a reliable statement about the character of the mutation, as too many proteins are still encoded in the candidate region. To narrow down the area that contains the mutated gene, a fine-resolution mapping is necessary. The size of the final region should be 40 kb or less (Jander *et al.*, 2002), which corresponds to approximately 0.16 cM genetic distance. The first step has already been done with the creation of a new mapping population, consisting of crosses between three mutant lines of the mapping population for the first-pass mapping that had revealed an above average number of recombination events on chromosome III and the corresponding 86L*er* plant line. However, it cannot be excluded that also the *sud*2 mutation encodes for a QTL and that the mapping process is therefore more labour-intense than estimated.

4.4 Identification and characterization of a new N-end rule pathway ligase in plants

The investigations of *Arabidopsis* gene At5g02310 and its protein product presented in this work showed that this gene is a sequelog of the yeast

ubiquitin ligase Ubr1 and, like Ubr1, At5g02310 encodes an E3 ligase involved in the N-end rule pathway of protein degradation. Furthermore, At5g02310 is not the *CER3* gene that is part of the wax biosynthesis as it has been reported mistakenly in former publications by Hannoufa and Eisner and therefore the At5g02310 locus was renamed in this work PRT6 for **PR**O**T**EOLYSIS **6**.

4.4.1 PRT6 degrades proteins with a basic amino acid at the amino terminus

The yeast gene *UBR1* encodes a ubiquitin ligase that degrades proteins with a destabilizing amino-terminal residue according to the N-end rule pathway (Bartel *et al.*, 1990; Varshavsky, 1996). Substrates carrying a stabilizing residue at the amino terminus do not bind to the ligase. Proteins that contain regions of sequence similarity to the Ubr1 protein of *S. cerevisiae* have been found in mice and humans (Kwon *et al.*, 1998). It was shown that components of the N-end rule pathway exist in plants (Bachmair *et al.*, 1993) but no N-recognin with sequence similarity to the *S. cerevisiae* Ubr1 protein has been found previously. A sequelog of the yeast *UBR1* gene in *Arabidopsis* has now been identified in this work, and in accordance with the nomenclature of an already described ubiquitin ligase that is involved in the N-end rule pathway of plants, it has been named PRT6. The N-recognin PRT6 was shown to recognize and to bind proteins carrying the basic amino acid Arg as amino-terminal residue, but unlike its yeast sequelog Ubr1, it has no influence on the stability of proteins with hydrophobic amino termini or aromatic ones. This result is in line with the existence of the already identified ubiquitin ligase involved in the plant N-end rule pathway, PRT1, which is responsible (only) for the degradation of proteins with aromatic amino acids at the amino terminus (Potuschak *et al.*, 1998; Stary *et al.*, 2003), demonstrating that an N-recognin can be responsible also for only a subset of destabilizing N-degrons.

It could be demonstrated in this work that *prt6* mutants stabilize proteins with basic amino acids at the amino terminus, and it could be confirmed that *prt1* mutants degrade proteins with aromatic amino acids at the amino

terminus (Fig. 3.24). It was shown that the degradation of the R-GUS test protein is not mediated by PRT1, as R-GUS test proteins accumulated to the same extent in *prt6* single mutants and in *prt1 prt6* double mutants. It could also be demonstrated that one of the test substrates used in this work, carrying an aliphatic hydrophobic amino acid (Leu) at the amino terminus, was metabolically unstable in every tested mutant line (*prt1* single mutant, *prt6* single mutant and *prt1 prt6* double mutant). The test protein with an aliphatic hydrophobic amino-terminal residue did not accumulate in any mutant line, suggesting the existence of at least one more plant ubiquitin ligase involved in the plant N-end rule pathway that degrades proteins with an aliphatic hydrophobic amino acid at the amino terminus.

4.4.2 N-recognins in plants and mammals and their functions

In yeast, the destabilizing residues can be divided in type 1 (basic amino acids) and in type 2 (bulky hydrophobic and aromatic residues) (Varshavsky, 1996). Proteins with destabilizing residues either of type 1 or of type 2 bind to the same N-recognin Ubr1. Although located on the same protein, the binding regions for type 1 and type 2 residues are functionally apart from each other (Du *et al.*, 2002). UBR1, the mammalian sequelog to the yeast N-recognin Ubr1, has at first been considered also to be the sole N-recognin in mammals as it could also degrade destabilizing residues both of type 1 and of type 2 (Reiss and Hershko, 1990). Later however, a homolog was identified (UBR2) with functionally overlapping activity and high sequence similarity (Kwon *et al.*, 2003) that could partly replace the function of UBR1 in *UBR1* mutants. Double mutant *UBR1-/- UBR2 -/-* mouse strains did not survive, however fibroblasts retained a significant fraction of the pathway's activity, indicating the existence of at least one more N-recognin (Tasaki *et al.*, 2005). Affinity-based proteomic screening and sequence alignment finally revealed five more genes with sequence similarity to the yeast N-recognin Ubr1. Interestingly, two proteins – UBR4 and UBR5 – that were identified by using the affinity-based proteomic screening both bound to Arg, a type 1 N-degron, on the matrix whereas UBR1 and UBR2 preferentially

bound to type 2 N-degrons (Tasaki *et al.*, 2005), supporting the hypothesis that UBR4 and UBR5 are involved in the degradation of type 1 amino-terminal residues. Besides this, it was shown that UBR4 and UBR5 are unrelated to UBR1 and UBR2 regarding the sequence and the size. The existence of several N-recognins in mammals supports the possibility that also in plants there is more than one ubiquitin ligase involved in the N-end rule pathway. Furthermore, it is also possible that also in plants there is more than one N-recognin responsible for the same type of N-degron.

The common motif that links all seven identified N-recognins is the UBR box, a zinc-finger-like domain; therefore the ligases were named UBR1-7. However, it is unclear so far whether this motif is characteristic for either the type 1 binding site, or the type 2 binding site. As the N-end rule pathway can be found both in eukaryotes and in prokaryotes, comparisons of homologous regions between the two kingdoms might help to answer this question. In eukaryotes, a region with essential amino acids for the type 2 binding site, which is responsible for aromatic and hydrophobic residues in N-recognins, has been identified (Kwon *et al.*, 1998). It was shown in prokaryotes that the *E. coli* protein ClpS, a protein that recognizes and binds substrates in bacteria with the type 2 destabilizing amino-terminal residues Phe, Tyr, Trp and Leu, shares considerable secondary structure with the predicted secondary structure of the putative type 2 binding site of N-recognins in eukaryotes (hypothesis by Lupas and Koretke, 2003; proof by Erbse *et al.*, 2006). It is supposed that due to this sequence similarity, ClpS can interact directly with amino-terminal destabilizing residues of proteins and target them to ClpAP for degradation (Erbse *et al.*, 2006). Besides this, it has been shown that the basic amino acids Arg and Lys are only secondary stabilizing residues in *E. coli* and not primary destabilizing ones as in eukaryotes (Mogk *et al.*, 2007), indicating that the identified region with the type 2 binding site motif in E.coli might be responsible for proteins with bulky hydrophobic amino-terminal residues of type 2. Interestingly, this type 2 binding site motif is not conserved in the *Arabidopsis* N-recognin PRT6 that was described in this work and shown to degrade substrates with basic amino-terminal residues

(Garzón *et al.*, 2007). As sequence alignments of the UBR box showed that this motif can also be found in the PRT6 gene of *Arabidopsis* (Tasaki *et al.*, 2005), the UBR box motif was hypothesized to be involved in the binding of basic amino termini (Garzón *et al.*, 2007). This assumption is supported by the function of PRT1, another N-recognin that has been identified in plants (Stary *et al.*, 2003). PRT1 was shown to degrade proteins with aromatic amino acids at the amino terminus, a subset of type 2 N-degrons. Structural analysis revealed that PRT1 carries two RING finger domains and one ZZ domain (Potuschak *et al.*, 1998), but not the UBR box motif. This can be seen as an additional hint that the UBR box motif is involved in the degradation of proteins with amino acids of type 1 at the amino terminus. Interestingly it was found that the ZZ domain of PRT1 weakly resembles a part of the UBR box motif, suggesting the possibility that there are variants of the UBR box motif that can recognize distinct subsets of N-degrons (Tasaki *et al.*, 2005).

4.4.3 Potential roles for additional plant N-recognins

Recently, two more proteins besides PRT6 with sequence similarity to the yeast N-recognin Ubr1 could be identified in *Arabidopsis*: At3g02260 and At4g23860. All three *Arabidopsis* proteins have the UBR box motif in common that has already been identified in mammalian N-recognins. According to their sequence similarity with already identified mammalian UBR genes, the new proteins were called UBR4 and UBR7 (Tasaki *et al.*, 2005). UBR7 has unknown function(s) as it has not been investigated so far, but UBR4 turned out to be a sequelog of the 560 kDa *Arabidopsis* BIG protein that was shown to be involved in auxin transport (Gil *et al.*, 2001). A possible involvement of *Arabidopsis* UBR4 in the plant N-end rule pathway still has to be demonstrated. Interestingly, it has been shown that BIG contains three zinc-finger domains and has therefore already been hypothesized to be involved in protein-protein interactions (Gil *et al.*, 2001).

Due to the common motif of the UBR box that has been identified in all mammalian UBR ligases so far, a potential role of the two newly identified

proteins as N-recognins in the plant N-end rule pathway can be hypothesized. However, it is unclear so far in which physiological process the two new candidate ligases might be involved. The N-end rule fusion protein assay with *prt6* mutants, presented in this work, could demonstrate that the PRT6 ligase is responsible for the degradation of a protein with a basic amino acid (Arg) at the amino terminus and also the involvement of the N-recognin PRT1 in the degradation of proteins with an aromatic amino acid (Phe) at the amino terminus was confirmed. Quantitative analysis of the results, however, showed differences in the extent of protein accumulation. Whereas the ratio of the band intensity of the test protein to its reference protein was above 3 in *prt6* mutants expressing the stable M-GUS control protein, the value of the basic R-GUS test protein to its reference protein in *prt6* mutant plants was at 1.2 (Table 3.15), indicating that in spite of a mutation of the PRT6 gene, a modest level of degradation of proteins with basic amino acids at the amino terminus can still take place. This finding supports therefore the possibility that proteins with basic amino acids at the amino terminus might be degraded not only by one but by several different N-recognins as it was found in mammals (Tasaki *et al.*, 2005). PRT6 is therefore one, but may not be the only E3 ligase for proteins with basic amino termini in plants. Similar to these results in *prt6* mutants, the quantitative analysis in the *prt1* mutant expressing the aromatic F-GUS test protein revealed a band intensity ratio of only 2.0 which was also below the expected value. One possible explanation why R-GUS test proteins are less stable in *prt6* mutant plants and F-GUS test proteins in *prt1* mutants when compared to the level of M-GUS test proteins might be the presence of conformational differences in the structure of the different test proteins, which might make R-GUS and F-GUS proteins more attractive to other (unknown) protein turnover pathways.

The observed differences in protein stability, however, support the assumption that also the plant N-end rule pathway contains N-recognins with overlapping functions as it has already been shown in the mammalian N-end rule pathway (Tasaki *et al.*, 2005). It could be shown in this work that PRT1 is not responsible for the degradation of proteins with basic amino acids

at the amino terminus and that proteins with aromatic amino-terminal residues do not serve as substrates for PRT6, suggesting that there are two, yet unknown N-recognins involved in the N-end rule pathway of protein degradation. Together with the other yet unknown ubiquitin ligase(s) that should be responsible for the degradation of proteins with hydrophobic amino-terminal residues, at least three additional ligases can be postulated to be involved in the degradation of proteins with destabilizing residues if no overlap exists. A potential role for the recently identified *Arabidopsis* sequelogs of mammalian N-recognins UBR4 and/or UBR7 might therefore be one option.

One reason for this assumption is the presence of the UBR box motif that has been found in these two ligases and which is hypothesized to bind peptides with basic amino acids at the amino terminus (Garzon *et al.*, 2007), which makes them candidates for the missing N-recognin that is responsible for amino-terminal residues of type 1. Especially UBR4, the formerly identified BIG protein is very promising: Comparison of the identified UBR box motif (Tasaki *et al.*, 2005) with the three putative zinc-finger domains in BIG (Gil *et al.*, 2001) revealed that one of the two identified cysteine-rich domains, CDR-1, corresponds to the UBR box motif - but besides this motif, BIG carries also the ZZ binding site that in turn was identified in PRT1 (Potuschak *et al.*, 1998), the plant N-recognin that is responsible for the degradation of proteins with aromatic amino-terminal residues (Stary *et al.*, 2003). Therefore, UBR4 is a very interesting candidate with the potential to function in the degradation of proteins both with basic amino acids (type 1) and with aromatic amino acids (type 2) at the amino terminus.

However, it has to be considered that a possible binding of a protein substrate not necessarily also leads to a subsequent ubiquitylation and degradation: In the yeast N-recognin Ubr1, where the UBR motif has been described for the first time, it was shown that Ubr1 can bind large protein substrates carrying a basic amino-terminal residue. However, the same binding site recognizes and binds dipeptides that

are not ubiquitylated but serve as allosteric regulators involved in peptide import (Baker and Varshavsky, 1991; Turner *et al.*, 2000).

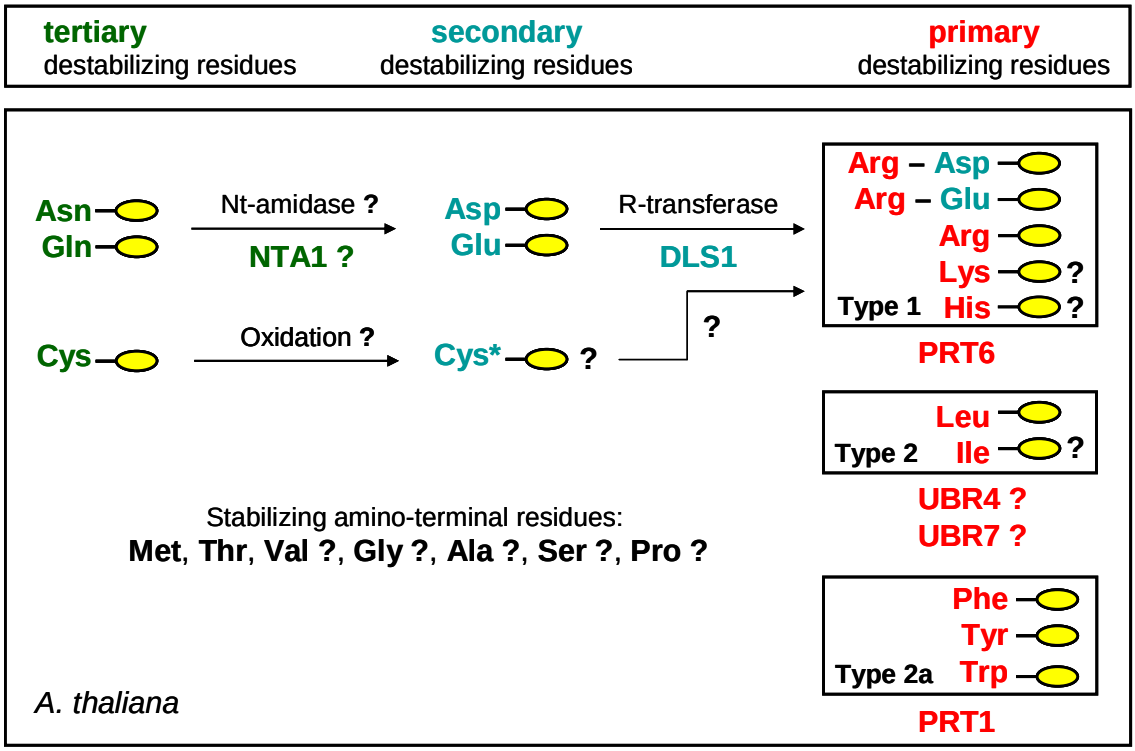

Fig. 4.1: The N-end rule pathway in plants (A. thaliana). *The N-end rule pathway in plants shows certain similarities to the one found in yeast and mammals (cf. Fig. 1.6). However, only two proteins have been identified so far with N-recognin function. PRT6 has been demonstrated to degrade proteins with Arg at the amino terminus and is postulated to recognize subtrates with the two other basic amino acids Lys and His, too. PRT1 degrades substrates with type 2 primary destabilizing residues, however its function is limited to aromatic amino acids at the amino terminus (type 2a), a subset of type 2 primary destabilizing residues in yeast and mammals. As it has been shown that also Leu confers destabilizing character in plants, at least one more N-recognin has been postulated to be responsible for the degradation of proteins with Leu at the amino terminus, together with Ile, the last remaining amino acid with destabilizing character in yeast and mammals. However, the role of Ile has not been proven yet. So far, only one more protein (DLS1) has been demonstrated to be involved in the plant N-end rule pathway, as DLS1 is necessary for degradation of proteins with Asp and Glu, two secondary destabilizing residues, at the amino terminus. Similar to the mammalian pathway, a destabilizing role is also postulated for the tertiary destabilizing residues Asn, Gln and Cys, however this still needs to be shown. Stabilizing character has been demonstrated for Met and Thr and still needs to be proven for Ala, Gly, Pro, Ser and Val.*

In Fig. 4.1, the N-end rule pathway components in *Arabidopsis* are summarized. Like in yeast and mammals (cf. Fig. 1.6), primary destabilizing residues have been identified also in plants. Proteins with Arg (type 1) at the amino terminus have been demonstrated to be a substrate for a plant N-recognin and PRT6 has been shown to mediate their turn-over

(Garzón *et al.*, 2007). As Arg is a basic amino acid, it was postulated that also Lys and His have destabilizing character at the amino terminus. However, this still needs to be shown. Whereas this is similar to the yeast and mammalian N-end rule pathway, it has been demonstrated in plants that primary destabilizing residues of type 2 with bulky hydrophobic character are divided into two groups, each group containing only a subset of type 2 residues. Whereas type 2 comprises in plants the aliphatic amino acids Leu and Ile, type 2a encompasses the aromatic amino acids Phe, Tyr and Trp. For the latter, an N-recognin – PRT1 – has already been identified (Stary *et al.*, 2003), in contrast to the amino acids of type 2, where test proteins with Leu at the amino terminus have been demonstrated to be destabilizing (Stary *et al.*, 2003, Garzón *et al.*, 2007), however, no N-recognin has been detected yet. Therefore, a potential role of UBR4 or UBR7 was suggested in this work as these proteins share the UBR box motif that has been shown to be part of the yeast N-recognin Ubr1. A destabilizing character is also postulated for Ile, as it is a primary destabilizing residue both in yeast and in mammals, but this also needs to be proven. Asp and Glu, two secondary destabilizing residues, have been shown to require DLS1 for degradation. This protein presumably functions as an R-transferase that is also involved in leaf senescence (Yoshida *et al.*, 2002). The tertiary destabilizing residues in yeast and mammals, Asn and Gln, are still only hypothesized to be involved also in the plant N-end rule pathway, as no Nt-amidase has been identified yet. Stabilizing character has been demonstrated for Met and Thr (Worley *et al.*, 1998) and was postulated for Val, Gly, Ala, Ser and Pro. Likewise, the role of Cys is also still hypothetical, although it is thought to be first oxidized and then arginylated like in the mammalian N-end rule pathway. As the mammalian N-end rule pathway differs to a certain extent from the yeast one (primary destabilizing residues of type 3, Cys as a tertiary destabilizing residue), it cannot be excluded that also the plant N-end-rule pathway has developed some distinct pathways that are specific only for plants.

In the near future, analyses of the two potential N-recognins UBR4 and UBR7 are certainly the most promising approaches for further investigations of the

plant N-end rule pathway. An assay similar to the one that was presented in this work would be desirable, including the two already identified N-recognins as overlapping functions cannot be excluded. In this way and in combination with double and triple mutants, a potential involvement of UBR4 and UBR7 into the N-end rule pathway can be revealed.

4.4.4 PRT6 is not identical with the previously described CER3 gene

The presented data in this work could clearly identify the locus At5g02310 as a ubiquitin ligase involved in the degradation of proteins with basic amino termini in the N-end rule pathway. Former annotations of the locus At5g02310 as corresponding to the *CER3* gene involved in the wax biosynthesis could not be confirmed. The *PRT6* gene is not identical with the *CER3* gene as it was postulated by Hannoufa and co-workers in 1996 and later by Eisner and co-workers in 1998. The sequence X95962 that was originally identified as the *CER3* gene corresponds to the carboxyl-terminal half of *PRT6*. Sequences of the 3´ half of the *PRT6* gene were shown to be able to complement a *cer3* mutation (Eisner *et al.*, 1998). Mapping analyses however placed the *cer3-1* mutation at the bottom of chromosome 5 (Koornneef *et al.*, 1989), whereas the gene At5g02310 is instead located at the top of chromosome 5. This discrepancy between the estimated location of *CER3* on the genetic map at approximately 82 cM and the genome sequencing data had already been mentioned by Meinke and co-workers (Meinke *et al.*, 2003). Besides this, *prt6* mutant plants display the normal *Arabidopsis* phenotype. All *prt6* mutants used in this work did not show any wax deficiency at the cuticle and expressed normal wax layers, whereas the *cer3-1* mutant was indeed defective in the wax metabolism with a diminished production of surface wax (Garzón *et al.*, 2007). This contradiction is now resolved, and in addition to the presented results that At5g02310 corresponds to *PRT6*, another group could show that *CER3* actually corresponds to At5g57800, representing the previously identified gene WAX2/YRE/FLP1 which has an incompletely characterized function in wax biosynthesis (Rowland *et al.*, 2007).

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