Modification of the Plant Retinoblastoma-Related Protein (RBR1) by SUMO: Structural and Functional Studies

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LIST OF ABBREVIATIONS

ATP: Adenosine Tri phosphate.

CDK: Cyclin-dependent kinase.

CycD: Cyclin D.

DNA: Deoxyribonucleic acid.

DP: DNA-binding heterodimerization partner protein.

E2F: Adenovirus E2 promoter-binding factor.

GFP: Green fluorescent protein.

GST: Glutathione-S-transferase.

HDAC: Histone deacetylase

LB: Luria-Bertani medium

PCR: Polymerase chain reaction.

PTM: Post translational modification

Rb: Retinoblastoma.

RBR1: Retinoblastoma-related protein.

SENPs: SUMO-specific proteases.

SUMO: Small ubiquitin-related modifier.

ABSTRACT

The retinoblastoma protein (Rb) is a transcription regulator and key component of the Rb/E2F/DP pathway which regulates progression of the cell cycle in plants and animals. Within the pathway, Rb blocks E2F transcriptional activity consequently ensuring restricted cell proliferation. Of great importance too, is a family of posttranslational modifiers referred to as small ubiquitin-related modifiers (SUMO), whose modification consequences include; sub cellular localization of proteins, alteration of protein to protein interaction and regulation of transcriptional activity.

In order to study and depict the plant retinoblastoma related protein (RBR1) as a SUMO substrate; its modification site was mutated to address the effect of the mutation on protein localization. Additionally, an *in-vitro* assay was used to further illustrate the consequences of the mutation. In protoplasts transfected with wild type RBR1 the protein was solely present in the nucleus while those transfected with mutated RBR1, the protein was seen in both the nucleus and the cytosol. From the *in vitro* SUMOylation assay it was evident that while wild type RBR1 could be modified by SUMO, its mutated version could not undergo modification.

The results from this study don't only show RBR1 as a SUMO substrate; they also suggest that modification by SUMO could be needed for its sub-cellular localization.

CHAPTER ONE

INTRODUCTION

1.0 Background

The growth, development and division of cells within multi-cellular organisms such as plants depends on a sequence of synchronized events which are spatially and temporarily tightly regulated within individual cells (Desvoyes *et al.,* 2006). Furthermore, organ development in plants is just about a post embryonic process; as a result organogenesis depends on constant ability of a given set of cells to grow prior to undergoing specific differentiation programs (Desvoyes *et al.,* 2006). Consequently the post-embryonic nature of organogenesis, multi-cellularity of plants requires precise linking of cell proliferation, differentiation and arrest of the cell cycle so that all processes are coordinated with the overall development program (Desvoyes *et al.,* 2006).

Unlike animals, development of new plant organs such as roots, leaves and flowers repeatedly occurs over the plants life span which can at times extend over thousands of years. As a result of continuous organogenesis, regulation of the cell cycle is of fundamental importance for plant growth and development (Wildwater *et al.* 2005; Inze *et al.*, 2006).

Gutierrez and co workers (2002) acknowledged that in addition to hormonal signals, a key regulator of the plant cell cycle is the Rb/E2F/DP pathway which consists of the retinoblastoma protein (Rb) and the E2F transcription factor. Rb functions by binding the E2F transcription factor consequently blocking transcription of cell related cycle genes; this in turn prevents uncontrolled proliferation of cells (Brehm *et al.,* 1999).

Like most proteins, Rb is subject to posttranslational modification by processes such as phosphorylation, glucosylation and acetylation resulting in chemical alteration of the amino acids within the protein (Verger *et al.,* 2003). Alternatively the protein can also be modified by addition of other polypeptides such as ubiquitin and the small ubiquitin related modifiers also referred to as SUMO (Verger *et al.,* 2003; Gill, 2004). The consequences of these modifications include sub-cellular localization of proteins, alteration of protein to protein interactions and degradation of proteins (Richards, 2008).

1.1 Objectives of the study

Conjugation of SUMO to substrate proteins has been implicated in regulation of a number of cellular processes ranging from nuclear transport, cell cycle control; transcription regulation and DNA repair (Gill, 2004). Earlier *in-vitro* experimentation in the Bako lab (UPSC) had shown that the plant retinoblastoma-related protein RBR1, whose function is crucial for plant cell division and development, is modified by SUMO. The site(s) of modification and functional consequence of SUMOylation on RBR1 function were however yet to be determined. Hence the objectives of this study were;

1. To indentify a site(s) on *Arabidopsis* RBR1 protein modified by SUMO followed by; PCR-directed mutagenesis of predicted conjugation site(s), as well as analysis of mutant proteins by *in vitro* SUMOylation assays.
2. Functional studies of RBR1 carrying mutation(s) of SUMO conjugation site(s) through; Transient expression of RBR1 mutant fused to GFP in plant cells to investigate the effect of mutation on intracellular localization using confocal microscopy.

CHAPTER TWO

LITERATURE REVIEW

2.1 The Plant Cell-cycle

The cell cycle also referred to as the cell-division cycle involves a sequence of four coordinated events culminating in replication of the cells genetic material. The four events include; Gap phases (G1 and G2) which separate DNA replication (S phase) and the M (mitosis) phase respectively (Ferreira *et al.,* 1994). An additional role of gap phases within the cycle is to serve as check points' ensuring each phase is successfully completed before the next is initiated (Ferreira *et al.,* 1994; Dewitte *et al.,* 2003).

Under the influence of hormonal signals and metabolic changes during G1 Phase, cells start preparing for the impending division (Dewitte *et al.,* 2003). At a certain point, the cell moves into S-phase during which genetic material is replicated and doubled resulting in chromosomes with two sister chromatids. The cell subsequently moves into G2 phase during which it continues to grow while assembling cytoplasmic material required for eventual division. The final phase of the cycle is M phase which involves nuclear division accompanied by cytoplasmic division (cytokinesis) resulting into two daughter cells (Dewitte *et al.,* 2003).

2.2 Regulation of the cell cycle

2.2.1 Cyclins and cyclin dependent Kinases (CDKs)

Regulation of the cell cycle is essential to ensure; division of cells never occurs until all DNA has been replicated and repaired in case of damages (Inze *et al.,* 2006). Hence cycle regulation occurs throughout the cell division machinery with a unique characteristic of

all regulation points being under the control of cyclin dependent kinases (CDKs) (Pines, 1999).

Cyclins are a multi group of proteins characterized by a poorly conserved region essential for interaction with partner CDKs. The conserved cyclin region is 250 amino acids long comprising of two folds of five helices (Dewitte *et al.,* 2003). Using sequence based classification; five types of cyclins (A, B, C, D and H) have been indentified in plants with significant roles at different stages of the cell cycle (Renaudin *et al.,* 1996; Vandepoele *et al.,* 2002). "A-type cyclins appear at the beginning of the S-phase and play a role in its progression, B-type cyclins are involved in G2 /M transition while D-type cyclins control progression through G1 and S phase" (Dewitte *et al.,* 2003).

Pines (1999), noted that progression of the cell cycle is dependent on activation and deactivation of cyclin dependent kinases (CDKs) which belong to a conserved serine/theorine protein kinase family. CDKs remain inactive until their partner cyclins bind them to form Cyc-CDK complexes; these are then activated by CDK-activating kinases (CAKs) by phosphorylating threonine residues contained in CDK T-loops (Pines, 1999). Consequently, CDK induced phosphorylation is responsible for onset of DNA replication and mitosis during S and M phases respectively (Pines, 1999; Inze *et al.,* 2006).

Four CDKs namely CDKA1, CDKA2 and CDKB1, CDKB2 have been indentified in *Arabidopsis thaliana* belonging to CDKA and CDKB families respectively. CDK related studies in *Antirrhinum* cells revealed that CDKA activity is up regulated during G1 and S

phase while CDKB activity is intense during S, G2 and M phases (Fobert *et al.,* 1994; Joubes *et al.,* 2000; Vandepoele *et al* 2002).

Once activated, Cyc-CDKs covalently add phosphate groups to serine or threonine residues within substrate proteins consequently altering their properties. One notable substrate of CDKs is the retinoblastoma protein (Rb) whose phosphorylation is essential for progression of the cell cycle (Dewitte *et al.,* 2003).

2.2.2 Rb/E2F/DP Pathway

The Rb/E2F/DP pathway controls transition from G1 to S phase of the plant cell cycle, it consists of the retinoblastoma protein (Rb) a transcription regulator and the E2F transcription factor (Gutierrez *et al.,* 2002; Inze *et al.,* 2006). According to Weinberg (1995), involvement of the Rb protein in regulation of cell division was originally exclusively associated with animals. However this view changed as a result of the discovery that Rb related proteins and components of the Rb/E2F/DP pathway do actually exist in plants too (Huntley *et al.,* 1998)

De Jager *et al.,* (1999) referred to the Rb protein "as being part of a conserved pathway controlling the activation of cell division in animals". It contains a number of functional domains two of which denoted A and B are conserved in humans and plants (Harbour *et al.,* 2000). Furthermore, interaction of the two domains results in formation of a central pocket which enables Rb to interact with partner proteins such as cyclin D, histone deacetylases via their LxCxE motifs (L-leucine, C-cysteine, E-glutamic acid and x being any amino acid) (Harbour, 1998).

Rb is a nuclear protein characterized by a number of potential binding sites for CDKs. Furthermore, the protein is crucial in regulation of the cell cycle, cell differentiation

during which it functions by blocking E2F transcription activity consequently preventing uncontrolled cell proliferation (Dewitte *et al.,* 2003). The importance of Rb in cell cycle regulation was elucidated by inhibiting its function through virus-induced gene silencing which resulted in prolonged cell proliferation and delayed differentiation of *Nicotiana benthamiana* leaf and stem cells (Park *et al.,* 2005). Additionally, the plants showed retarded flower formation highlighting a possible role for Rb in flower development (Park *et al.,* 2005).

The second component of the cell cycle regulating pathway is a family of transcription factors referred to as E2Fs. E2Fs contain a DP heterodimerization domain enabling them to combine with DP proteins resulting in an active E2F/DP transcription complex which induces expression of genes involved in cell cycle progression (Desvoyes *et al.,* 2006). Furthermore, E2Fs also contain binding domains to which the Rb protein binds to block transcription activity. Six E2Fs (E2Fa, E2Fb, E2Fc, DEL1, DEL2, and DEL3), two DP proteins (DPa and DPb) and a single Rb homolog (*RBR1*) have been indentified in *Arabidopsis thaliana* (Desvoyes *et al.,* 2006; Hirano *et al.,* 2008).

2.2.2.1 Regulation of the Rb/E2F/DP Pathway

Rb functions by binding E2F transcription factors (Figure 1A) consequently blocking transcription of cell cycle related genes (Brehm *et al.,* 1999). Repression of E2F activity is due to the Retinoblastoma protein recruiting histone deacetylase (HDAC) enzymes which are co-repressors of transcription. HDACs remove acetyl groups from histones within DNA resulting in chromatin modification, condensation of DNA and inhibition of transcription (Brehm *et al.,* 1999).

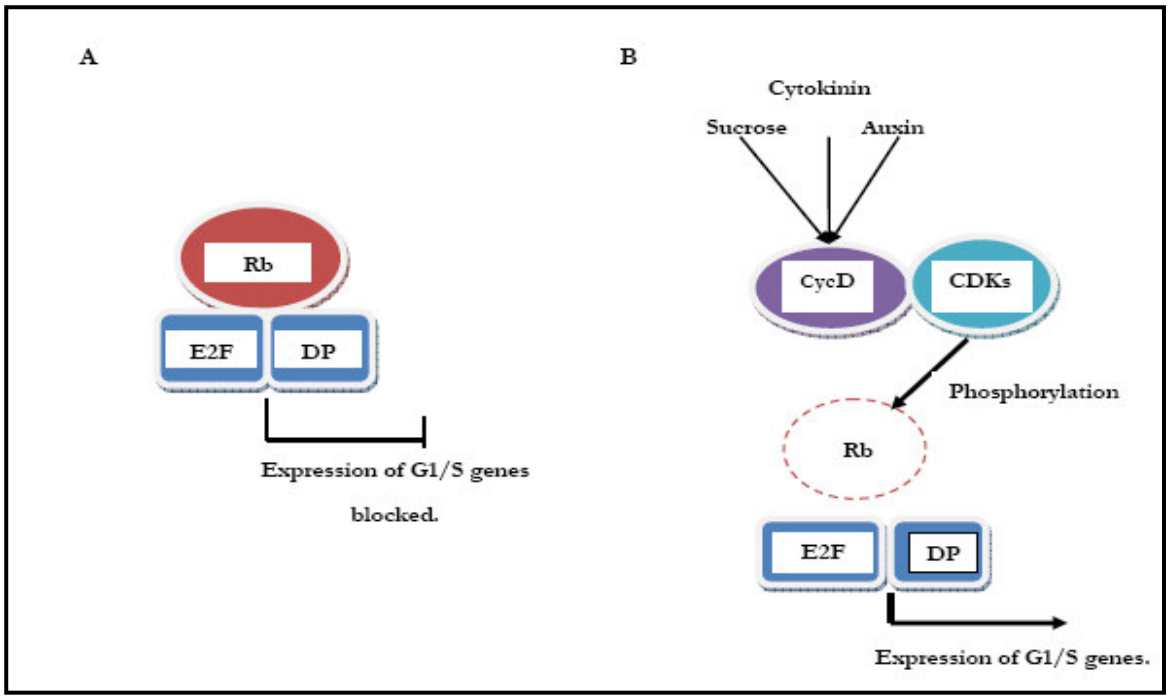

Figure 1 Transition from G1 to S phase of the cell cycle in plants. (**A**) The retinoblastoma protein binds to the E2F/DP transcription factor blocking expression of cell cycle related genes. (**B**) Expression of D type cyclins in response to metabolite and hormonal signals leads to activation of cyclin dependent kinases. Activated CDKs go on to phosphorylated Rb, once Phosphorylated Rb activity is terminated leading to progression of the cell cycle (Modified from De Jager *et al.,* 1999).

However, in response to signals induced by metabolites (sucrose) and hormones (auxin and cytokinins), D-type cyclins are expressed (Figure 1B). CycDs subsequently bind their partner CDKs forming CycD-CDK complexes which are activated by CDK-activating kinases (CAKs). Active Cyc-CDKs are capeable of phosphorylating target proteins resulting in altered protein properties (Dewitte *et al.,* 2003).

The Retinoblastoma protein being a CDK target is phosphorylated leading to termination of its repression activity on the E2F transcription factor (De Jager *et al.,* 1999). In absence of Rb induced repression (Figure 1B), E2Fs are eventually capeable of inducing the expression of genes involved in G1/S transition of the cell cycle (De Jager *et al.,* 1999; Dewitte *et al.,* 2003).

Xie and co workers (1995) reported that interaction between viral proteins and Rb induces direct entry of the cell cycle into the S phase. This is attributed to ability of viral proteins to mutate the LxCxE motif on the HDACs terminating their co-repression activity (Park *et al.,* 2005).

2.3 Posttranslational modification of proteins

Posttranslational modification (PTM) is the chemical alteration of proteins after biosynthesis; it may involve a change in the chemical nature of amino acids through processes such as phosphorylation, acetylation, hydroxylation, glycosylation, alkylation and methylation. Alternatively, proteins can also be modified by addition of other polypeptides (Verger *et al.,* 2003; Gill, 2004). The consequences of these modifications include localization and degradation of proteins (Richards, 2008).

The most common polypeptide involved in PTM is the 76 amino acid (Ubiquitin) present in most eukaryotic species whose biological function is marking proteins for eventual degradation by the 26S proteasome (Kurepa *et al.,* 2002). It is covalently attached to lysine residues within substrate proteins via an isopeptide bond formed between the lysine residues and a poly ubiquitin chain (Kurepa *et al.,* 2002). The ubiquitination pathway is ATP dependent and relies on an activating enzyme (E1), conjugating enzyme (E2) and a ligase (E3) which enable poly-ubiquitination of target proteins (Kurepa *et al.,* 2002).

In an ATP dependent reaction, E1 activates the ubiquitin precursor via a thiol ester bond formed between a glycine in ubiquitin and the cysteine end in the E1 enzyme (Smalle *et al.,* 2004). Activated ubiquitin is then transferred to E2 or ubiquitin conjugating enzyme, eventually, ubiquitin is conjugated on to target proteins via an isopeptide bond between

the C-terminal glycine of ubiquitin and the lysine residue of the substrate protein with the help of an E3 ligase enzyme (Smalle *et al.,* 2004).

2.3.1 SUMO

In addition to ubiquitin, families of ubiquitin like proteins (Ubls) have over the years been discovered and found to be involved in post translational of proteins in animals and plants. Key among Ubls is a family referred to as SUMO with functions spread across a variety of biological processes (Johnson, 2004).

SUMO is an acronym for small ubiquitin-related modifier, a family of conserved proteins present in all eukaryotes. SUMOs are approximately 11 KDa in molecular weight, 25 amino acids longer than ubiquitin but with a 20% sequence identity with ubiquitin (Johnson, 2004). Initial identification of SUMO was in 1997 after unearthing its conjugation to the GTPase activating protein RanGAP1, a protein involved in nuclear transport and the cell cycle in animals (Matunis *et al.,* 1998; Verger *et al.,* 2003; Marx, 2005). As is the case with ubiquitination, SUMOylation also involves addition of SUMO to lysine residues within substrate proteins. "SUMO modifies a number of proteins which participate in diverse cellular processes such as; transcriptional regulation, nuclear transport, maintenance of genome integrity, and signal transduction" (Verger *et al.,* 2003; Johnson, 2004; Bossis *et al.,* 2006).

Three SUMOs have so far been indentified in animals namely; SUMO-1, SUMO-2 and SUMO-3 while a total of 9 genes (*SUM1 to SUM9*) with strong resemblance to animal and fungal SUMOs have been identified in *Arabidopsis thaliana* (Kim *et al.,* 2002; Novatchkova *et al.,* 2004). Although referred to as ubiquitin like proteins, there are notable differences between SUMO and ubiquitin; for instance SUMO-1contains a long

and flexible N-terminal which is absent in ubiquitin (Bayer *et al.,* 1998). Secondly, unlike ubiquitin, SUMO-1 lacks Lysine-48 hence its inability to form multiple SUMOylation chains as is the case with ubiquitin (Bayer *et al.,* 1998).

Additionally, while ubiquitination is associated with marking proteins for degradation, SUMOylation has been implicated in influencing protein to protein interactions, regulating protein stability and sub-cellular localization of substrate proteins (Bossis *et al.,* 2006). In plants, animals SUMO modification occurs at all development stages and their respective tissues. This is made possible by Specific SUMO proteases which de-conjugate SUMO precursor from substrate proteins making it available to be reused, although the long term fate of sumoylated proteins remains altered even after the modifier has been de-conjugated (Verger *et al.,* 2003; Gill, 2004; Hay, 2005; Bossis *et al.,* 2006).

2.3.2 SUMO conjugation pathway

SUMOylation (Figure 2) occurs in the nucleus and cytoplasm in a pathway relying on enzymes similar to those in ubiquitination but specific to SUMO. The pathway makes use of an activating enzyme (E1), conjugating enzyme (E2) and a ligase (E3) (Johnson, 2004).

2.3.2.1 SUMO activating enzyme E1

The activating enzyme consists of two un-identical protein sub units which must combine to form an active heterodimer. In *Arabidopsis thaliana* the enzyme is denoted SAE (SUMO activating enzyme) with two subunits SAE1a and SAE1b whose gene

annotations are At4g24940 and At5g50580 respectively (Johnson *et al.,* 1997; Novatchkova *et al.,* 2004).

2.3.2.2 SUMO conjugating enzyme E2

Contrary to ubiqutination in which E3 ligases ensure substrate specificity, previous *in vitro* studies have highlighted the SUMO cojugating enzyme as being able to attach SUMO on to substrates without the E3 ligase (Rodriguez *et al.* 1999 and 2001). A single E2 enzyme exists in *Arabidopsis thaliana* denoted SCE or SUMO conjugating enzyme (Novatchkova *et al.,* 2004).

Melchior *et al.,* (2003) and Johnson (2006) attributed SUMO specificity to a short precise sequence which the conjugating and ligase enzymes recognize in all substrate proteins. "The sequence denoted ΨKXE or Ψ-Lys-X-Glu; where Ψ is a large hydrophobic amino acid; K the lysine residue which is modified; X is any residue; and E/Glu is glutamic acid (Johnson, 2006).

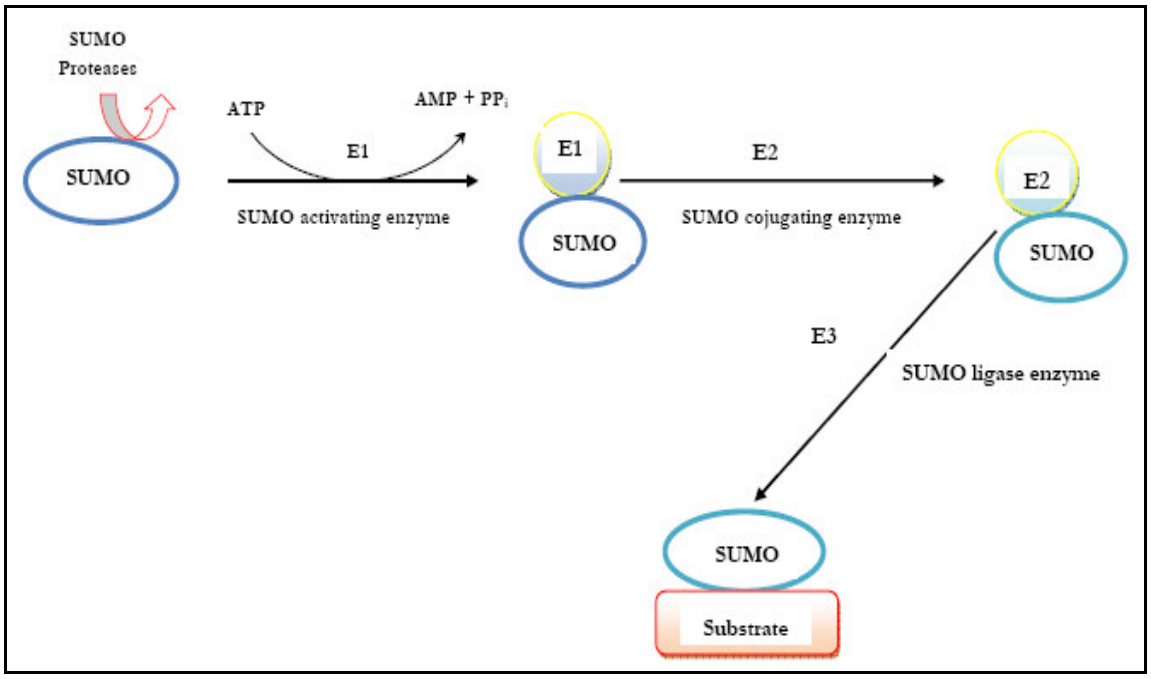

Figure 2 The SUMO conjugation pathway. SUMO precursor is cleaved by SUMO proteases to expose the glycine at its C-terminal end. Cleaved SUMO is then energetically activated by the E1-activating enzyme and transferred to the conjugating enzyme (E2). The final step of the pathway involves attaching SUMO on to the substrate protein in the presence of an E3 ligase (Modified from Marx, 2005).

Prior to activation (Figure 2), the SUMO precussor is processed by SUMO-specific proteases (SENPs) to expose the glycine at its C-terminal end. Then, in an ATP dependent reaction a thioester bond is formed between the exposed glycine and the catalytic cysteine of the activating enzyme (E1). Next, activated SUMO is transferred to the cysteine end of the E2 cojugating enzyme. The final step involves attachment of SUMO to lysine residues on target proteins a reaction that is aided by SUMO-E3-ligases (Johnson, 2004; Gill, 2004; Bossis *et al.,* 2006).

2.4 Regulation of SUMO conjugation

Reversibility of the SUMOylation pathway is ensured by SUMO-specific proteases or isopeptidases which remove SUMOs from modified proteins making it available for re-

use as a precursor (Johnson, 2006). Though effects of SUMOylation have clearly been established, not much is known about how the pathway is regulated. However, Johnson (2004) suggested that regulation of the pathway could be occurring during conjugation or de-conjugation of SUMO leading to change in amount of modified proteins.

Studies in animals have however revealed a role for Gam1 protein in regulating SUMO conjugation and de-conjugation (Bossis *et al.,* 2006). Gam1 is a viral protein residing in CELO (Chicken embryo lethal orphan virus); it is involved in transcriptional activation of cellular and viral genes that inactivate HDACs (Boggio *et al.,* 2005).

In an earlier study, Boggio and co workers (2004) showed that Gam1 targets SUMO E1 and E2 enzymes. Consistent with this finding, cellular expression of Gam1 resulted in decreased levels of SUMO conjugates, implying that the protein could be targeting the respective SUMO enzymes for proteasome degradation (Boggio *et al.,* 2005). Furthermore, abiotic factors such as heat shock, osmotic and oxidative stress have also been implicated in regulating the SUMOylation pathway in animals (Bossis *et al.,* 2006).

2.5 Biological effects of SUMO

Various nuclear proteins with roles related to transcriptional regulation, DNA repair and signaling have been identified as targets of post translational modification by SUMOs resulting in sub cellular localization of the proteins and alteration of protein-protein interaction (Gill, 2004). SUMOylation also affects processes including DNA repair, hormonal signaling and stress response.

2.5.1 Modulation of transcriptional activity

The majority of proteins modified by SUMO include signaling proteins, enzymes and transcription factors or regulators (Johnson, 2004). SUMOylation of the latter proteins results in increased repression of transcription activity, an assertion supported by studies in which mutation of SUMO acceptor lysines resulted in increased transcription activity. Additionally over expression of de-conjugating enzymes has also shown increased transcription activity in the previously affected transcription factors (Gill, 2005).

According to Johnson (2004), inhibition of transcription activity could be due to SUMO induced interactions with proteins that co-repress transcription. Furthermore, some transcription factors could require post translation modification by processes such as phosphorylation and ubiquitination. However SUMOs have the added ability of preventing such modifications by blocking lysine residues where they would normally occur (Johnson, 2005).

2.5.2 Sub-cellular localization of proteins

Studies involving the first indentified SUMO substrate protein RanGAP1 have revealed a role of SUMOylation in sub cellular localization of proteins. RanGAP1 is a GTPase activating protein with a significant role in protein transportation into the nucleus via the nuclear pore complex (Melchior *et al.,* 1993; Gill, 2004). In animals unmodified RanGAP1 is located in the cytoplasm, however subsequent to SUMOylation the protein is present in the nuclear pore. Additionally, the modified protein shows increased interaction with the nuclear protein RANBP2 (RAN binding protein2) which could imply structural alterations within sumoylated RanGAP1 resulting in elevated levels of interaction with RANBP2 (Matunis *et al.,* 1996). Consistent with this suggestion, protein

sequencing showed that interaction between RanGAP1 and RANBP2 required prior modification of RanGAP1 by SUMO (Matunis *et al.,* 1996 and 1998)

2.5.3 DNA repair

Involvement of SUMOs in DNA repair was highlighted in experimentation using thymine DNA glycosylate (TDG). TDG is a DNA repair enzyme which removes uracil and thymine from U-G or T-G mismatched base pairs respectively, resulting in DNA devoid of a pyrimidine site (s) which is then repaired by downstream enzymes (Hardeland *et al.,* 2002).

In vitro studies with unmodified TDG show that the enzyme could only perform a single round of base removal as a result of being tightly bound to the reaction products (Johnson, 2004). Contrary to this, sumoylated TDG did perform multiple base removal reactions an indication that modified TDG was not being impeded by the end products (Johnson, 2004).

2.5.4 Cell cycle regulation

In addition to being involved in DNA repair, sub-cellular localization of proteins and modulation of transcription activity; SUMO modification has also been implicated in regulating progression of the cell cycle (Johnson *et al.,* 1997). *Saccharomyces cerevisiae SUMO- E1* and *SUMO- E2* mutants have cell cycle defects and arrest at the G2 to M boundary (Johnson et al., 1997). Similarly, mutation of SUMO- E1 and SUMO- E2 in *Schizosaccharomyces pombe* resulted in cells with stern impaired growth and mitotic defects, leading to suggestions that SUMOylation is crucial for cell cycle progression (Hay, 2005).

2.5.5 A biotic stress response

Saitoh *et al.,* (2000) reported that in animals SUMO2/3 is involved in cellular response to environmental stress. Similarly SUMOs do indeed play an equivalent role in plant abiotic stress response. Exposure of *Arabidopsis* cells to ethanol and hydrogen peroxide (H_2O_2), caused a striking increase in SUMO conjugates while withdrawal of the two stress factors resulted in a reduction of SUMO conjugate levels (Kurepa *et al.,* 2003). This observation could be an indication of involvement of SUMOs in plant stress response.

2.5.6 Abscisic acid (ABA) signaling

In addition to the aforementioned role in stress response, biological consequences of SUMOylation in plants extend to hormonal signaling in particular with the stress hormone ABA (Lois *et al.,* 2003). *Arabidopsis SUM1* over expressers have superior insensitivity towards ABA induced root growth inhibition (Lois *et al.,* 2003). However, when SCE1 (sumo conjugating enzyme) activity is suppressed, the plants succumb to the ABA effect resulting in a stunted root growth phenotype (Lois *et al.,* 2003).

CHAPTER THREE

MATERIALS AND METHODS

The study was conducted within the Department of Plant Physiology at Umea University (UmU).

3.1 Construct

The construct pRT104-GFP-RBR (7.2 kb) was used as the raw template for this study. It consisted of; vector pRT104, green fluorescent protein (GFP) and the full length RBR1 protein from *Arabidopsis thaliana*.

3.1.1 Mutation of SUMOylation site

In order to generate RBR1 with a mutated SUMOylation site, nucleotides coding for the lysine at the SUMOylation site (ΨKXE) in the wild type protein were substituted for alanine using Silent Site Selector web tool (http://rana.lbl.gov/SSS/). A forward and reverse primer pair was designed and synthesized to introduce the desired mutation by PCR- site directed mutagenesis

3.2 PCR-site directed mutagenesis of SUMO conjugation site

A 720bp RBR1 fragment denoted "RBR1-mtSUMO" with a mutated SUMOylation site was amplified by polymerase chain reaction using the primer pair; R: 5'-ATAGGTACCCTATGAATCTGTTGGCTCGGTCGCGAGGGGTGCGGCACCAC-3' and F: 5'-TTCAGTCGACACATTGACCAGATCATTCTCTGTTGCTTCTACGGAGTGC-3'.

PCR amplification was performed in 200µL thermal tubes using a MJ mini thermo cycler (Bio-Rad, CA, USA), with each tube containing a 50µL reaction mixture containing; 0.5µL of DNA template (15ng/µL) mixed with 2.5µL of each 0.5mM primer, 5µL of 10X PCR buffer (Invitrogen, Oxon, UK), 2.5µL of 2.5mM Invitrogen $MgCl_2$, 1.25µL of 0.25mM dNTPs, 0.75µL of *Taq* DNA polymerase (Invitrogen) and 35µL of water. The mixture was subjected to an amplification program with an initial denaturation step at 95^{o}C for 4 minutes followed by 30 cycles consisting of; annealing of primers with template at 55 ^{o}C for 1 minute, extension of primers at 72 ^{o}C for 1 minute and final elongation at 72 ^{o}C for 5 minutes.

After PCR, 10µL of amplified product were resolved by gel electrophoresis on a 1% agarose gel containing 0.25µg/uL ethidium bromide while making use of a 1Kb DNA ladder (Invitrogen) to determine fragment size. After electrophoresis, fragment size was viewed using a UV documentation camera (Techtum Lab, Umea, Sweden).

Next, 40uL of the remaining PCR product were treated with proteinase K (Fermentas, Vilnius, Lithuania) and purified by phenol/chloroform extraction (15:24:1, Sigma, Germany), precipitated, washed with 70% ethanol and finally resuspended in 15µL of water. Treatment with proteinase K was to remove tightly bound Taq polymerase that would interfere with restriction digestion.

3.3 Cloning RBR-mtSUMO

The construct pRT104-GFP-RBR was double digested with appropriate restriction enzymes to rid it of the C-terminal part of RBR1 protein which was subsequently replaced with amplified RBR1 containing a mutated SUMOylation site.

3.3.1 Restriction and ligation

3µg of the cloning vector were digested with enzyme SalI (Fermentas) for 2hrs at 37˚C after which enzyme activity was terminated with 65˚C incubation on a heating block. After 15 minutes the vector was purified by phenol/chloroform extraction, precipitated, washed with 70% ethanol and finally resuspended in 15µL of water. It was re-digested with KpnI (Fermentas) for 2hrs at 37˚C after which enzyme activity was terminated by incubating at 80˚C on a heating block.

Next, the vector was dephosrylated in a reaction containing; 25µL of vector, 2.9µL 10X dephosphorylation buffer and 1µL of shrimp alkaline phosphate (Fermentas). The reaction was performed at 37˚C for 30minutes followed by termination of SAP activity at 70˚C for 15 minutes. The DNA was resolved on a 1% agarose gel and isolated using the NA45 membrane (Schleicher and Schuell Bioscience, Dassel, Germany).

Prior to ligation, restriction of the PCR product was done with the same enzymes and conditions used for the vector. The vector and insert were then ligated together in a 10uL reaction; 5uL of insert, 3uL of vector, 1uL 5X ligase buffer and 1uL T4DNA ligase (Invitrogen). Ligation was done over night at 12˚C in a thermo cycler (Bio-Rad).

3.3.1.1 Transformation of *Escherichia coli*

100µL of thawed XLI blue *E.coli* competent cells in a 1.5mL Eppendorf tube were mixed with 10µL of ligation mix and left on ice, after 30 minutes the cells were heat shocked in a water bath at 42˚C for 90 seconds. 900uL of SOC medium (5X SOB media, glucose and water) were immediately added to the heat shocked cells to aid cell recovery. Next, the transformed cells were placed on a rotary shaker at 37˚C for 1hour after which 200µL

of the cells were spread on LB plates and grown overnight at 37˚C. From the overnight LB plates, a single colony was used to inoculate 5ml of LB media (containing 50mg/L carbenicillin) in a 15mL falcon tube overnight at 37˚C.

Mini-preparation was performed using a QIAprep spin mini-prep kit (Qiagen, Solna, Sweden) following the manufacturer's guidelines. To detect presence of the mutation, mini-preps were double digested in a single reaction with SalI and NruI (Fermentas). The NruI restriction site had been integrated into the reverse primer used to induce the mutation. Furthermore, the minipreps were sequenced using a 35S terminal antisense and a pRT104 35S primer.

3.4 Transient expression of GFP-RBRmtSUMO in suspension culture cells

3.4.1 Transfection of protoplasts

4 day old *Arabidopsis thaliana* root suspension cells were collected in a 50mL falcon tube by centrifugation at 1200rpm, 5 minutes in a ZK380 centrifuge (Hermle, Wehingen, Germany), after which the supernatant was discarded. In order to rid the cells of their cell walls, they were resuspended in 25mL of enzyme solution which was topped up with 25mL of B5-0.34M glucose mannitol (Table 1). Re-suspended cells were then transferred to petri-dishes and subjected to vigorous shaking on a rotary shaker.

After 4hrs, the cell suspension was centrifuged, supernatant discarded and the pellet resuspended in 25mL of B5-0.34M glucose mannitol followed by further centrifugation. Viable cells were collected by re-suspension in 5mL of B5-0.28M sucrose in which they floated.

Table 1: Composition of solutions used in transfection of *Arabidopsis* protoplasts.

B5- 0.28M Sucrose (S)	B5- 0.34M Glucose Mannitol (GM)	Enzyme solution	PEG Solution
- 3.16g/L B5 powder (Duchefa).	-3.16g/L B5 powder (Duchefa).	-1% Cellulase (Serva).	-25% PEG 6000.
-96g/L Sucrose.	-30.5g/L Glucose.	-0.2% Macerozyme (Yakult)	-0.45M Mannitol.
-PH 5.5 (with KOH).	-30.5g/L Mannitol.		-0.1M $Ca(NO_3)_2$
	-PH 5.5 (with KOH).		-PH 9.0 (with KOH)

**PEG (Polyethylene glycol), KOH (Potassium hydroxide), $Ca(NO_3)_2$ (Calcium nitrate).

Prior to transformation of protoplasts, a single positively sequenced colony was used to inoculate 2mL of fresh LB medium for a scale up culture. After 2.5hrs, 2mL of scale up culture were added to 50mL of fresh LB medium (100µg/mL carbenicillin) and grown overnight at 37˚C with shaking. Plasmid DNA was prepared using a plasmid mid kit (Qiagen) as described by manufacturer.

3-5µg of plasmid DNA (GFP-RBRmtSUMO) were gently mixed with 50µL of protoplasts followed by 150µL of PEG solution, the transformation mix was left in the dark at room temperature for 30 minutes. Protoplasts were twice washed with 0.275M $Ca(NO_3)_2$ to clear them of PEG solution after which they were collected by centrifugation and mixed with 0.5mL of B5-0.34M glucose mannitol. The cells were placed in a microtiter plate and incubated overnight in the dark; localization of GFP in the transfected cells was performed by confocal laser scanning microscopy

3.5 Sub cloning RBR-mtSUMO into vector pGEX-5X-1

Fragment RBR-mtSUMO was digested with SalI (Fermentas) while expression vector pGEX-5X-1 was first digested with XhoI (Fermentas), next the ends were filled with Klenow polymerase (Takara, Saint-Germain-en-Laye, France) to clear the vector of protruding 3' overhangs produced by XhoI. The Klenow filling reaction contained 20uL of vector, 3uL 10X Klenow buffer, 0.5µL 2.5mM dNTP, 0.5µL Klenow polymerase and 26uL of water. The mixture was incubated at room temperature for 10 minutes followed by incubation at 70˚C on a heating block to terminate Klenow activity.

Next the vector was digested with SalI, dephosphorylated with shrimp alkaline phosphate and purified from a 0.8% agarose gel using the NA45 membrane (Schleicher and Schuell). The purified vector was ligated with the insert (RBR-mtSUMO) after which the ligation was used to transform *E. coli* XL1blue competent cells.

Mini-preparation was done using a QIAprep spin mini-prep kit (Qiagen). To verify presence of the insert, mini-preps were digested with NruI (Fermentas), SalI (Fermentas) and resolved by electrophoresis on a 0.8% agarose gel. Positive mini-prep samples were further sequenced with the sequencing primers GEX lower (5'-CCGGGAGCTGCATGG TCAGAGG-3') and GEX upper (5'-GGGCTGGCAAGCCACGTTTGGTG-3').

3.6 Protein Expression and purification

3.6.1 Expression of RBR-mtSUMO

0.5uL of mini-prep DNA (pGEX-5X-1-RBR-mtSUMO) were used to transform 100uL of *E.coli* BL21 cells by heat shock, the cells were spread on LB plates containing Carbenicillin (100µg/mL) and grown overnight at 37˚C. A single colony was used to

inoculate 5ml of LB medium (100µg/mL carbenicillin) overnight at 37˚C. Next, a scale up culture containing 3mL of overnight culture and 200ml fresh LB medium was grown without induction.

After 2.5hrs, 200mL of scale up culture were added to 600mL of fresh LB medium (100µg/mL carbenicillin); the overall culture was grown at 37˚C until its OD_{600} reached 0.7. 80uL of isopropyl-b-D-1-thiogalactopyranoside (IPTG) were then added to the culture to a final concentration of 0.1mM after which growth was continued at 28˚C for 2.5hrs. The cells were eventually harvested by centrifugation at 3000g for 20minutes in a J-20xp centrifuge (Beckman coulter, CA, USA); the resulting pellet was retained while the supernatant was discarded.

3.6.2 Purification

Each gram of pelleted cells was re-suspended in 5mL of lysis buffer (25mM Tris-HCl pH7.8, 100mM NaCl, 2mM EDTA, 1mM DDT, 0.5mM PMSF and 1mM Benzamidin) followed by 0.5mg/mL of lysozyme and 0.2% Triton X-100 with gentle stirring at 4˚C. The cell suspension was agitated for 12 minutes with a Branson sonifier (Kebo Lab, Sweden), before adding 0.1mg/mL of DNase and $MgCl_2$ to a final concentration of 10mM.

Next the cell lysate was centrifuged at 12000g for 30 minutes; the resulting supernatant was filtered through a 0.45µ cartridge (Sarstedt, Nümbrecht, Germany) after which 5M NaCl was added to the filtrate culminating in a final salt concentration of 200mM. In order to bind the protein, 0.5mL of Glutathione-Sepharose resin equilibritaed in lysis buffer were next added to the filtrate with gentle stirring at 4˚C.

After 45 minutes the resin solution was thrice washed with lysis buffer, 10 protein fractions were then eluted each with 1mL of 10X elution buffer (50mM Tris-HCl pH8.0, 200mM NaCl, 1mM DDT, 0.02% Triton X-100 and 10mM reduced glutathione). The protein fractions were left in dialysis buffer (25mMTris-HCl ph 7.8, 100mM NaCl, 1mM EDTA, 1mM DDT, 10% glycerol, 0.2mM PMSF and 0.5mM Benzamidine) overnight at 4˚C. After dialysis proteins were concentrated using micron YM-30 filters (Millipore), protein concentration was measured and aliquots were flash frozen in liquid Nitrogen before storage at -80˚C for later use.

3.6.3 Electrophoresis of protein fractions

SDS-polyacrylamide gel electrophoresis was performed at 20Amps in a glass encased gel consisting of a 10% resolving gel (30% Acrylamide/bisacrylamide mix, 1.5M Tris pH8.8, 10% SDS, 10% Ammonium persulfate, Temed and water) and a 1.5 cm stacking gel (30% Acrylamide/bisacrylamide mix, 1M Tris pH6.8, 10% SDS, 10% Ammonium persulfate, Temed and water). To 16µL of each protein fraction, 5X SDS loading buffer (250mM Tris-HCl pH6.8, 10% SDS, 30% Glycerol, 5% β-mercaptoethanol, & 0.02% bromophenol blue) was added. The samples were boiled at 95˚C for 5 minutes and loaded for electrophoresis together with a Dalton VIIL molecular marker (Sigma).

After electrophoresis, the gel was incubated in coomassie brilliant blue (CBB) for 30 minutes. It was then repeatedly washed with destaining buffer (20% methanol and 7% acetic acid) until the protein bands were clearly visible.

3.7 *In-vitro* SUMOylation

The *in-vitro* assay was performed using a SUMOylation kit (Enzo, NY, USA). Four separate reactions were set up in 1.5mL Eppendorf tubes as shown in table 2. The reaction tubes were then incubated on a heating block at 30˚C, after 1hr the reaction was stopped by adding 60µL of stop buffer (1X PBS, 25mMEDTA and 0.2mg/mL BSA) to each tube. 10µL of GST-magnetic beads were then added to each tube to bind the proteins, the reaction was conducted at 4˚C with gentle shaking. Next the magnetic beads in each tube were thrice washed with 150µL of wash buffer (1X PBS and 0.1mg/mL BSA) followed by protein elution with 35µL of 1X SDS sample buffer. The samples were boiled for 5 minutes at 95˚C.

Table 2: Composition of reactions used for the *in-vitro* SUMOylation assay

Reactants	Reaction content (uL)			
	wt	wtØ	mut	mutØ
10X SUMOylationBuffer	2	2	2	2
20X MgATP	1	2	1	1
E1	1	-	1	-
E2	1	-	1	-
SUMO1	1	1	1	1
GST_RBR_wt	1.5	1.5	-	-
GST_RBR_mut	-	-	0.5	0.5
H_2O	17.5	14.5	13.5	15.5

**wt:- wild type, mut:- mutant and Ø:- negative control

3.7.1 Electrophoresis and western blot analysis

SDS-polyacrylamide gel electrophoresis was performed in a glass encased gel consisting of an 8% resolving gel and a 1.5 cm stacking gel. 15uL of each sample were loaded into the gel wells together with 5uL of protein molecular marker (All blue).

After electrophoresis, proteins were electrophoretically transferred to a 6.5 by 9 cm PVDF membrane (Millipore, MA, USA) in transfer buffer (50mM Trisbase and 50mM Boric acid) overnight at 4˚C in a trans-blot apparatus (Bio-Rad).

Subsequent to overnight protein transfer, the PVDF membrane was stained with Ponceau S solution for 1 minute to detect protein bands after which it was destained using 1% acetic acid. Next the membrane was incubated in blocking solution at room temperature, after 2hrs it was rinsed with 1X TBST (50mM Tris-pH8.0, 150mM NaCl and 0.05% Tween) and re-incubated in 4mL of blocking solution containing an anti-RBR antibody for 2hrs. This was followed by re-incubation with a peroxidase-conjugated rabbit anti-chicken (IgG) for 1hr.

After antibody incubation, the membrane was washed with 25mL of TBST; 25mL blocking solution diluted with TBST, 25mL of TBST and lastly with Milli-Q water each wash lasting 10 minutes. Finally, the immunoreactions present on the PVDF membrane were developed onto x-ray film using enhanced chemiluminescence (ECL, Bio-Rad).

CHAPTER FOUR

RESULTS

4.1 Mutation of *Arabidopsis* RBR1 SUMO modification site

Modification by SUMO occurs at the ΨKXE sequence motif which is present in almost all substrate proteins. The motif is made up of a large hydrophobic amino acid (Ψ), a Lysine (K) which is modified, an amino acid residue (X) and a glutamic acid denoted E (Johnson, 2006). Hence in order to study the effect of SUMOylation on the Retinoblastoma related protein (RBR1), a mutation was introduced in the nucleotides coding for the Lysine amino acid at the SUMOylation site.

Alignment of C-terminal sequences (Fig. 3a) of RBR proteins from different plants shows the presence of the SUMOylation motif (ΨKXE). The *Arabidopsis* RBR1 protein carrying a mutation at the SUMOylation site was amplified by PCR-site directed mutagenesis resulting in a 720bp fragment (Fig. 3b, Lane 2).

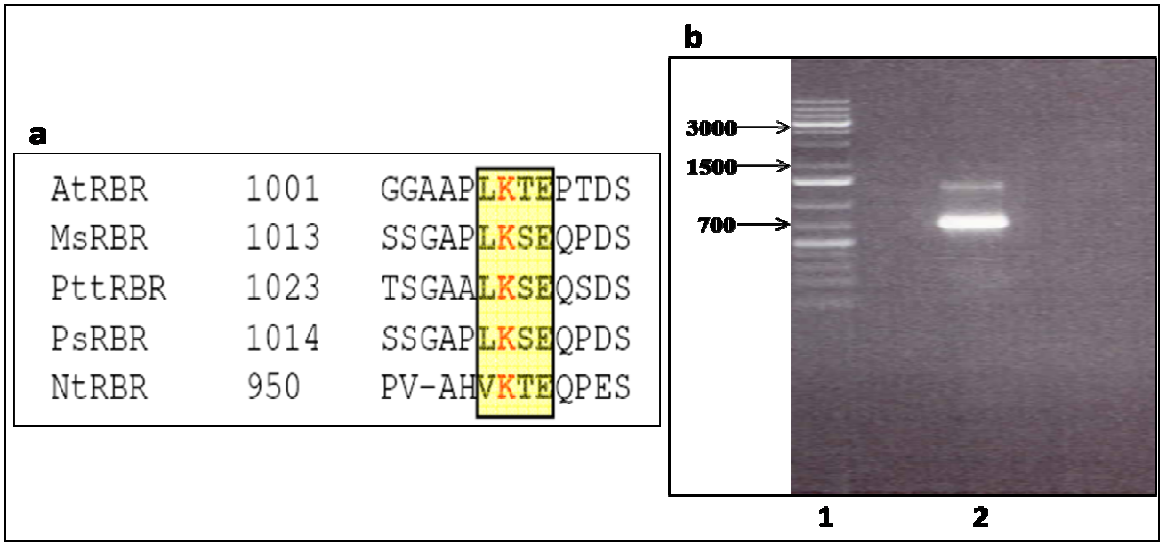

Figure 3 (**a**) Alignment of C-terminal sequences of different plant RBR proteins showing the presence of a SUMOylation motif (ΨKXE). (**b**) 720bp RBR1 fragment (Lane 2) with a mutated SUMOylation site amplified by PCR, in lane 1 is the 1Kb DNA ladder.

4.2 Effect of SUMO modification on RBR1 localization

In order to assess the effect of SUMO modification on RBR1 localization, *Arabidopsis* protoplasts were transfected with plasmid DNA coding for wild type RBR1 and RBR1 carrying a mutated SUMOylation site. Both constructs contained the green fluorescent protein to aid in protein localization.

In protoplasts transfected with wild type DNA, the protein was exclusively localized in the nucleus (Fig.4; a-c). However, protoplasts transfected with the protein carrying a mutated SUMOylation site, localization was not exclusive to the nucleus but also in the cytosol (Fig.4; d-f).

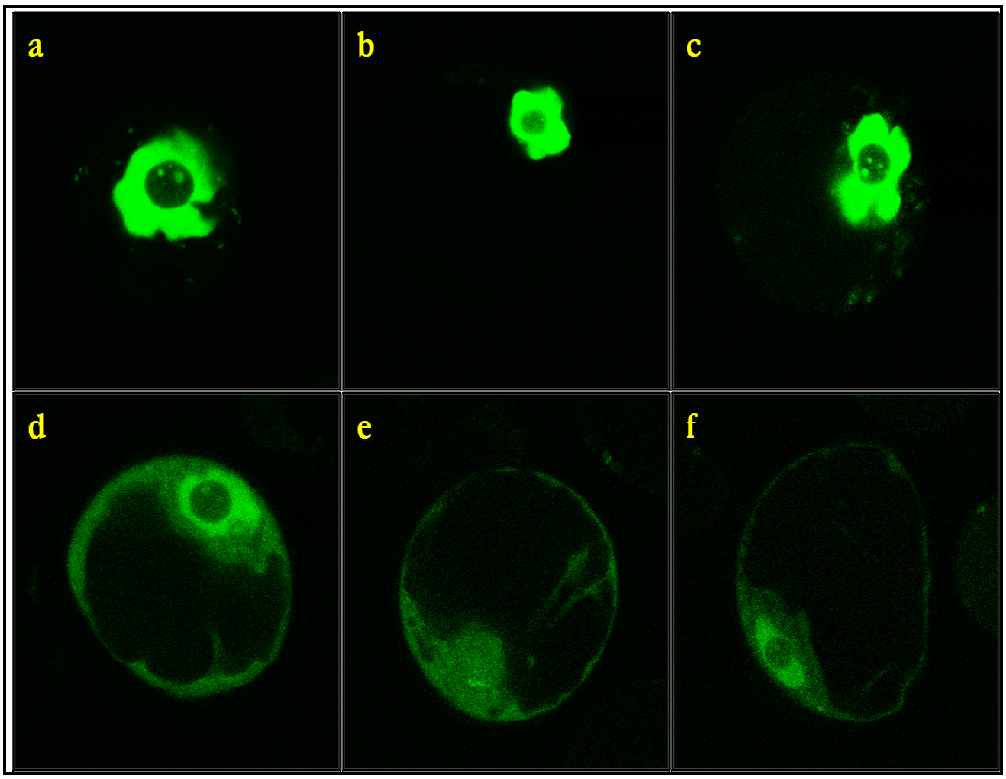

Figure 4 Effect of SUMO on RBR1 localization in *Arabidopsis* protoplasts, 14hrs after transformation. (**a-c**) In protoplasts transformed with the wild type protein (wtRBR1-GFP), the green fluorescent protein is exclusively localized in the nucleus. (**d-f**) Transformants with RBR1-mtSUMO containing a mutated SUMOylation site however show that the GFP is not exclusive to the nucleus but also in the cytosol.

4.3 Expression and Purification of protein RBR1-mtSUMO

To assess the effect of mutating Lysine 1007 on RBR1 SUMOylation, the amplified PCR product (RBR1-mtSUMO) carrying a mutated SUMO modification site was sub cloned into a glutathione-s-transferase (GST) gene fusion vector (pGEX5X-1) for protein expression. Cell pellets were obtained from LB media before and after induction of protein expression. After induction of protein expression with IPTG, RBR1-mtSUMO (52KDa) was clearly expressed (Figure 5a, Lanes 3 and 5). The protein was subsequently purified using glutathione Sepharose resins (Figure 5b, Lanes 3 and 4).

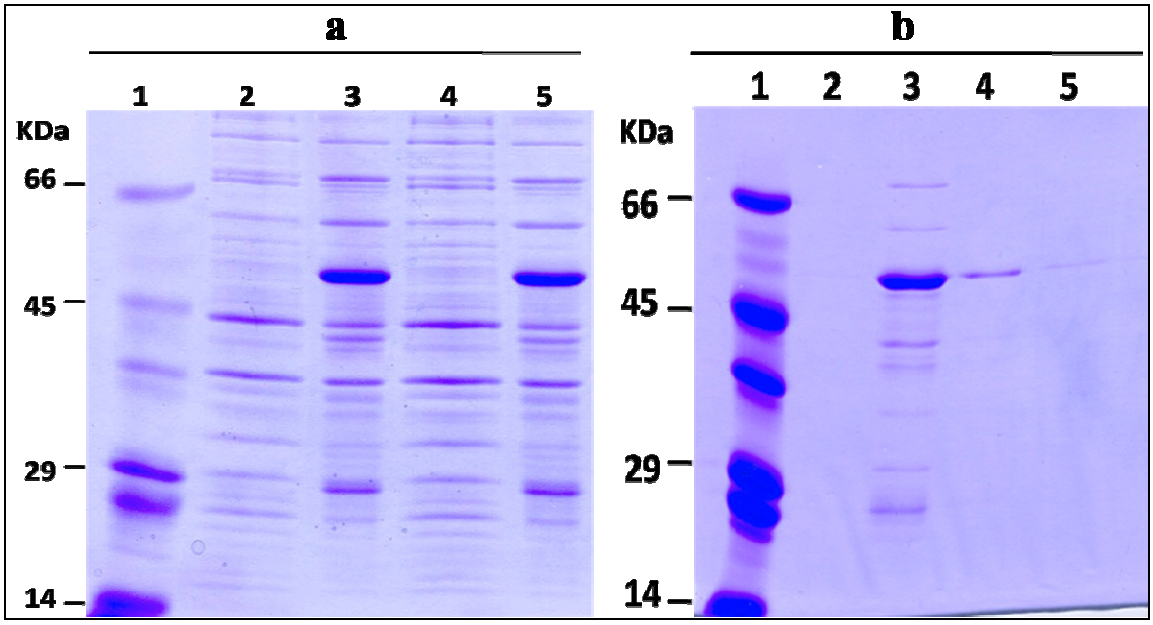

Figure 5 Protein expression and purification. (**a**) Expression of the protein RBR-mtSUMO; Lanes 2 and 4 are from samples before induction of protein expression while lanes 3 and 5 are samples taken after induction showing the expressed protein ~52KDa. (**b**) Protein purification; Lanes 3 and 4 show the purified protein. In both figures, lane 1 contains the molecular weight marker.

4.4 Mutation of Lysine 1007 prevents modification of RBR1 by SUMO

The purified protein was tested using an *in vitro* SUMOylation assay kit to determine if it could be modified by SUMO. The assay contained RBR1-mtSUMO or RBR1-wt, SUMO precursor, E1 enzyme, E2 enzyme and Mg-ATP. Figure 6a, shows protein transfer on to the PVDF membrane after staining in Ponceau S solution for 1 minute. When the wild type protein was incubated with all the components of the SUMOylation assay (Fig 6b, Lane 2), a SUMO modified protein was observed slightly less than 75KDa. On the contrary, the wild type protein which was incubated in the assay lacking the E1-activating and E2-conjugating enzymes was not modified (Figure 6b, Lane 1). Even in the presence of all assay components, the protein carrying a mutated site could not be SUMOylated (Figure 6b, Lane 4). The same result was observed when the mutated protein was incubated in the assay lacking the activating and conjugating enzymes (Figure 6b, Lane 3).

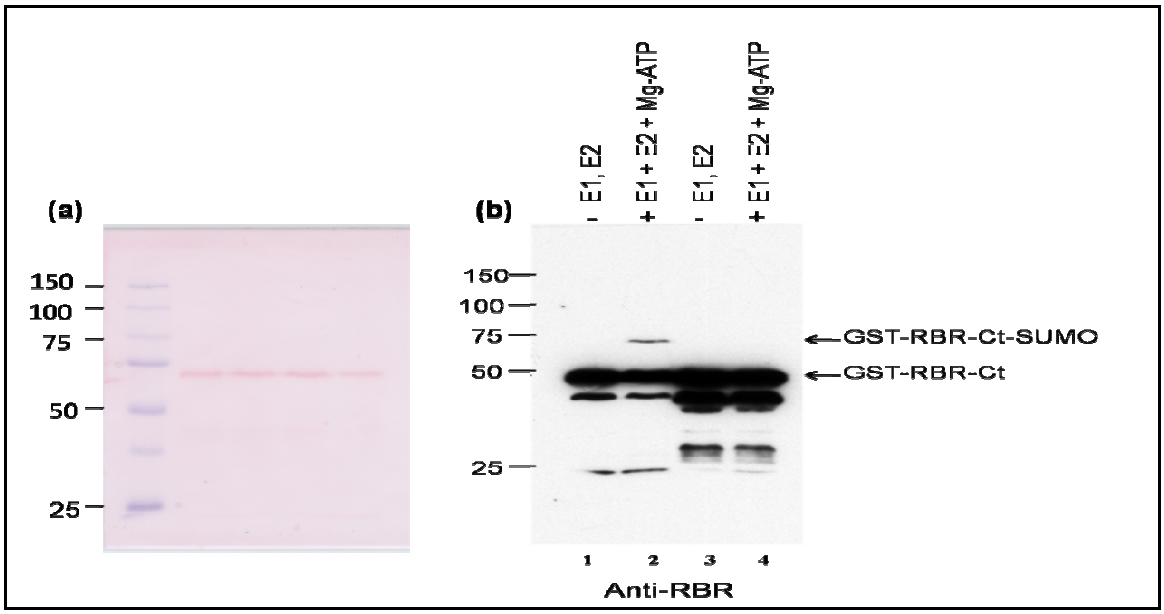

Figure 6 Ponceau staining and *in-vitro* SUMOylation. (**a**) Ponceau staining of the PVDF membrane showing protein transfers (**b**) *In-vitro* SUMOylation; Lane 1 contained the wild type protein (wt-RBR1) in the absence of the E1 and E2 enzymes. Lane 2 had the

wild type protein in the presence of all assay components; the protein was subsequently modified to yield the protein (GST-RBR-Ct-SUMO). Lane 3 contained the mutated protein negative control lacking E1 and E2 enzymes while Lane 4 had the mutated protein with all assay components.

Both the negative and positive controls (Figure 6b, Lanes 3 and 4) of the protein carrying a mutation were not SUMOylated, implying that the mutation prevented modification.

CHAPTER FIVE

DISCUSSION

5.1 Discussion

Before 1997, ubiquitin was perhaps the most known polypeptide to be involved in post translational modification of proteins (Marx, 2005). However a number of other polypeptides have since been indentified SUMO being among them (Kurepa *et al.,* 2002). Substrate proteins contain a characteristic ΨKXE sequence motif specific for SUMOylation; mutation of the lysine within the motif has been shown to affect modification and localization of RanGAP1 a nuclear transport protein in animals (Matunis *et al.,* 1998).

Alignment of the *Arabidopsis* retinoblastoma related protein (RBR1) sequence with other plant sequences, revealed the presence of the unique SUMOylation motif in all sequences. Hence in this study Lysine 1007 located within the motif was mutated in order to address the effect of SUMO modification on RBR1. The outcome of the mutation was highlighted by restricted localization of the wild type protein in the protoplast nucleus while the protein carrying a mutation was seen to be present in both the cytosol and the nucleus.

Johnson (2004) noted that in addition to sub cellular localization of proteins, modification by SUMO alters protein to protein interaction and protein interaction with other substrates. Hence localization of the wild type protein in the nucleus observed in this study could most likely be a post SUMO modification effect caused by; interactions between SUMO (the modifier) and nuclear proteins resulting in sub cellular localization of the modified wild type protein.

Additionally as noted by Gill (2005), modification of transcription factors by SUMO enhances their interactions with proteins that would normally have less or no interaction with the unmodified protein. Hence there is still a likely possibility that after modification of RBR1, there is an onset of interactions between the modified protein and nuclear proteins resulting in the eventual sub-cellular localization of the protein.

Alternatively it is also possible that once sumoylated, the wild type protein undergoes conformation changes that could expose or conceal certain binding sites. These changes in protein conformation could play a major role in facilitating eventual localization of the protein in the nucleus. Unlike the wild type protein, the absence of these SUMO induced effects on the mutated protein could be the cause of its presence in the cytosol.

It is however important to highlight the fact that, a fatal error was noticed during the course of the study. The delivered forward primer which was used for site directed mutagenesis deviated from the native sequence as it contained a point mutation in form of a missing T nucleotide. Putting that in consideration it is likely that the construct GFP-RBR1-mtSUMO had an additional mutation instead of the required one at the modification site. This without doubt results in a truncated protein, unable to be sumoylated and hence ending up in the cytosol giving the impression that it's the unmodified full length protein.

Having rectified the primer problem prior to protein expression, the results from the *in vitro* SUMOylation assay did clearly show that the wild type protein was SUMOylated. However in the absence of an acceptor lysine, the protein RBR1-mtSUMO could not be

SUMOylated. This result is similar and in agreement with results obtained from a related study by Matunis *et al.,* (1998) where they showed that, substituting the acceptor lysine for arginine in RanGAP1 prevented modification of the protein by SUMO.

5.2 Conclusions

In conclusion, transfection results from this study do not provide undisputed confirmation that modification by SUMO affects sub-cellular localization of RBR1. However, the *in-vitro* study did certainly confirm that, the plant retinoblastoma-related protein can't be modified by SUMO in the absence of the acceptor lysine contained in its modification site.

Additionally it can also be concluded that failure of the mutated protein to undergo modification, means that the C-terminal of RBR1 contains a single SUMOylation site.

5.3 Future Direction

Though protein localization seen in this study is in agreement with results from related mutational studies, the first course of action would be to re-transform protoplasts with a corrected construct in order to confirm that the mutated protein is indeed localized in both the nucleus and the cytosol unlike the wild type protein.

In order to confirm the presence of the transiently expressed GFP-tagged protein, it can be extracted from the transfected cells and subjected to immuno blot analysis with GFP and RBR1 specific antibodies.

It would also be interesting to do a structural study to get a clear picture of the structures of the wild type protein, its SUMO modified form and the mutated protein using studies such as nuclear magnetic resonance (NMR) or X-ray crystallography. This would shade more light on the occurrence of SUMO induced conformational changes.

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