Molecular Cloning and Functional Characterization of *Brassica* *UBC13* Genes

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By

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ABSTRACT

Lysine63 (K)-linked polyubiquitination of target proteins is a fundamentally different process from conventional K48-linked polyubiquitination that targets proteins for degradation via the 26S proteosome. Lys63-linked polyubiquitination regulates numerous cellular processes. The unique feature of Ubc13 compared to other ubiquitin-conjugating enzymes (Ubcs) is its ability to form a stable complex with a Ubc-E2 variant (Uev), which promotes the formation of Lys63-linked polyubiquitination. Ubc13 functions in DNA damage tolerance in budding yeast and is involved in several pathways in mammalian cells. *Arabidopsis* contains two *UBC13* genes and four *UEV1* genes that are involved in various developmental processes and stress responses including DNA damage response, root development and immunity. Recent studies imply that AtUbc13s contribute to plant susceptibility against soil-borne pathogen such as clubroot, a major disease in *Brassica napus*. However, there is no published information regarding characterization of *B. napus* Ubc13s (BnUbc13s). This project aims to understand functions of Ubc13 and Ubc13-Uev1 complexes in canola. As canola is a polyploid and often contains many homologous genes, this study aims to provide guidelines to selectively target a subset of homologous genes by gene editing to protect from clubroot disease. Twelve *BnUBC13* genes were identified through genomic data analysis, eight of which encode proteins different from AtUbc13s were cloned and characterized. All eight BnUbc13s were able to physically interact with AtUev1 to form stable complexes. Furthermore, *BnUBC13* genes functionally complemented the yeast *ubc13* null mutant defects, suggesting that *BnUBC13*s can replace yeast *UBC13* in DNA damage tolerance. Furthermore, a CRIPSR/Cas9 construct was designed to simultaneously target five *BnUBC13* genes and was used to transform *B. napus* cv. Westar (DH12075). Twenty-eight out of thirty regenerated lines were found to contain homozygous or heterozygous mutations in 5 targeted *BnUBC13* genes, validating our genomic editing approach in canola. In addition, *BnUBC13* transcript levels in resistant and susceptible canola before and after clubroot infection were analyzed based on the in-house RNA-seq data and were found to not fluctuate drastically. This study provides convincing data to support notions that *B. napus* Ubc13s promotes Ly63-linked polyubiquitination, that BnUbc13s are involved in error-free DNA damage tolerance and that *BnUBC13*s are housekeeping genes.

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LIST OF ABBREVIATIONS

At	*Arabidopsis thaliana*
3-AT	3-aminotriazole
a.a.	amino acids
AD	activation domain
Ade	adenine
amp	ampicillin
ATP	adenosine 5'-triphosphate
Bn	*Brassica napus*
BD	binding domain
BLAST	basic local alignment search tool
bp	base pair
Da	Dalton
dd	double distilled
DDT	DNA damage tolerance
DMSO	dimethyl sulfoxide
E1	ubiquitin activating enzyme
E2	ubiquitin conjugating enzyme
E3	ubiquitin ligating enzyme
EDTA	ethylenediaminetetraacetic acid
Glu	glutamate
Gly	glycine
GST	gluthathione-S-transferase
h	human
HECT	homology to the E6-AP carboxyl terminus
His	histidine
Ile	isoleucine
IκB	Inhibitor of κB protein kinase
IKK	IκB kinase

IPTG	isopropyl-β-D-thiogalactopyranoside
K	lysine
LB	Luria broth
Leu	leucine
MMS	methyl methanesulfonate
MW	molecular weight
nt	nucleotide
NEMO	NF-κB essential modulator
NF-κB	nuclear factor kappa B
OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
PRR	post replication repair
PEG	polyethylene glycol
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PVDF	polyvinylidene difluoride
RING	really interesting new gene
SD	synthetic dextrose
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Sg	single guide
TLS	translesion DNA synthesis
UV	ultraviolet
Wt	wild type
y	yeast (budding)
YPD	yeast extract-peptone-dextrose
TRAF2	tumor necrosis factor receptor-associated factor 2
TRAF6	tumor necrosis factor receptor-associated factor 6
Ub	ubiquitin
Ubc	ubiquitin-conjugating enzyme
Uev	ubiquitin-conjugating enzyme variant

CHAPTER 1: INTRODUCTION

1.1 Ubiquitin and ubiquitination process

Ubiquitin (Ub) (Figure1-1) is a highly conserved protein composed of 76 amino acids and is prominent in eukaryotes. Ub is one of the most highly conserved proteins from yeast to humans. Its amino acid sequence is identical among animals, while *Saccharomyces cerevisiae* Ub differs by only three amino acids from the animal form (Ozkaynak et al., 1987). It is well known for its ability to target a short-lived protein for regulated degradation by a large intracellular protease known as 26S proteosome (Hershko and Ciechanover, 1998). Ubiquitin is able to conjugate with other proteins by forming an isopeptide bond using its C-terminal carboxylate and eventually produces a monoubiquitinated substrate (Komander and Rape, 2012). Ub has two key features. One is that the carboxyl group of the last C-terminal glycine residue (Gly76) can form an isopeptide bond with a substrate lysine residue. Another is that seven lysine residues in Ub, i.e. Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63 (Figure 1-2) can be potentially used to form distinct types of poly-Ub chains, at least five of which have been observed *in vitro* or *in vivo* (Volk et al., 2005). Multigene family including monomeric and multimeric *UB* genes encodes Ub. Monomeric *UB* genes encode Ub monomers that fuse to one of two ribosome proteins (Callis, 1995), while multimeric *UB* genes encode N-to-C linked Ub repeat polypeptides (Ozkaynak et al., 1987) that are subsequently cleaved to form Ub monomers. In addition, Ub can exist as a free cellular monomer or covalently is covalently attached to other proteins. Cellular Ub homeostasis is maintained by two means: either from newly synthesized polyproteins by proteolysis or by recycling Ub molecules linked to other proteins (Kalderon, 1996).

Ubiquitination, the attachment of Ub to targeted proteins, regulates diverse processes such as proteosomal and lysosomal degradation, subcellular localization (Kerscher et al., 2006), DNA damage response (Jentsch et al., 1987; Pastushok and Xiao, 2004), ribosomal biogenesis (Finley et al., 1989), cell cycle progression (Harper, 2002), apoptosis (Zhang et al., 2004), mitochondrial inheritance (Fisk and Yaffe, 1999) and transcriptional regulation (Kao et al., 2004). Ub conjugated in the target protein can alter the protein stability, localization or activity (Dorval and Fraser, 2007).

The ubiquitination (Figure1-3) requires three basic enzymatic activities, E1, E2, and E3, which work in concert to transfer Ub to client substrates and to form poly-Ub chains. Firstly, high energy thioester bond is formed with the C terminus of Ub by a Ub activating enzyme (Uba or

E1). The Ub molecule is then transferred to the active-site Cys of a Ub-conjugating enzyme (Ubc or E2). The subsequent transfer of Ub to the ε-amino group of a Lys side chain within the substrate is catalyzed by the Ub-charged E2 after binding to an E3 ligase. Poly-Ub chain is formed through sequential ubiquitination cycles by ligating additional Ubs to the initial Ub molecule. Ub can be conjugated to itself via specific Lys residues, resulting in diverse types of chain linkages. Substrate degradation is mainly carried out by the linkage through Lys48. 26S proteosome degrades protein substrates carrying Lys48-linked poly-Ub chains (Cohen et al., 2015). Substrate specificity of ubiquitination is mainly determined by the interaction of E2 and E3. Formation of poly-Ub chains is thought to be essential for targeting the Ub-tagged protein to the 26S proteosome (Eytan et al., 1989).

Only one E1 is found in many organisms while deletion of that gene in yeast becomes lethal (McGrath et al., 1991). Two E1 isoforms have been found in human cells resulting from alternative translation initiation sites (Handley-Gearhart et al., 1994). Anyways, multiple E2 and E3 are available in almost all multicellular organisms. Plants known E2s contain a conserved catalytic core domain with the active site cysteine residue to form a thiolester bond with Ub (Pickart, 2001). Genomic analyses reveal that yeast, human and *Arabidopsis thaliana* have 11, 50 and 38 E2s. Individual E2s can interact with different E3s, and a single E3 might interact with more than one E2. The opportunity for the target protein to be recognized by the ubiquitination system is getting enhanced because of the diversity of relationship between E2s and E3s. E3s play a major role in recognition of substrate and, as a result, E3s differ in size and functional domains that are classified into three distinct families. HECT (homologous to the E6-AP carboxyl terminus) domain and RING (really interesting new gene) domain families are the two main subfamilies (Pickart, 2001). For the HECT-domain E3, Ub is first transferred from E2 to a highly conserved cysteine residue in the HECT domain, and then the Ub is conjugated to a lysine of the substrate bound to an E3. However, E3s of RING-domain subfamily directly transfer Ub from E2 to the target protein as thioester intermediate formation with Ub is not happening in E3s of the RING domain subfamily.

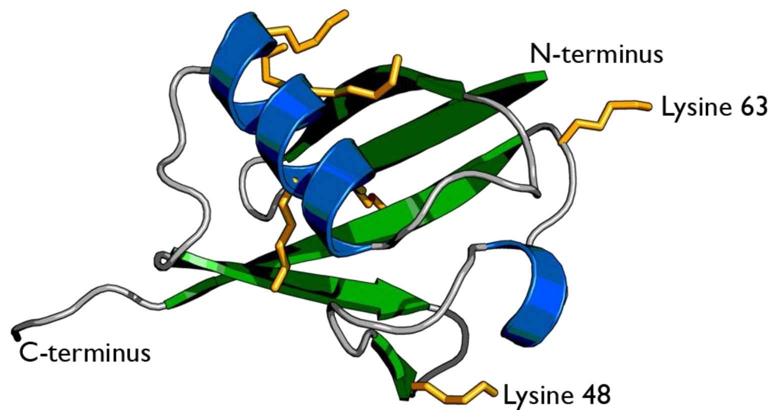

Figure 1-1: The structure of ubiquitin protein. Alpha-helices are coloured in blue and β-strands in green. The orange sticks indicate seven lysine residues. The two best-characterized lysine residues lysine 48 and 63 in polyubiquitin chain formation are marked. The image was taken from http://en.wikipedia.org/wiki/Ubiquitin.

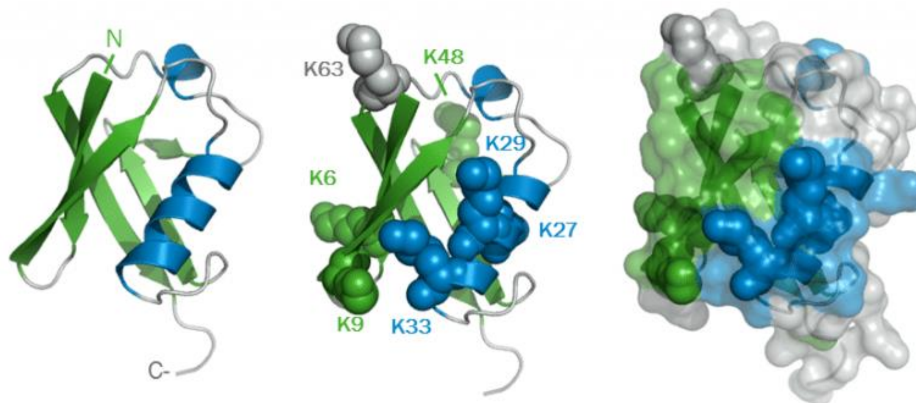

Figure 1-2: Models of Ub. Ub's seven lysine residues (K6, K9, K27, K29, K33, K48 and K63) are highlighted. The image was taken from http://flipper.diff.org/app/pathways/Ubiquitination

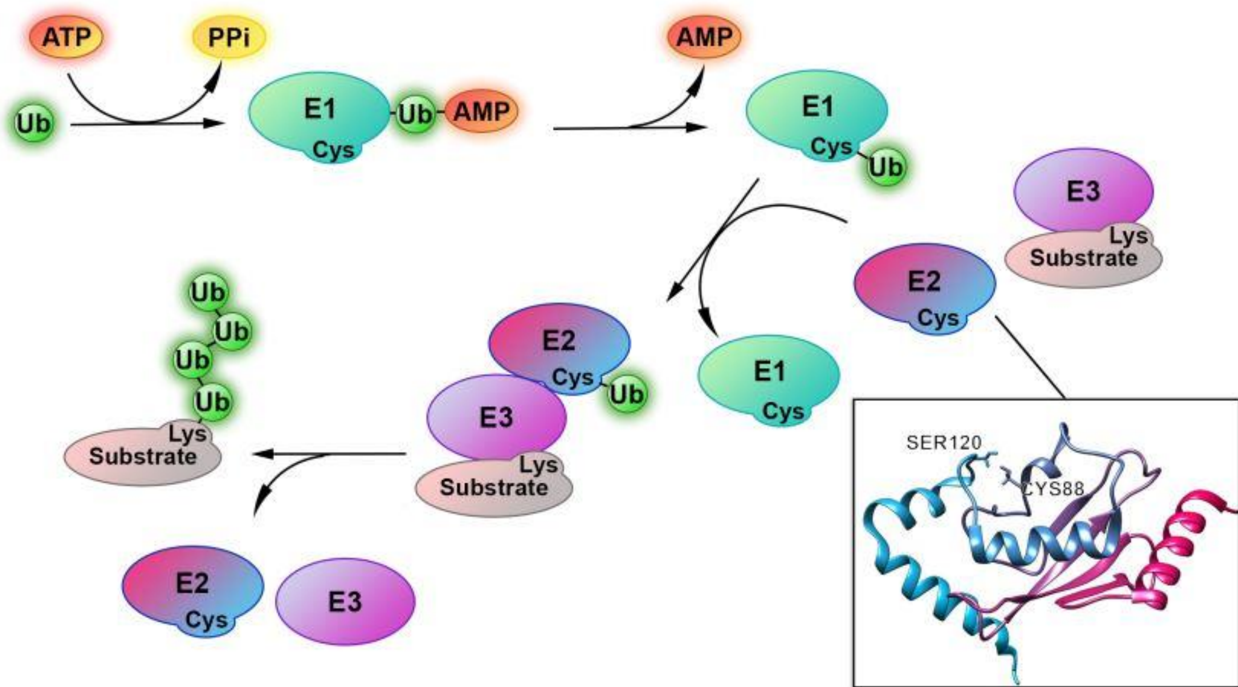

Figure 1-3: The ubiquitin (Ub) conjugation machinery employs three basic enzymatic activities, E1, E2, and E3, that work in concert to transfer Ub to target substrates and to form polyUb chains (Valimberti et al., 2015).

1.2 Functions and importance of Ub chains

The attachment of Ub molecule or chain of Ub molecules to a target substrate protein is an ATP dependent reversible process. There are three types of ubiquitination: mono-ubiquitination, multiple mono-ubiquitination, and poly-ubiquitination. Mono-ubiquitination is the attachment of a single Ub to a substrate (Hicke, 2001). Multiple mono-ubiquitination is when two or more lysine residues in a substrate are appended with single Ub molecules (Haglund et al., 2003; Mosesson et al., 2003), while attachment of a chain of Ub, formed by a repeat process to a substrate is known as poly-ubiquitination (Pickart, 2001). Poly-Ub chains can be formed by each of seven lysine residues found in the Ub surface, as observed in budding yeast (Peng et al., 2003). In addition, linear Ub chain or Met1-linked Ub chain (M1 chain) is generated by attaching Ub to the N terminus of the proximal Ub (Iwai et al., 2014). The length of the Ub oligomer(s) and the configuration of Ub-Ub linkages in the Ub chain decides the fate of a Ub-protein conjugate. Binding of the modified protein to the 26S proteosome is efficiently promoted by chains of four

or more Ubs, in which the C-terminus of one Ub is attached to Lys48 of the next Ub (Pickart, 2001).

Different Ub modifications can play different roles in the regulation of cellular processes (Weissman, 2001). For example, chromatin structure and transcription are regulated by Histone H2B mono ubiquitination that leads to methylations on another core histone H3 (Briggs et al., 2002). Receptor endocytosis and degradation is promoted by membrane receptor mono-ubiquitination in vacuole (Bonifacino and Traub, 2003). Moreover, endocytosis of plasma membrane proteins, sorting of proteins to multivesicular bodies (MVB), DNA repair, histone activity, and transcriptional regulation are some of the processes that protein mono-ubiquitination gets involved in (Gupta-Rossi et al., 2004). Cbl-mediated mono-ubiquitination at several sites (multi-ubiquitinated) modifies the epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR), which is significant, adequate and compulsory for endocytosis and receptor degradation (Bakowska et al., 2007). Targeting proteins for degradation by the 26S proteosome is mediated by Lys48-linked poly-Ub chains. Poly-Ub chains formed via Lys6-linkage on BRCA1 is involved in the formation of nuclear focus for DNA repair (Morris and Solomon, 2004). Protein kinase activation and lysosomal localization of Jun requires Lys27 linked poly-Ub chain (Ikeda and Kerppola, 2008; Okumura et al., 2004), while its proteosomal targeting and protein turnover requires Lys11-linked poly-Ub chain (Baboshina and Haas, 1996; Johnson et al., 1995). Finally, Lys63-linked chains have been implicated in DNA repair and NF-κB activation (Dwane et al., 2017; Zhang et al., 2001a).

The decision taking on mono-ubiquitination or poly-ubiquitination by the ubiquitination machinery of the substrate is unclear. Many realizable explanations are available. Firstly, there are specificities in terms of the type of ubiquitination in E3 ubiquitin ligase. For example, PCNA mono-ubiquitinaton at the Lys164 residue is mediated by E3 Rad18 whereas PCNA poly-ubiquitination at the same site is promoted by E3 Rad5 (Hoege et al., 2002). As another example, mono-ubiquitination and poly-ubiquitination of p53 are mediated by E3s Mdms2 and p300, respectively (Grossman et al., 2003). Secondly, Ub modification type is specified by Ub-binding proteins. For example, mono-ubiquitination is controlled by Ub-interacting motif (UIM) and the Cue1-homologous (CUE) domain in the endocytic proteins (Di Fiore et al., 2003). Thirdly, the decision of mono-ubiquitination or poly-ubiquitination of a substrate is specified by Ubcs. For example, to date the only recognized E2 enzyme capable of catalyzing Lys63-linked poly-Ub

chains is Ubc13, which is believed to play a vital role in cell signaling. Studies have identified an E2 enzyme, UBE2S/E2-EPF, that assembled Lys11 linkages in vitro (Baboshina and Haas, 1996). Short Lys11-linked chains get extended into long Lys11-linked ubiquitin polymers on APC/C (anaphase-promoting complex/cyclosome)-bound substrates by the Lys11-specific 'elongating' E2 enzyme UBE2S (Garnett et al., 2009; Williamson et al., 2009; Wu and Karin, 2015). Single substrate-binding event takes place when ubiquitin chain extends with up to 13 ubiquitin molecules (Wickliffe et al., 2011) and the proteosome degrades subsequently released substrates rapidly. Lys48-linked poly-ubiquitination for protein degradation is mediated by other E2 enzymes such as Ubc4 and Ubc5 in yeast and Ubc8, Ubc9 and Ubc10 in *A. thaliana* (Kraft et al., 2005). Moreover, Ub removal from substrates is mediated by de-ubiquitinating enzymes (DUBs) as ubiquitination is considered as a dynamic and reversible process (Wilkinson, 2000). Hence, homeostasis of selected substrate mono- or poly-ubiquitination is regulated by a balance between DUB and ubiquitination machinery (Haglund et al., 2003).

1.3 The structure of Lys48- and Lys63-linked poly-Ub chains

It is well known that protein degradation by 26S proteosome is carried out mainly by the Lys48-linked poly-Ub chains. As opposed to Lys48-linked poly-Ub, Lys63-linked poly-Ub is involved in a variety of cellular events independently of degradative signaling (Weissman, 2001). Most importantly, the difference in cellular functions is caused obviously with regard to the linkage-specificity of poly-Ub chains. The question why Lys63-linked and Lys48-linked poly-Ub chains have very different functions is answered with their structures.

Distinct functions are probably because of the very different conformations formed by these two poly-Ub chains as revealed by structural and modeling analyses. The presence of a hydrophobic patch is shown by the crystal structure of Lys48-linked di- and tetra-Ub. This hydrophobic patch is composed of Leu8, Ile44 and Val70 in each Ub molecule on interface between two subunits (Cook et al., 1992). A closed conformation is formed by Lys48-linked di-Ub as revealed by NMR studies. This conformation has a hydrophobic patch that is stacked at the interface at neutral pH, but the conformation is open at lower pH (Varadan et al., 2002). Lys48 poly-Ub chains have zigzag topology.

However, covalent hydrophobic interface is absent in Lys63-linked di- and tetra-Ub chains. An array of beads on a string resembles the conformation of Lys63-linked chains (Tenno et al.,

2004). The inter subunit interface of Lys63-linked chains lack the hydrophobic patch that is a major surface for proteosome to contact with Ub (Tenno et al., 2004). The topology of the poly-Ub chain is shown in Figure 1-4.

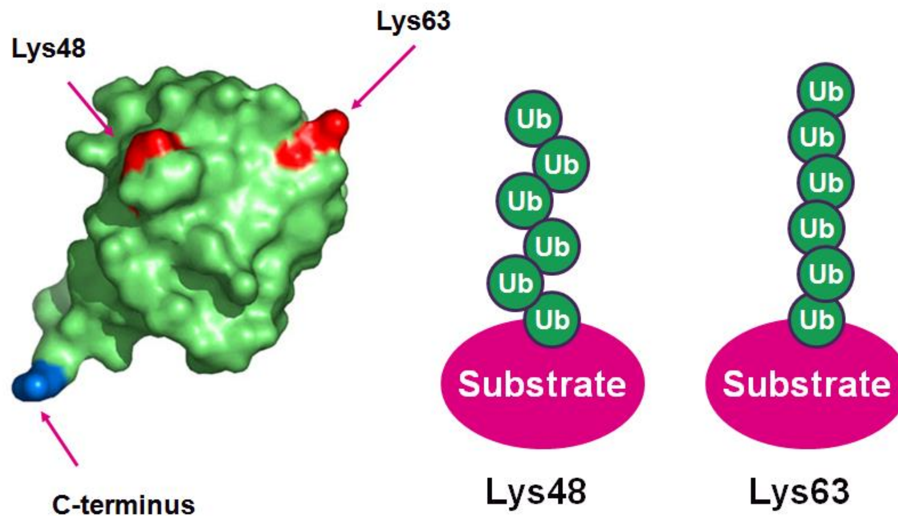

Figure 1-4: The structure and topology of Ub and poly-Ub chains. Red coloration shows the sites of Lys48 and Lys63, and blue coloration shows the C-terminus of Ub. Lys48-linked chain appears like zigzag, while Lys63-linked chain likes a string in their topologies.

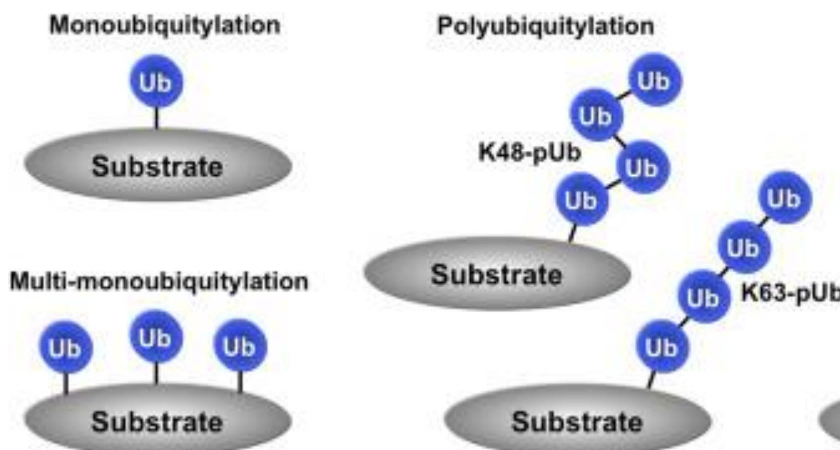

Figure 1-5: Different types of ubiquitination. Three general types of Ub modifications: mono-ubiquitination, multi mono-ubiquitination and poly-ubiquitination (Emmerich and Cohen, 2015).

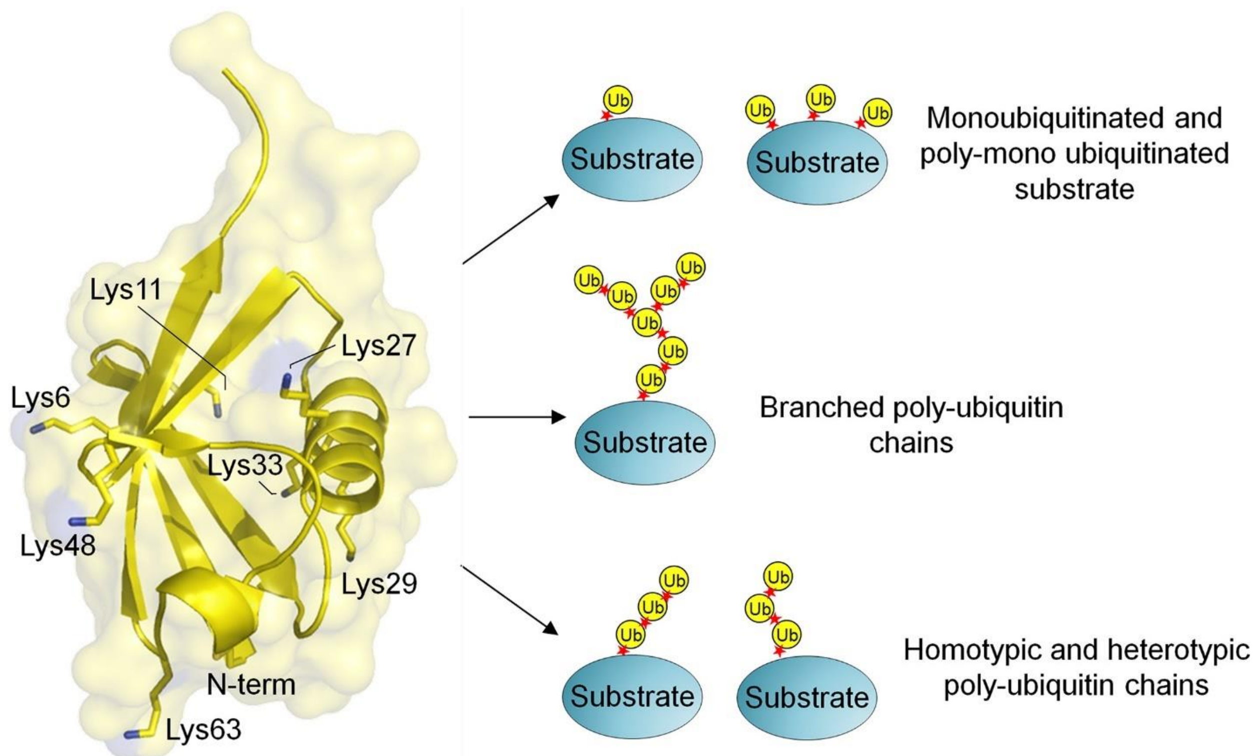

Figure 1-6: Ub structure. Ub modification is carried out through the seven lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63, rendered as sticks) and the N-terminal Met (Veggiani and Sidhu, 2019).

1.4 Functions of Lys48- and Lys63-linked poly-Ub chains

It is well-known that distinct structural and functional information is conveyed by poly-Ub chains bearing different linkages. In addition, a well-known fact that degradation of proteosome is targeted by the Ub chains linked by Lys48. In contrast, Lys63-linked chains represent non-proteolytic functions commonly in pathways including DNA damage repair, cellular signaling, intracellular trafficking, and ribosomal biogenesis.

The traditional view of the function of Lys48-linked polyubiquitination is that degradation of polyubiquitinated substrates can be targeted by a chain consisting of a minimum of four Ub moieties that can interact with the proteosome with high affinity (Thrower et al., 2000). Lys48 poly-Ub chains function predominantly as a marker for the proteolytic attack by the 26S proteosome (a eukaryotic ATP-dependent proteolytic complex) (Baumeister et al., 1998; Coux et al., 1996). Nevertheless, non-proteolytic functions for Lys48-linked polyUb chains have also been

reported. For example, budding yeast Cdc48 (also called p97) works together with characteristic cofactors to differentially act on numerous substrates conjugated with Lys48-linked poly-Ub chains (Jentsch and Rumpf, 2007; Ye, 2006). A Cdc48 complex recognize Lys48-linked poly-Ub chains and ATPase, which extracts ubiquitinated substrates from an immobilized cellular compartment or a large protein complex is activated. Degradation of misfolded proteins of the endoplasmic reticulum (ER)-associated degradation (ERAD) and a membrane-bound transcription factor Spt22 activation are some segregase functions of Cdc48 (Bays et al., 2001; Braun et al., 2002; Jarosch et al., 2002; Rabinovich et al., 2002; Rape et al., 2001; Ye et al., 2001). Poly-ubiquitinated substrates released from ER membrane are shuttled to the proteosome for degradation by ERAD. On the other hand, released Spt23 loses of most of its Ub conjugates and becomes stable in cells (Rape et al., 2001).

Lys48 linked polyubiquitination function in preventing self-pollinating in plants. The majority of plants produce flowers that contain both male and female reproductive organs and there is a chance of self-pollination if plants take no action (Kao and Tsukamoto, 2004). Self-pollination leads to gradual decrease in genetic diversity and as a results plants have evolved mechanisms known as self-incompatibility to prevent self-pollination and promote outcrosses (Gaudeul and Till-Bottraud, 2004). Rejection of the plants' own pollen is achieved by Lys48 poly-Ub chain mediated protein degradation. The plant Antirrhinum protein encoded by self-incompatibility gene interacts with S-RNase that is polyubiquitinated and degraded by proteosome in compatible pollination but not in incompatible pollination (Qiao et al., 2004). Another important function of Lys48-linked ubiquitination is the regulation of cell cycle. G1, S, G2, and M are the four distinct phases of a typical eukaryotic cell cycle (Bicknell and Brooks, 2008). A set of protein kinases called cyclin-dependent kinases (CDKs) regulate the transition from one cell cycle phase to the next one. Cyclins positively regulate CDK whereas CDK inhibitors negatively regulate CDK (Hochegger et al., 2008). Cyclins and CDK inhibitors are ubiquitinated and degraded by Lys48 poly-Ub chains to ensure the unidirectional and irreversible progression of the cell cycle (Santopietro et al., 2006). The G1/S transition is mediated by SCF E3 complex, composed of Skip1, Culin and F-box, to ubiquitinate and degrade G1/S cyclin and CDK inhibitors (CKIs) like p27 and (Nakayama and Nakayama, 2005). The metaphase-anaphase transition is mediated by a multimeric E3 anaphase promoting complex (APC) to boost sister chromatids separation (Vodermaier, 2004). In addition, Lys48 poly-Ub chain mediated protein degradation pathway

supports to degrade many other cell cycle regulators, such as Cyclin B or Plk1 (Stegmeier et al., 2007). Moreover, Lys48 poly-Ub chain mediated protein degradation is involved in auxin promoted lateral root formation. *A. thaliana* TIR1 is rich in leucine repeats and contains an F-box motif to serve as a key component of SCF^{TIR1}. TIR1 captures auxin signals and promotes degradation of Aux/IAA (indole-3-acetic acid) proteins, the repressors of auxin-responsive transcription, with the support of Lys48 poly-Ub chain-mediated proteosome pathway (Dharmasiri and Estelle, 2004; Gray, 2001). Aux/IAA degradation allows ARF (auxin response factor) binding to the promoters of many auxin-responsive genes, leading to the lateral root development (Guilfoyle et al., 1998; Tiwari et al., 2003).

Lys63-linked polyubiquitination is involved in numerous cellular events that do not depend on degradative signaling through the proteosome (Marx, 2002). A well-characterized example is Lys63-linked polyubiquitination of proliferating cell nuclear antigen (PCNA) in the DNA-damage response (DDR), which will be described in detail in Section 1.6.

Another important function in Ly63-linked polyubiquitinated is its involvement in the nuclear factor kappa-lighter-chain-enhancer of activated B cells (NF-κB) pathway. The NF-κB family of transcription factors is involved in many cellular processes such as stress-induced immunity, cell cycle progression, inflammatory response, oncogenesis, viral replication and various autoimmune diseases (Zhang et al., 2001b). NF-κB proteins in dimeric form are sequestered in the cytosol in cells through non-covalent interaction with IκBs proteins. NF-κB activating signals phosphorylate IκBs by an IκB kinase (IKK) complex, leading to their ubiquitination and subsequent degradation (Wegener and Krappmann, 2008). IKK is composed of IKKα, IKKß, and a regulatory subunit IKKγ/NEMO (NF-κB essential modulator). The nuclear localization signals (NLSs) on the NF-κB subunits are then exposed, resulting in the NF-κB nuclear translocation, binding to a consensus sequence (5'-GGGACTTTCC-3') of various promoters and activation of these genes. During this process, IKK and Jun amino-terminal kinase (JNK) are activated by a E3 protein TRAF6 (Tumor-necrosis factor (TNF)-receptor associated factor 6), which interacts with Ubc13 through its RING-finger domain (Wooff et al., 2004) to catalyze Lys63-linked poly-Ub chains (Deng et al., 2000a; Wang et al., 2001a). In addition, another RING-finger protein TRAF2-mediated NF-κB activation also requires Ubc13-Uev for Lys63-linked poly-Ub (Shi and Kehrl, 2003). It appears that the cellular target of the above Lys63 poly-Ub is NEMO in T- and B-cells and possibly in other cells as well (Zhou et al., 2004a). The

Lys63 polyubiquitination in the NF-κB pathway mediated by Ubc13-Uev1 is shown in Figure 1-7.

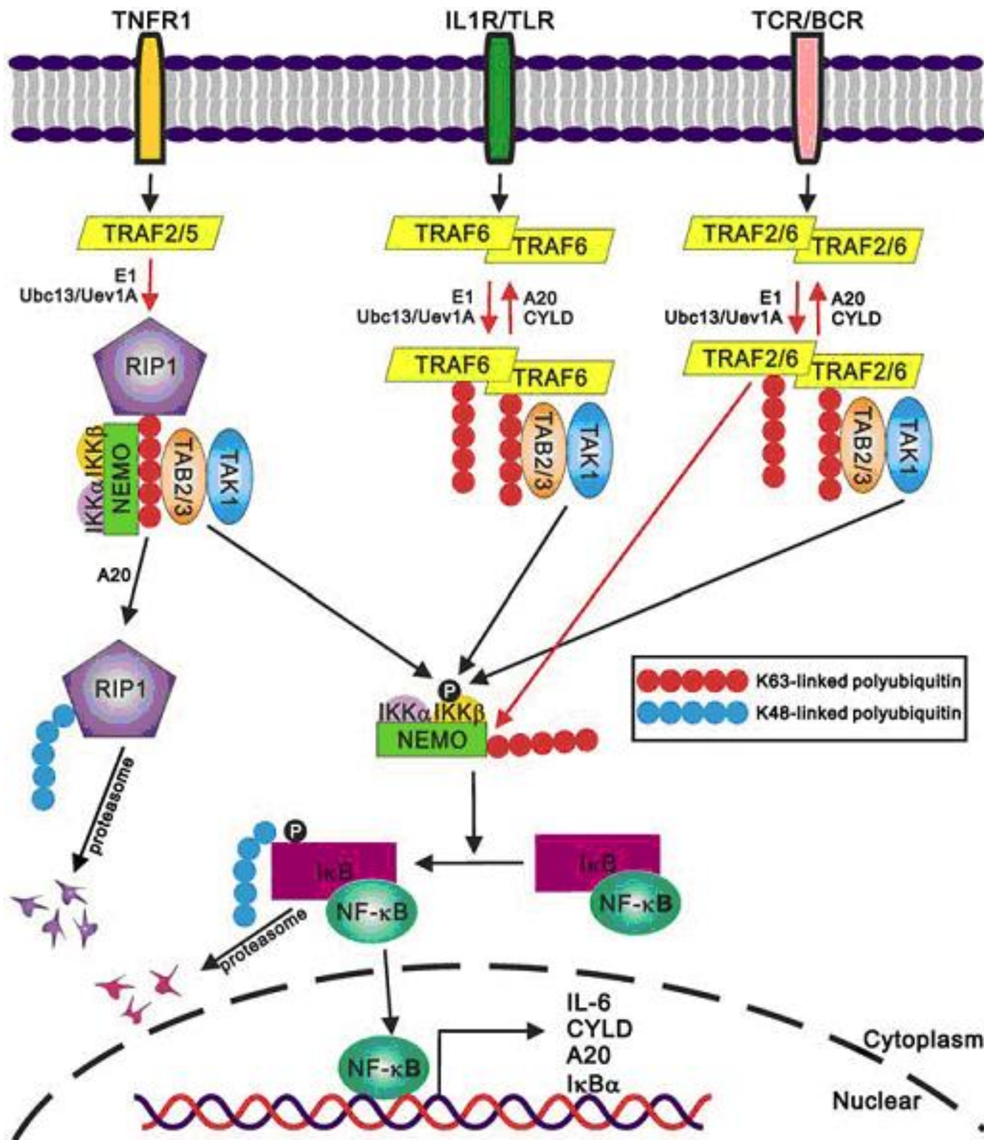

Figure 1-7: NF-κB transcription factor activation is promoted by the Ubc13-Uev1A mediated Lys63-linked polyubiquitination. Lys63-linked poly-Ub chain formation on substrate NEMO is catalyzed by a Ubc13-Uev1A complex together with TRAF2/5 or TRAF6. This process leads to phosphorylation and degradation of IκBs. After that, transcription of the corresponding genes is activated by the released and nucleus translocated NF-κB subunits (Adhikari et al., 2007).

Although some literature only reveals a few scattered reports on Lys63-linked ubiquitin chains being involved in proteosome-dependent degradation (Babu et al., 2005; Kim et al., 2007), some other reports believe that Lys63-linked polyubiquitination is equally competent in creating a destructive fate to substrates, although through autophagasom-mediated autophagy that is a proteosome-independent mechanism. Here, double membrane vesicles engulf damaged organelles or part of the cytosol, which subsequently fuses with lysosomes to degrade the sequestered contents (Reggiori and Klionsky, 2005). It helps to adapt cells to support with many stress conditions such as amino acid starvation. In addition, this removes misfolded protein aggregates and damaged organelles that are usually oversized to be taken cared by cellular proteases such as the proteosome in an effective way. Recent studies reveal that missing link between ubiquitin and autophagy is carried out by a protein termed p62 or sequestosome 1 (Li and Ye, 2008).

1.5 Ubc13 proteins

In eukaryotes, the only known Ub-conjugating enzyme that catalyzes the Lys63-linked polyubiquitination reaction is Ubc13, which is highly conserved in the evolution process. However, a Ubc-like, or Ubc/E2 variant (Uev) is also required to form a stable heterodimer with Ubc13 (Hofmann and Pickart, 1999). Uev can interact with an acceptor Ub noncovalently (McKenna et al., 2001) and orients its Lys63 residue proximal to the C-terminus of the donor Ub that binds to the active site of Ubc13 by a thioester bond (Lewis et al., 2006; McKenna et al., 2003). According to previous studies only one *UBC13* gene was discovered from yeast and mammal genomes, while two *UBC13* genes are present in *Arabidopsis* and Zebrafish genomes (Li et al., 2010; Li and Schmidt, 2010; Wen et al., 2006). A Uev protein is encoded by *MMS2* in budding yeast (Broomfield et al., 1998). Mms2 and Ubc13 cooperation is required for error-free DNA-damage tolerance (DDT) (Brusky et al., 2000; Hofmann and Pickart, 1999). It is plausible that the reason Ubc13 is dedicated to Lys63-linked polyubiquitination is due to its unique feature to bind a Uev (Pastushok et al., 2005).

1.5.1. Ubc13 functions in mammals

Regulation of both innate and adaptive immune responses in mammalian immune systems requires Ubc13-mediated Lys63 polyubiquitination. This includes the regulation of important processes such as signal transduction and activation of NF-κB, a key immunity regulator (Wu and

Karin, 2015). In addition, involvement of the Ubc13–Uev complex is detected in TRAF6-mediated regulation of IκB kinase (Deng et al., 2000a) and Bcl10/MALT-mediated Lys63-linked polyubiquitination of NEMO/IKK (Zhou et al., 2004a), both leading to NF-κB activation.

Ubc13 plays an important role in attaching target proteins with Lys63–linked poly-Ub chains, that are crucial for the immune receptor signals transmission culminating in activation of the transcription factor NF-κB. Recent studies have found, almost normal NF-κB activation is showed by Ubc13-deficient cells but considerably impaired activation of mitogen-activated protein kinase. Finally, Ubc13 plays a significant role in mammalian immune response (Yamamoto et al., 2006).

Furthermore, it was shown that suppression of synapse differentiation in the mammalian brain is operated by *UBC13* in concert with a E3 protein RNF8, bringing up a novel Ubc13-RNF8 ubiquitination signaling to establish neuronal connectivity in the mammalian brain. Knockdown and conditional knockout in granule neurons of the mouse cerebellum inhibit RNF8 and the parallel fiber presynaptic bouton numbers and functional parallel fiber/Purkinje cell synapses *in vivo* is enhanced drastically (Valnegri et al., 2017).

1.5.2. Ubc13 functions in plants' root development and immunity

Thus far, *UBC13* is the only known ubiquitin-conjugating enzyme (E2) specialized in Lys63-linked polyubiquitination. The functions of *UBC13* and *UEV* suggested to be conserved in plants as *UBC13* and *UEV* homologs have been found in all sequenced plant genomes and isolated in several plant species (Guo et al., 2016; Mural et al., 2013; Wen et al., 2006; Zang et al., 2012).There are two *UBC13* genes in *Arabidopsis thaliana* namely, *UBC13A* and *UBC13B* and the studies have found the products of these genes can interact with yeast Mms2 or human Uev1/Mms2 and can functionally complement the yeast ubc13 null mutant in terms of spontaneous mutagenesis and sensitivity to DNA-damaging agents (Wen et al., 2006).*UBC13* genes of *Arabidopsis thaliana* were implicated in DNA damage tolerance (Wen et al., 2006), apical dominance (Yin et al., 2007), iron metabolism (Li and Schmidt, 2010) and auxin signaling (Wen et al., 2014) In addition, it was found that plant immunity is regulated by *UBC13* and *UEV* homologs of tomato (*Solanum lycopersicum*), through interaction with the kinase protein Fen, which plays a role in effector triggered immunity (Mural et al., 2013). Cell death which is triggered by Fen overexpression in *Nicotiana benthamiana* and by several NLR/effector pairs requires Ub-

conjugating activity of tomato *UBC13* and interaction with Fen (Mural et al., 2013). Moreover, it was revealed that *UBC13A* and *UBC13B* play an important role in the response to pathogen infection and low temperature stress by regulating programmed cell death (Wang et al., 2019). After all, the study demonstrated that *UBC13* is a key regulator in plant response to low temperature and pathogens (Wang et al., 2019). Furthermore, *UBC13* is involved in root-hair development (Li and Schmidt, 2010) according to recent studies. Finally, *UBC13* functions in root development by affecting auxin signaling is revealed (Wen et al., 2014).

There is a suggestion that the Lys63-linked ubiquitination is involved in the modification of an auxin transporter, PIN2, and affects its vacuolar sorting (Leitner et al., 2012). Inability to form branched root hairs upon Fe deficiency was observed in some experiments in a *ubc13a* mutant. In addition, reduction in root-hair density was marked by *ubc13* double mutant. This result indicates that *UBC13* is involved in root-hair development (Li et al., 2010). Moreover, root development is positively regulated by *UBC13* as it affects auxin signaling and auxin responsive protein stability (Wen et al., 2014). The roles of Lys63-linked polyubiquitination is implied by these results in addition to DNA-damage response, suggesting that *UBC13* is critical for all major aspects of root development: primary root, lateral roots and root hairs by affecting auxin signaling and Aux/IAA protein stability (Wen et al., 2014). *UBC13* genes of *A. thaliana* were 3 involved in DNA-damage tolerance (Wen et al., 2006), apical dominance (Yin et al., 2007), iron metabolism and auxin signaling (Wen et al., 2014). Genetic studies have revealed that plant immunity and defense responses, such as hypersensitive response (HR) associated cell death, ROS burst, and expression of defense related genes are controlled by *UBC13* in *Arabidopsis* (Wang et al., 2019). The ubiquitination-mediated posttranslational modification system plays regulatory and essential roles in plant immunity. Recent studies have found that the immune response in *Solanum lycopersicum* (tomato) is regulated by *UBC13*-mediated ubiquitination against the pathogen *Pseudomonas syringae* (Mural et al., 2013). It is clear that Ub-mediated degradation of proteins is also critical for plant defense mechanisms (Yang et al., 2006). The soybean Ubc2 gene has been shown to regulate gene expression levels for abiotic stress tolerance in a previous study (Zhou et al., 2010). It was revealed that the *UBC13* gene is a housekeeping gene in plants, since the transcript levels of *UBC13* genes is relatively stable in *Arabidopsis* and rice, regardless of plant development, tissue distribution and response to stresses (Zang et al., 2012).

1.6 DNA damage and DNA damage tolerance

Plants are continuously exposed to various environmental stress factors, including high solar radiation containing UV light, ionizing and other cosmic radiations, desiccation, rehydration, soil salinity and various soil pollutants including heavy metals as they are sessile and depend on sunlight for photosynthesis (Britt, 1996; Manova and Gruszka, 2015; Roy, 2014; Sinha and Hader, 2002; Tuteja et al., 2009; Waterworth et al., 2011). Not only the above-mentioned exogenous factors but also the endogenous factors like reactive oxygen species (ROS), as metabolic byproducts of chloroplast and mitochondria, often induce various forms of DNA lesions and cause genome stability in plants. Normal cellular functions are disrupted by unrepaired damage in DNA in actively dividing cells and finally plant growth and development are affected. DNA lesions, such as single- and double-strand breaks (SSBs and DSBs), intra- and inter-strand crosslinks and generation of apurinic and apyrimidinic sites are created by different stresses (Gill et al., 2015). Moreover, out of several forms of DNA damage, DSB is recognized as one of the most serious forms (Charbonnel et al., 2010; Li and Xu, 2016; Puchta, 2005; West et al., 2004). Animals and plants possess the same kind of post embryonic development where a pool of undifferentiated meristematic stem cells localized in plant meristems generate cells (Perianez-Rodriguez et al., 2014; Peris et al., 2010; Sozzani and Iyer-Pascuzzi, 2014). Actively dividing cells in the meristematic zone are most vulnerable to DSBs (Roy, 2014). When the DNA with DSBs get replicated during mitosis, it leads to lose of chromosome fragments. These chromosomal defects are inherited by the actively dividing stem cells and create a mutant cell population. Chromosomal deletion creates mutations and stall replication and transcription, leading to loss of cell viability and eventually plant growth, development and productivity (Fulcher and Sablowski, 2009). To maintain genome integrity under abiotic and biotic stress conditions, DNA damage in the genome should be detected and repaired rapidly. Therefore, DNA damage response (DDR) is the process to maintain genome integrity as it has the ability to detect and repair DNA damage associated with highly coordinated cellular processes (Hu et al., 2016; Reisman et al., 2012; Yoshiyama et al., 2013). DNA damage sensors, signal transducers, mediators, and effectors are included in plant DDR pathway as key regulatory components similar to animals. DNA damage sensors and signal transducers are well conserved between animals and plants. Anyhow, plant genomes do not possess any homolog of p53, the central effector of the DDR system in animals and, as a result,

DNA damage response cascade diverged downstream of the transducers in plants (Yoshiyama et al., 2013). p53 plays key roles in controlling cell division and cell death with damaged DNA and in suppressing tumor formation (Beckerman and Prives, 2010; Gimenez and Manzano-Agugliaro, 2017). DNA synthesis over a damaged template is carried out via a process known as DNA damage-tolerance (DDT), also named DNA post-replication repair (PRR) in budding yeast. This process is known to be highly conserved from yeast to human, and biochemically diverse proteins are being involved. There are two sub-pathways in DDT pathway: the error-prone and the error-free.

Lys63 polyubiquitination mediated by Ubc13-Mms2 plays an important role in DNA repair. In yeast and mammalian cells, the *RAD6* pathway is central to PRR (Saffran et al., 2004). In the yeast PRR pathway the two E2s implicated are Rad6 and the heteromeric Ubc13-Mms2 complex (Broomfield et al., 1998; Xiao et al., 2000). Mms2 is a Uev and it was initially discovered in yeast (Broomfield et al., 1998). Physical contacts between members of the *RAD6* pathway are mediated by two RING-finger type E3s Rad18 and Rad5. The Ubc13–Mms2 complex is recruited by Rad5 to the stalled replication fork with the support of its RING finger domain. In addition, Ubc13-Mms2 is brought into contact with the Rad6–Rad18 complex by Rad5 which is associated with Rad18 (Ulrich and Jentsch, 2000). The formation of a heteromeric complex is promoted by the interaction between the two RING-finger proteins, and the enhancement Lys63 poly-Ub chain formation can take place by the coordination of the two E2s Rad6 and Ubc13–Mms2. Excitingly it was found that DNA damage triggers the distribution of Ubc13 and Mms2 proteins to the nucleus although they are largely cytosolic proteins (Ulrich and Jentsch, 2000). There are three sub-pathways in the *RAD6* pathway**:** *REV3* which is an error-prone sub-pathway and two error-free sub-pathways represented by *RAD5* and *POL30* (Xiao et al., 2000). *RAD6*/*RAD18* control the two independent error-free PRR pathways (Xiao et al., 2000). It was evident that one (Ulrich and Jentsch, 2000) or both (Xiao et al., 2000) of the error free pathways are promoted by Ubc13-Mms2. In the *RAD6* pathway, PRR is composed of PCNA (proliferating cell nuclear antigen). Lys164, which is an important residue in PCNA can be modified in three ways: monoubiquitination by Rad6 and Rad18, Lys63-linked polyubiquitination by Ubc13-Mms2 in complex with Rad5, and conjugation of SUMO (small ubiquitin-related modifier) by Ubc9 (Hoege et al., 2002). It has been evident that these modifications have different implications in DDT. Figure 1-8 shows roles of Ubc13-Uev mediated Lys63 poly-Ub chain in yeast PRR.

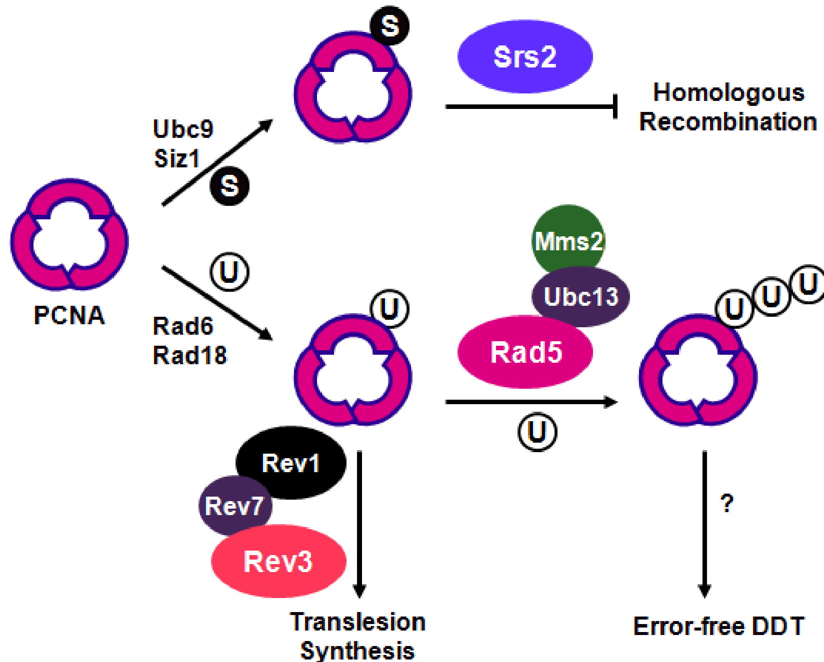

Figure 1-8: Roles of Ubc13-Uev mediated Lys63 poly-Ub chain in yeast PRR. Rad6 and Rad18 monoubiquitinate the Lys164 residue of PCNA, and the Ubc13-Mms2 complex further polyubiquitinate with Rad5. The poly-ubiquitinated PCNA then functions in the error-free PRR pathway (Pickart, 2002). Modification of the same lysine residue can be done via SUMO (S) (small ubiquitin-related modifier), conjugation and antagonistic functions with Ubc13-mediated polyubiquitination are getting competed by ubiquitination and sumoylation.

1.6.1 Error-prone translesion DNA synthesis

In translesion synthesis (TLS) mechanism, specialized damage-tolerant DNA polymerases is used to bypass the DNA lesion. As a result, damage-induced mutations are caused by TLS (Andersen et al., 2008; Friedberg, 2005). Monoubiquitination of PCNA is carried out as the first step in eukaryotic TLS mechanism, and this modification of the PCNA recruits damage-tolerant polymerases including Polζ (Rev3 and Rev7), Polδ, and Rev1, which are required for TLS to be recruited to stall replication forks (Bienko et al., 2005; Kannouche et al., 2004) and stimulate their ability to polymerize across lesions (Garg and Burgers, 2005). Stepwise covalent modifications of

the PCNA takes place in yeast TLS which is encoded by *POL30*. PCNA is monoubiquitinated by Rad6-Rad18 at the Lys164 residue when exposed to DNA damage to promotes TLS.

1.6.2 Error-free lesion bypass

Prevention of spontaneous and DNA damage–induced mutagenesis is carried out via error-free lesion bypass which is one branch of PRR mediated by the Ubc13-Mms2 complex in yeast (Barbour and Xiao, 2003; Broomfield et al., 2001). The first step of the error free lesion bypass is monoubiquitination of PCNA by Rad6-Rad18 at the Lys164 residue, followed by further polyubiquitination through Lys63-linked chains by the Mms2-Ubc13-Rad5 complex (Hoege et al., 2002). It is supposed that error-prone TLS is promoted by monoubiquitinated PCNA, whereas error-free bypass of damaged templates is promoted by polyubiquitinated PCNA (Pastushok and Xiao, 2004; Stelter and Ulrich, 2003). SUMO (small ubiquitin-related modifier) modifies the same Lys164 residue during normal replication. SUMO requires the Siz1-Ubc9 complex where the DNA helicase Srs2 gets recruited by the sumoylated PCNA to stalled replication forks to prevent inappropriate recombination (Papouli et al., 2005; Pfander et al., 2005).

1.6.3 DNA damage tolerance in plants

Plants, as they do not possess any mobile functions, have to tolerate and even thrive wide range of environmentally harmful conditions such as excessive sunlight radiation, chemical mutagens, fungal toxins, high and low temperatures, and water stress. Under these circumstances, it is vital for plants to occupy an efficient system to maintain genome stability. There are different mechanisms involved in this system not only to prevent damage to DNA but also to remove or repair the damage when damage to DNA occurs. Less information is known about the pathways involved in DNA repair in plants although they have been extensively investigated in yeast and mammals.

Plants use sunlight for photosynthesis although it becomes stressful under different conditions. For example, overproduction of reactive oxygen species can result in damaging the photosystems in the chloroplasts when exposed to strong sunlight (Asada, 1999). It is known that UV light, which comes from sunlight as a subcomponent has serious effects on plants. There are several types of damage caused to cellular compounds, membranes, and phytohormones by UV light inducing various DNA lesions. The major lesions on DNA are cyclobutane pyrimidine

dimmers (CPDs) and photoproducts, and minor lesion includes oxidized or hydrated bases, single-strand breaks, and others (Ballare et al., 2001; Rousseaux et al., 1999). As a result, two main protective strategies have been adopted by plants with evolution to avoid the adverse effects of UV light. Flavonoids and phenolic compounds shield the effects from UV light as one strategy whereas DNA repairs such as photoreactivation and dark repair takes place as the second strategy (Britt, 1999; Hays, 2002; Tuteja et al., 2001). In higher plants, the major DNA repair pathway for CPDs is photoreactivation, which is mediated by a photolyase (Dany et al., 2001). Light in 300-600 nm range (Hays and Pang, 1999) is absorbed by photolyases to monomerize UV-induced CPDs.

NER (Nucleotide Excision Repair), BER (Base Excison Repair), MMR (Mismatch Repair) and other DNA repair pathways are included in the dark repair which is observed in several plant species. Sequenced *A. thaliana* and rice genomes revealed that most of the genes involved in NER and BER are present in higher plants (Kimura et al., 2004), suggesting that these DNA repair mechanisms are highly conserved in yeast, human and plants.

Although there is a constant threat to the DNA sequence in mitochondria and chloroplasts in plants by oxidative damage, very low rate of changes is observed, indicating efficient DNA repair mechanisms in these organelles. The BER pathway is found in plant mitochondria by recent studies. The mitochondrial DNA is replicated, proofread, and repaired in inner membrane-bound nucleoids (Boesch et al., 2009).

Northern and *in situ* hybridization were used to investigate the expression patterns of DNA repair genes encoding CPD photolyase, UV-DDB1, CSB, PCNA, RPA32, and FEN-1 in rice (*Oryza sativa* L. cv. Nipponbare) (Kimura et al., 2004). According to the results, the expression of all the genes were found in tissues rich in proliferating cells. Excitingly, mature leaves express more MMR genes than the SAM (shoot apical meristem), implying the importance of DNA repair in mature leaves (Kimura et al., 2004). The overview of types of DNA damage in plants is shown in Figure 1-9.

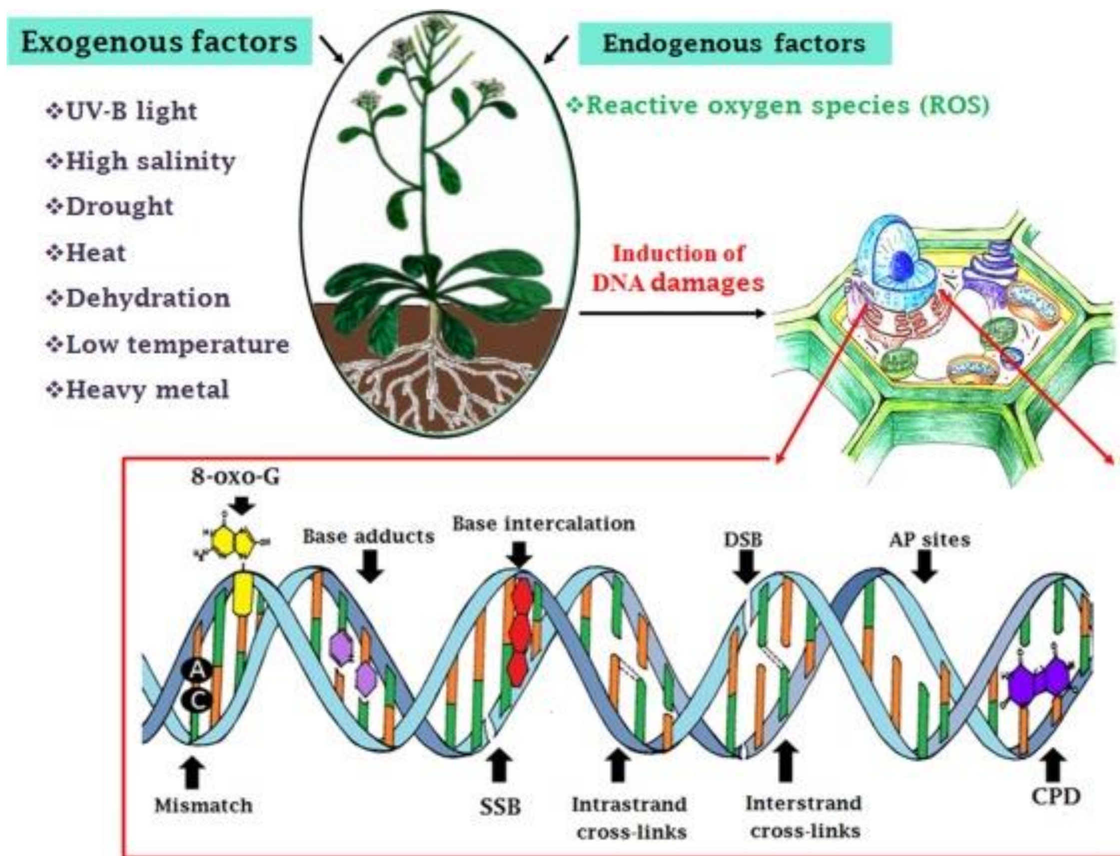

Figure 1-9: Types of DNA damage. Schematic representation of various types of exogenous factors such as UV and ionizing radiation, salinity, desiccation, heavy metals etc, and endogenous factors mainly reactive oxygen species (ROS) induce major DNA lesions including DNA single and double-stranded breaks (SSBs and DSBs), CPD, intra and interstrand cross-links, AP sites and 8-oxo-G (Mahapatra and Roy, 2020).

RAD6 genes are found in *Arabidopsis* (Sullivan et al., 1994; Zwirn et al., 1997), which imply the existence of a plant DNA damage tolerance pathway. Plant genes such as *AtPOLH* (Santiago et al., 2006), *AtPOLK* (Rodriguez-Rojas et al., 2004) , *AtREV3* (Sakamoto et al., 2003), *AtREV1* and *AtREV7* (Takahashi et al., 2005) involved in the error-prone DDT pathway have been isolated and characterized, while the error-free damage tolerance have not been well characterized in plants.

1.7 Canola and clubroot disease

Canola carries a bright yellow flower and falls into the Brassicaceae family (Zhang et al., 2018). Three different species: *Brassica napus*, *B. rapa*, and *B. juncea* are included in this family (Zhang et al., 2015). *B. napus*, also known as rapeseed, originated from the Mediterranean area and Northern Europe in 2000 BC. Rapeseed was recognized as a high-erucic acid crop, containing >40% erucic acid in the oil. In North America, high-erucic acid rapeseed oil used to be produced in small quantities for industrial nonfood usage. The quality of the earlier rapeseed cultivars was improved by some Canadian scientists in 1976 using traditional plant breeding and this conversion created commercially consumable canola cultivars. The new word "canola" was registered in Canada in 1979 to describe a new seed which carries oil that was low in both erucic acid and glucosinolates. By definition, canola has specific cut-off levels of erucic acid (<2%) and glucosinolates (<30 umol/g) for both human and animal consumption. In 1977, Europe introduced edible rapeseed oil (low-erucic acid rapeseed (LEAR) oil) that contains <5% erucic acid and low glucosinolates. Canola oil was granted "generally recognized as safe" (GRAS) status as a dietary component by the U.S. Food and Drug Administration in 1985 (Lin et al., 2013).

Canola has reached its success as one of the most significant oilseeds crops worldwide over the past 40 years. At the present time, canola is the third largest vegetable oil by volume followed by palm and soybean oil. In 2010/2011, Canada produced 31% of the canola oil while Europe produced 63% when the production was 38 million metric tons worldwide. In the United States canola oil obtains the first place in most widely used consumed oil, whereas soyabean placed in the second (Lin et al., 2013).

Being one of the top canola-producing countries, Canada is ranked third in oil production. Therefore, it is significant for Canada to optimize agronomic treatments in this key agricultural industry. There have been various approaches to potentially enhancing canola yield or reduce disease outbreaks such as increasing seeding density, adapting fertilization regimes, and selecting optimal rotation crops and rotation sequences (Guo and Fernando, 2005; Harker et al., 2003).

The obligate parasite *Plasmodiophora brassicae Woronin* causes clubroot disease in Brassicaceae. This soil-borne pathogen infection in susceptible host genotypes, causes the formation of large galls or club-shaped swellings on the roots, which hinders water and nutrient uptake by the plant. When the symptoms get severe, it causes stunting of the above-ground organs, as well as yellowing, wilting and premature senescence. Therefore, these symptoms lead to reduced crop yield and quality. Losses to clubroot is conservatively estimated to range from 10 to

15% on a global scale (Tso et al., 2021). This soil-borne pathogen can survive in soil as resting spores, which have a half-life of approximately 4 years and remain viable for up to 20 years (Hwang et al., 2012). Due to this reason, it has become difficult to eradicate the *P. brassicae* pathogen once it spreads and cause infections in the field. European settlers have introduced clubroot to Canada with *P. brassicae*-infected fodder turnips (*Brassica rapa L. var. rapa*) that was taken to use as animal fodder (Hwang et al., 2012). It is evident that even since as early as 1916 Nova Scotia has conducted clubroot research and indicated that the disease was probably well established in parts of Canada by the late 19th or early 20th centuries (Estey, 1994). The fields in the Maritimes, Quebec and British Columbia reported the occurrence and spread of clubroot disease on cruciferous vegetables from the 1920s to the 1950s nearly every year and occasionally from Ontario fields (Howard et al., 2010). The production of cabbage and other crucifers in areas was reported to lose in a greater quantity due to clubroot outbreaks reported occasionally in the 1960s (Creelman, 1965), and in the 1970s where the disease remained the most important limiting factor (Howard et al., 2010). Despite these outbreaks, until the 1970s and 1980s there were no reports on clubroot disease occurrence outside of the traditional vegetable growing regions in the Maritimes, Quebec, Ontario and British Columbia, when a few home gardens in Alberta and in a market garden in Manitoba reported to have its occurrence in unpublished reports. Clubroot was observed for the first time on the Canadian canola in 1997, in Quebec (Morasse et al., 1997) but the first identification of canola clubroot in in the prairie provinces (Alberta, Saskatchewan and Manitoba) which contribute to the canola production as more than 98% of the harvested hectares of Canadian canola are grown in these provinces (Canola Council of Canada, Winnipeg, Manitoba) happened in 2003 (Tewari et al., 2005). In north and northwest of the city of Edmonton, in the central part of Alberta, 12 clubroot-infested fields were identified in 2003 and impacts were concerned that would result in large economic losses on the multi-billion-dollar canola industry (Strelkov et al., 2006; Tewari et al., 2005)). As a result, large, coordinated research was initiated leading to understand and manage this disease as canola clubroot identified in Alberta.

1.8. Subject background and project objectives

1.8.1. Subject background

Numerous important cellular processes in all eukaryotes are regulated by Lys63-linked polyubiquitination. Ubc13 promotes Lys63-linked polyubiquitination, which is distinct from the conventional Lys48-linked polyubiquitination that leads to the degradation of target protein. A unique heterodimeric E2 complex Ubc13-Uev is required for the formation of Lys63-linked polyubiquitination. In yeast, Ubc13 has been shown to function in error-free DDT, whereas in mammalian cells, Ubc13 is involved in several cellular processes including but not limited to DDR and innate immunity.

Previous studies found two *UBC13* genes and four *UEV1* genes in *Arabidopsis.* It has been found that *Arabidopsis UBC13* genes function in plant immunity and regulate defense responses. These responses include hypersensitive response (HR) associated cell death, ROS burst and expression of defense related genes. Previous studies provide convincing data that plant *UBC13* contributes to root growth and development as well as disease resistance against soil-borne pathogens. Based on an *Arabidopsis* clubroot disease model, we hypothesize that *UBC13* is involved in plant response to clubroot. Clubroot disease management in Canola is difficult, as resting spores has the ability to remain viable in soil for more than 15 years. Therefore, one of the best ways to control clubroot in canola is to have resistant varieties because it is effective and environmentally friendly.

However, there is no published information regarding *Brassica napus UBC13* and its role in disease resistance. This project aims to understand functions of *BnUBC13* genes and BnUbc13-BnUev1 complexes in canola. Furthermore, it aims to provide guidelines to target *BnUBC13* genes by genome editing to protect canola from the clubroot disease.

1.8.2. Project objectives

The two major objectives of the study were:

- To characterize functions of different canola *UBC13* genes
- To provide guidelines to target certain *UBC13* genes by genome editing

They were further divided into four specific aims:

- To identify and clone *B. napus UBC13* genes

- To test whether *BnUBC13* genes are able to functionally complement the yeast *ubc13* null mutant for DNA-damage response

- To test whether BnUbc13s are able to physically interact with *A. thaliana* Uev1

- To assess whether *BnUBC13* genes contribute to plant disease resistance against soil-borne pathogens such as clubroot

CHAPTER 2: MATERIALS AND METHODS

2.1 Plant genomic DNA extraction

A DNeasy Plant Mini Kit (QIAGEN#69104) was purchased for the plant genomic DNA extraction. First, *Brassica napus* cv. Westar plant samples were disrupted using a mortar and pestle. Then 400 µL of buffer APi and 4 µL of RNase was added and incubated for 10 min at 65 ℃ followed by adding 130 µL of buffer P3 and incubating for 5 min on ice. After that, the lysate was centrifuged for 5 min at 20,000 *g*. Once the lysate was collected into a QIAshredder spin column placed on a 2 mL collection tube, it was centrifuged for 2 min at 20,000 *g*. The flow through was transferred into a new tube without disturbing the pellet. Next, buffer AW1 was added in 1.5 volumes with mixing. Once done, 650 µL of the mixture was transferred into a DNeasy mini spin column followed by centrifugation for 1 min at 6,000 *g*. The flow through was discarded, the spin column was placed into a new 2 mL collection tube, and 500 µL of buffer AW2 was added and centrifuged at 6,000 *g* for 1 min. After that, 500 µL of buffer AW2 was added and centrifuged for 2 min at 20,000 *g*. Finally, the spin column was transferred to a new 1.5 mL centrifuge tube and 100 µL of ddH_2O was added followed by centrifugation at 6,000 *g* for 1 min.

2.2 Total RNA extraction and concentration measurement

Seedlings of *B. napus* were used to extract total RNA using the TRIzol reagent (Invitrogen). Motor and pestle were used to homogenize *B. napus* tissues in the presence of liquid nitrogen. 1 mL of Trizol was mixed with the homogenized tissue (the amount of the tissue should be less than 100 mg per mL TRIzol), incubated for 5 min at 15-30 ℃ and spun down at 12,000 *g* for 10 min at 4 ℃ to remove proteins and polysaccharides. Next, 200 µL chloroform was added to the aqueous phase. Then it was shaken vigorously by hand for 15 seconds and incubated at 15-30 ℃ for 2-3 min followed by centrifugation at no more than 12,000 *g* for 15 min at 4 ℃. After that aqueous phase was separated. It was transferred to a new tube containing 0.5 mL isopropanol. Then it was proceeded with incubation at room temperature or 10 min and centrifugation at 12,000 *g* for 10 min at 4 ℃ to precipitate RNA. The supernatant was discarded, and the pellet was washed with 1 mL 70% ethanol, centrifuged again at 7,500 *g* for 5 min at 4 ℃. The RNA sample was then air dried for 5-10 mins then stored in a -80 ℃ freezer. Finally, a spectrophotometer (Thermo Scientific

GENESYS 20) was used to measure the concentration of RNA following the manufacturer's instructions.

2.3 RT-PCR

Total RNA was treated with DNaseI (Promega) for RT-PCR analysis. SuperScript RT-PCR III system (Invitrogen) was used to synthesize first-strand cDNA by reverse transcriptase following the protocol as described (Karsai et al., 2002). Briefly, 2 to 4 mg of total RNA from each sample was treated according to manufacturer's guidelines (Invitrogen ThermoScriptTM RT-PCR #1682307). *BnUBC13* gene-specific primer pairs (Table 2-1) were used for the rest of experiments to make sure that the amount of PCR product was not excessive and that the difference among different samples was not interfered by saturation of PCR amplification. Agarose gel electrophoresis was carried out with 8 µL of each reaction. All RT-PCR series were done at least three times for statistical analysis.

Table 2-1: Primers for amplifying *BnUBC13*s.

BnUBC13A-F	5' CTC GGATCC AA ATG GCG AAT AGT AAT CTT CCT C
BnUBC13A-R	3' CAGT GTCGAC TCA AGC GCC ACT TGC ATA TAG AC
BnUBC13B-F	5' CTC GGATCC AA ATG GCG AAT AGT AAT CTA CCG
BnUBC13B-R	3' CAGT GTCGAC TCA AGC GCC ACT TGC ATA CAG AC
BnUBC13C-F	5' CTC GGATCC AA ATG GCG AAT AGT AAT CTA CCG
BnUBC13C-R	3' CAGT GTCGAC TCA AGC GCC ACT TGC ATA CAG AC
BnUBC13D-F	5'CTC GGATCC AA ATG GCT AAT AGC AAT CTA CCG
BnUBC13D-R	3' CAGT GTCGAC TCA AGC GCC ACT TGC ATA TAG AC
BnUBC13E-F	5' CTC GGATCC AA ATG GCC AAC AGC AAT CTT CCC
BnUBC13E-R	3' CAGT GTCGAC TCA TGC GCC GCT TGC GTA CAG AC
BnUBC13F-F	5' CTC GGATCC AA ATG GCG AAT AGC AAT CTA CCG
BnUBC13F-R	3' CAGT GTCGAC TCA AGC GCC ACT TGC ATA TAG AC
BnUBC13G-F	5' CTC GGATCC AA ATG GCG AAT AGT AAT CTT CCT C
BnUBC13G-R	3' CAGT GTCGAC TCA AGC GCC ACT TGC ATA TAG AC
BnUBC13H-F	5' CTC GGATCC AA ATG GCC AAC AGC AAT CTG CCC
BnUBC13H-R	3' CAGT GTCGAC TTA TGC GCC GCT TGC GTA CAG AC
BnUBC13I-F	5' CTC GGATCC AA ATG GCC AAC AGT AAT CTC CC
BnUBC13I-R	3' CAGT GTCGAC TCA TTC GCC GCT TGC GTA AAG AC
BnUBC13J-F	5' CTC GGATCC AA ATG GCC AAC AGT AAT CTA CCC

BnUBC13J-R	3' CAGT GTCGAC TCA TGC ACC GCT TGC GTA AAG AC
BnUBC13K-F	5' CTC GGATCC AA ATG GCG AAC AGT AGT CTA CCA
BnUBc13K-R	3' CAGT GTCGAC TTA TGC GCC GCT GGC GTA AAG AC
BnUBC13L-F	5' CTC GGATCC AA ATG GCC AAC AGT AAT CTC CCC
BnUBC13L-R	3' CAGT GTCGAC TCA TTC GCC GCT TGC GTA AAG AC

Table 2-2: Primers for screening canola *Bnubc13* mutant lines after genome editing

BnaC6g20310D-F(K)	5′ AAAGTGTTCTCTTGATCATCGG
BnaC6g20310D-R	3′ CGAGATAAACATCTTGTAAACC
BnaC2g2560D-F (I)	5′ AACTCTTATCGATCGAGCGAG
BnaC2g2560D-R	3′ CCAATATCATTAAACACAGCCC
BnaA7g38410D-F (J)	5′ CCCAAATCTATCTCATTTAGACG
BnaA7g38410D-RP	3′ CAAAATCATCAAAAGAGCCCG
BnaA2g1970D-F (L)	5′ AGCGTGCGGATCTTCTCTCTC
BnaA2g1970D-R	3′ GAATGGTGGTACATAGGTGAGG
BnaC6g39290D-F (H)	5′ GATTCCTGATTCTCATTTTTTGTC
BnaC6g39290D-R	3′ CAGTCTGCGTAACACAACCAG

2.4 Yeast techniques and experiments

2.4.1 Yeast cell preparation and storage

Table 2-3 shows all haploid yeast strains used in this study. Rich YPD (1% Bacto-yeast extract, 2% Bacto-peptone and 2% glucose) and temperature at 30 ºC were used mainly to grow yeast cells. In addition, they were also cultured in a synthetic dextrose (SD) medium (0.67% Bacto-yeast nitrogen base without amino acids, 2% glucose) supplemented with necessary nutrients including 30 mg/L L-isoleucine, 150 mg/L L-valine, 20 mg/L adenine hemisulfate salt, 20 mg/L L-arginine HCl, 20 mg/L L-histidine HCl monohydrate, 100 mg/L L-leucine, 30 mg/L L-lysine HCl, 20 mg/L L-methionine, 50 mg/L L-phenylalanine, 200 mg/L L-threonine, 20 mg/L L-tryptophan, 30 mg/L L-tyrosine, 20 mg/L L-uracil as recommended (Sherman, 1991). If it is necessary to provide a selection medium for yeast transformation, any of the above auxotrophic supplements can be omitted. Auxotrophic supplements were made into 100 × stocks and always added before autoclaving the SD medium. When making plates, 2% agar was added to either YPD

or SD medium prior to autoclaving. Plates with yeast cells can be stored up to 4 months at 4 ^{o}C when sealed with parafilm. For the long-term storage, yeast cells were grown at 30 °C overnight in an appropriate liquid medium (rich or minimal selective) and 0.7 mL of the culture was mixed with 0.3 mL of 50% sterile glycerol before storing at -70 °C. When preparing methyl methanesulfonate (MMS)-containing media, MMS (Aldrich, Milwaukee, USA) was added immediately before pouring the plates. MMS plates were always made and used freshly on the same day to avoid loss of MMS activity.

Table 2-3: Haploid *Saccharomyces cerevisiae* strains used in the study

Strain	Genotype	Source
PJ69-4a	*MAT**a** trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ MET2::GAL7-lacZ LYS2::GAL1-HIS3 GAL2-ADE2*	P. James
HK580-10D	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	H. Klein
DBY747	*MAT**a** his3-1 leu2-3,112 trp1-289 ura3-52*	D. Botstein
WXY849	DBY747 with *ubc13Δ::HIS3*	Lab stock

2.4.2 Yeast transformation

Yeast transformation was carried out by dimethyl sulfoxide (DMSO)-enhanced method as described (Hill et al., 1991). Overnight grown yeast cells from YPD were poured into a fresh YPD in 1:50 ratio to carry out the subculture. Once yeast cells reached an OD of 0.6 indicative of mid-logarithmic phase of growth, they were precipitated by centrifugation, washed in 400 µL LiOAc solution (0.1 M lithium acetate, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and resuspended in 100 µL of the same solution. It was then mixed with 5 µL of denatured carrier DNA (single-stranded salmon sperm DNA) and 1-5 µL of transforming DNA. Next, it was incubated at room temperature for 5 min until 280 µL of 50% PEG4000 (50% polyethylene glycol 4000 in LiOAc solution) was added. Mixture was inverted 4-6 times to mix everything properly and incubated for 30 min at 30°C, followed by addition of 40 µL of DMSO. The mixture was heat shocked for 5 min in a 42 °C water bath. The yeast cells were pelleted by centrifugation, washed with sterile ddH_2O, resuspended in 100 µL ddH_2O, plated in a selective medium and incubated at 30 °C for 3 days to allow colony formation.

2.4.3 Yeast two-hybrid (Y2H) assay

Different combinations of $Gal4_{BD}$ and $Gal4_{AD}$ constructs were used to co-transform the Y2H strain PJ69-4A (James et al., 1996). At least five independent co-transformed colonies were grown in SD-Leu-Trp plates for each transformation in order to select them and then replica plated onto either SD-Leu-Trp-His alone or SD-Leu-Trp-His with various concentrations of 1,2,4-amino triazole (3-AT) to test the activation of the P_{GAL1}-*HIS3* reporter gene. In addition, they were also grown on SD-Ade to test the activation of the P_{GAL2}-*ADE2* reporter gene. Plates were incubated for 48 hours at 30 °C or as specified.

2.4.4 Cell survival assays

2.4.4.1 Gradient plate assay

The gradient plate assay was carried out to check the sensitivity of yeast cells to DNA damaging agent MMS. To perform this semi-quantitative measurement, each strain was inoculated in 1 mL of SD minimal medium having at least three replicates from each transformation. The cell density of each sample was calculated after growing them overnight at 30 °C and equal number of cells from the transformants as well as untransformed controls were imprinted onto YPD alone or YPD gradient plates containing 0.025% MMS and 0.001% MMS accordingly. A square petri dish was tilted and 30 ml of YPD + MMS medium was poured to create MMS gradient and once the agar was solidified, 30 mL YPD was poured to make the top layer by placing the petri plate in a flat position. The sample printing was done by mixing 0.1 mL of overnight culture, 0.4 mL sterile water and 0.5 mL of molten YPD agar on a glass slide. The mixture was taken by another sterile microscope glass slide and printed onto the plates across the gradient. Finally, they were incubated at 30 °C for three days or as indicated.

2.4.4.2 Serial dilution assay

A serial dilution assay was carried out to identify the sensitivity of yeast cells to DNA damaging agents such as MMS, 4NQO and UV. To perform this semi-quantitative measurement, each strain was inoculated in 1 mL of SD minimal media having at least three replicates from each different transformation and incubated at 30 °C overnight with shaking at 150-200 rpm.

Each strain was maintained at the same level of cell density after adjusting by either dilution with SD minimal medium or concentration by centrifugation. This culture was considered as 10^0 dilution and afterwards tenfold dilutions were obtained from this original sample. For each culture, a few 1.5 mL microcentrifuge tubes were marked as 10^0, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. Firstly, 1 mL of the original culture was added to the 10^0 labelled microcentrifuge tube and rest of the tubes were added with 900 µL ddH_2O. 100 µL of culture from the 10^0 dilution tube was added to the 10^{-1} dilution tube, mixed by gentle vortexing. Secondly, 100 µL was taken out of 10^{-1} tube and then added it into the 10^{-2} dilution. This was repeated with all tubes. Finally, 5 µL was taken out from the most diluted tube and carefully dropped onto the desired plate with different concentrations. This was repeated with all the dilution tubes. Plates were not moved until the spots are absorbed and incubated at 30 °C for 3 days before photography.

2.4.5 Spontaneous mutagenesis assay

Luria and Delbruck fluctuation test was used to measure the spontaneous Trp^+ reversion rates of DBY747 derivatives (Von Borstel, 1978). Trp^+ can be generated when several different mutation events happened to the *trp1-289* amber mutant (Xiao and Samson, 1993). WXY849 was transformed with pGAD-*BnUBC13s* or the pGAD424E empty vector control. SD-Leu plates were used to select the transformants. The experiment was carried out with at least five independent cultures of each strain. Yeast cultures were grown overnight, before it was counted with a hemocytometer. 5-mL fresh SD-Leu liquid medium was inoculated to a final density of 20 cells/mL and incubated at 30°C until the cell titer reached $2x10^7$ cells/ml. Cells were collected by centrifuging at 4,000 rpm followed by resuspension in sterile ddH_2O. At the end, they were plated in triplicates each and incubated at 30 ºC for three days. Colonies on the YPD plates were used to score total survivors and, on the SD-Trp plates to score Trp^+ revertants. Calculation of spontaneous mutation rates (number of revertants per cell per generation) was done as previously described (Williamson et al., 1985).

The formula to calculate the frequency of spontaneous mutagenesis is as follows:

Frequency (F) = total TRP^+ colonies/ total colonies on YPD

The following formular was used to calculate of spontaneous mutagenesis:

Rate = 0.4343 x Frequency/log (total cell number) – log (initial cell number)

2.5 Bacteria techniques and experiments

2.5.1 Bacterial plasmids preparation and storage

Firstly, total RNA was extracted from *B. napus* seedlings using the TRIzol reagent to clone the full-length coding cDNAs of these genes. RT-PCR was performed using total RNA with ThermoScript RT-PCR kit (Invitrogen) according to manufacturer's instructions. *B. napus UBC13* genes were obtained using the designed gene-specific primers. Table 2-1 shows all the primers were used in the study. *BnUBC13* homologs were cloned into the vector pGBT9E, while *AtUEV1D* was cloned into the vector pGAD424E (Bartel and Fields, 1995) to determine protein-protein interactions by the yeast two-hybrid assay. The cloned genes were confirmed by sequencing at the Eurofin Sequencing Laboratory. Finally, they were named as *BnUBC13A*, *BnUBC13B, BnUBC13C, BnUBC13D, BnUBC13E, BnUBC13F, BnUBC13G, BnUBC13H* and *AtUEV1D* based on the relatedness of sequences.

In addition, ORFs of these *BnUBC13* genes were isolated from the pGBT9 based plasmids and cloned into pET30+ (Invitrogen), while *AtUEV1D* was cloned into pGEX6p (Amersham Biosciences) to overexpress these genes in *Escherichia coli* as N-terminal His_6- and GST-fusions, respectively.

E. coli DH10B (Gibco BRL) was used for DNA plasmid propagation and isolation. As the plasmids used in the study uses the kanamycin (Kan)-resistance gene for selection, LB liquid or agar media (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.5% NaCl and 1.2% agar for plates) containing 50 μg/mL of Kan was used to culture the transformed cells. If samples needed to be stored for short period (2 or 3 months), they were kept at 4 ^oC in plates sealed with paraffin film. For a long-term storage, LB plus Amp/Kan liquid medium was used to grow cells overnight and 900 μL of culture was mixed with 100 μL DMSO to store at –70 ^oC.

2.5.2 Bacterial transformation

Bacteria transformation was carried out either by electroporation or a chemical method. For electroporation, 25 μL of *E. coli* competent cells was mixed with <2.5 μL plasmid DNA in an ice-cooled electroporation cuvette (Bio-Rad), which was left on ice for 45 sec. Cells were then

exposed to a voltage of 1.8 kV (for cuvettes with 0.1 mm width) using a *E. coli* Pulser (Bio-Rad). 500 µL of SOC was added to each sample as the nutrient rich medium. The cells were then transferred to a 1.5-mL centrifuge tube, incubated for 45 minutes at 37 °C and spread onto the LB plates containing appropriate antibiotics. The plate was incubated at 37 °C overnight for single colonies. The chemical transformation was carried out using the chemical competent cells and manufacturer's protocol (One shot Top 10F chemically competent cells, Invitrogen #2183968).

2.5.3 Preparation of competent cells

Bio-Rad *E. coli* Pulser manual was used to prepare the *E. coli* competent cells for electroporation. Briefly, 1 L of *E. coli* cells was cultured at 37°C until an optical density (OD) at 600 nm reached 0.6. Then it was centrifuged at 3,500 rpm for 15 minutes in a Beckman GSA rotor and 500 mL of 10% ice-cold sterile glycerol was used to resuspend the final pellet. After it was repeated for four times with gradually reduced glycerol volume, the pellet was resuspended in 4 mL of ice-cold, sterile 10% glycerol. The competent cells were aliquoted into 25 µL each in Eppendorf tubes and immediately placed in -70°C freezer for storage.

2.6 Protein extraction

2.6.1 Recombinant protein expression

The BL21-CodonPlus (DE3) bacterial strain used for the protein expression study was purchased from Thermo Scientific (2287225). pET30-*AtUBC13s* and pGEX-*AtUEV1D* were either separately or co-transformed into BL21(DE3)-RIL cells and the transformants were grown in the selective LB media overnight at 37°C. The culture was sub-cultured in a 1:50 ratio in the LB medium until it reaches OD_{600nm} of 0.6-0.8. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the final concentration of 0.2 mM and the incubation continued for 6 hr. The sample was harvested 8,000 rpm centrifugation in an Beckman Coulter Avanti JA17 rotor for 1 hr at 4 °C followed by immediate processing or storage at -70 °C.

2.6.2 Preparation of cell extract

The cell pellet was resuspended in phosphate-buffered saline (PBS, 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3). Then, the solution was passed through Constant Systems one shot cell disrupter at 25 PSI. The resulting crude extract was centrifuged at 17,000 rpm in an Beckman Coulter Avanti JA17 rotor for 30 min, and the soluble fraction was used for protein purification by chromatography.

2.6.3 Recombinant protein purification

The cell extracts were subjected to flow through the column with GST beads and His beads for GST-AtUev1D and His-BnUbc13s, respectively. After washing beads with the respective lysis buffers, the affinity-purified recombinant proteins were eluted from the column by using 20 mL of His elution buffer for His-BnUbc13 and GST elution buffer for GST-AtUev1D. Samples were collected, and concentration was measured. Eluted samples were subjected to SDS-PAGE and visualized by Coomassie Blue staining.

2.6.4 Western blot

The same SDS-PAGE gel with all required samples was used for the western blot. Here the gel was equilibrated for 20 min in a transfer buffer along with equal-sized polyvinylidene difluoride (PVDF) membrane and 3 M filter papers. All the components were prepared according to the manufacturer's manual of Bio-Rad trans-blot semi-dry transfer cell, which was used for the transfer of proteins onto the PVDF membrane. To transfer the samples, a constant current of 1 mA/cm^2 was used for 2 hr. Then, the membrane was transferred to a blocking solution overnight at 4°C. Primary antibody (His-tag rabbit, Cell Signaling Technology #D3110) was added to the membrane on the following day and it was incubated at room temperature for 1 hr with gentle shaking. After that membrane was washed three times with PBST. The secondary antibody (goat anti-rabbit, Bio-Rad #1706515) was diluted at 1:10000 in 10 mL PBST and incubated with the membrane under the same condition as the primary antibody. The membrane was washed 3 times for 5 min each with PBST. After that, it was again washed with PBS two times to prepare for the detection. Supersignal™ West Pico PLUS Stable Peroxide (Thermo Scientific #34577) was used as the substrate for the visualization of secondary antibody.

2.6.5 GST pull down assay

Bio-Rad poly-prep chromatography column (731-1550) was used for the GST pull-downs assay. The purpose of this assay was to determine the protein-protein interaction between BnUbc13s and AtUev1D. Co-transformed cells with His-BnUbc13s and GST-AtUev1D were grown at 37°C overnight in the LB selective medium and diluted 1:50 with the fresh medium to resume incubation. Once the cells OD_{600nm} = 0.6, 0.2 mM IPTG was added, and cells were collected after 6-hr incubation at 37°C. 2 g of the pelleted cells were lysed in 30 mL of lysis buffer using the cell disruptor, and the lysed cells were centrifuged for 1 hr at 4°C. The supernatant went through a 0.2 µm filter and was loaded to a column containing GST beads. The column was incubated for 1 hr at 4°C with gentle rocking, and the proteins were eluted after incubation. Finally, wash buffer (1x PBS buffer with 350 mM NaCl) was used to wash the beads by running through the column. The eluted samples were subjected to SDS-PAGE and visualized by Coomassie Blue staining.

2.6.6 Ub conjugation reaction

Purified His-BnUbc13s and GST-AtUev1D were used for the i*n vitro* Ub conjugation reaction. The ubiquitination assay kit containing Ub thioester/conjugation initiation reagents were purchased from Abcam (ab139467). 20-mL reaction mixture contained E1, Ub, MgATP, reaction buffer from the kit, plus BnUbc13 and AtUev1D prepared from this study. The Ub-K63R protein was purchased from Abcam (UM-K63R). The conjugation reactions were performed at 37°C for 4 hr according to the manufacturer's instructions, followed by running of 15% SDS-PAGE and western blotting using polyclonal goat anti-Ub antibodies (Bio-Rad).

2.7 Molecular biology techniques

2.7.1 PCR techniques

The polymerase chain reaction (PCR) was used throughout the study to amplify DNA fragments to be used in cloning or to be sent for sequencing. Instruction manual for the PrimeSTAR Max Premix (Takara #R045) was used to create the PCR reaction mixtures. The amplification was carried out in the SimpliAmp Thermal Cycler (Applied Biosystems by Life Technologies). The timelines used were a denaturing temperature at 98 °C for 40 sec, annealing

temperature at 58 °C for 20 sec, and primer extension at 72 °C for 2 min per kilobase of DNA to be amplified, for a total of 35 cycles.

2.7.2 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate plasmid DNA and amplified DNA fragments based on size. Agarose gel was prepared using 1x TAE buffer (40 mM Tris-acetate, 2 mM Na_2EDTA) mixed with 0.6-1% agarose. Then it was allowed to solidify with inserted set of combs to create wells. Next, the gel was loaded to the electrophoresis tank filled with 1x TAE buffer and a current of <100 mA was allowed until the proper migration distance was attained. Once the band has migrated enough distance, gel was stained in 0.5 µg/mL ethidium bromide (EtBr) for 5-10 min and finally observed under a UV trans-illuminator.

2.7.3 DNA fragment isolation

After restriction enzyme digestion, the DNA sample was run through a 0.6% agarose gel and stained with EtBr. The band of interest was identified using an UV trans-illuminator and cut out of the gel. Once the cut section is inserted to a 1.5 mL Eppendorf tube, it was proceeded with manufacturers instruction protocol included in the QIAquick gel extraction kit (QIAGEN #28706).

2.7.4 DNA sequencing

DNA sequencing was performed by the Eurofins DNA Sequencing Laboratory

2.7.5 DNA sequencing chromatograms analysis

One sgRNA was designed to target five *BnUBC13* genes and constructed into a pYLCRISPR/ $Cas9_{ubi}$-H (16419 bp) binary vector. Thirty genomic edited plants along with *Brassica napus* (DH12075) as a control sample were used for the analysis. Canadian spring type DH12075 was sequenced as part of Canadian Canola Sequencing Initiative. DH12075 is a double haploid line derived from a cross between the French spring type cresor and Canadian spring type westar. PCR reactions were proceeded for the target five *BnUBC1*3 genes. These samples were confirmed by agarose gel electrophoresis based on the anticipated product size and they were purified by using a QIAquick PCR purification kit (QIAGEN) according to manufacturer's

instructions and sequenced by Eurofins. Those sequences were analyzed for the target site with the assistance of sequence chromatograms.

2.7.6 RNA-Seq data analysis

RNA -sequence analysis was carried out for the *B. napus* lines carrying CR genes. RNA was extracted from the root samples using a RNeasy Plant Mini Kit (Qiagen) following manufacturer's instructions, including a DNase digestion using an RNase-Free DNase Set from Qiagen. The quality and concentration of RNA were assessed using the Experion automated electrophoresis system (Bio-Rad) and Nanodrop 2000c, to ensure that the quality and amount of RNA in each sample was sufficient for the cDNA library preparation.

The cDNA library was prepared using the TruSeq RNA Sample Preparation Kit v2 (Illumina), and the quality and concentration of cDNA were assessed using Experion DNA Analysis Kit and Nanodrop 2000c. The cDNA libraries were sent to McGill University and Génome Québec Innovation Centre for Illumina Hiseq 2500 RNA sequencing.

RNA-Seq data processing and DEG analysis were completed following the protocol from (Chu et al., 2015). Raw sequencing data were processed and analyzed using CLC Genomics Workbench version 10.1.1 (Qiagen). The raw reads were subjected to a quality control check and then trimmed to remove the Illumina adapters and low-quality reads. The clean reads were aligned to the *B. napus* reference genome (v4.1; http://brassicadb.org/brad). Gene expression levels were expressed in reads per kilobase of exon model per million reads (RPKM). Differently expressed genes (DEGs) were identified when the RPKM was |>4| and the threshold *P*-value of false discovery rate (FDR) was ≤ 0.01. DEG comparisons were made between the inoculated treatment and the negative control (mock inoculation) for each cultivar.

2.7.7 qRT-PCR

Total RNA was extracted from plant root samples after 14 days from germination/inoculation of pathogen according to manufacturer's instructions for RT-PCR analysis. Samples from three different cultivars (lines 15, 16 and 20) before and after clubroot infection were used. SuperScript RT-PCR III system (Invitrogen) was used to synthesize first-strand cDNA by reverse transcriptase following the protocol as described (Karsai et al., 2002).

Briefly, 2 to 4 mg of total RNA from each sample was treated according to manufacturer's guidelines (Invitrogen ThermoScript^{TM} RT-PCR #1682307). PCR was carried out with the DNA Master SYBR Green kit using 2 µL of cDNA (100 ng), in a 20 µL final volume with 0.5 µL each primer (10 µg and 10 µL of SYBR Green. Briefly, quantitative PCR was performed using CFX96 rtPCR Thermocycler (Bio-Rad) for 40 cycles at 95 °C for 15 s, specific annealing temperature 55 °C for 30s and 72 °C for 30s. Amplification specificity was checked using melting curve following the manufacturer's instructions. Results were analyzed using the Cq mean values and Induction fold was calculated according to the following equation.

$$\left\{\text{Average Cq value of Gene After treatment} - \text{Average Cq value of Gene Before treatment}\right\} - \left\{\text{Average Cq value of Reference Gene After treatment} - \text{Average Cq value of Reference Gene Before treatment}\right\} = e$$

Induction fold = $2^{(-e)}$

CHAPTER 3: RESULTS

3.1 *Brassica napus UBC13* genes

To identify *B. napus UBC13* genes, *Arabidopsis* Ubc13 protein sequence was used to search the *Brassica napus* protein database (through TAIR). Twelve highly similar proteins (E-values at or below 10^{-56}) were found. The respective proteins were named BnUbc13A-L. All twelve BnUbc13 proteins contain 153 amino acids and their alignment with AtUbc13s is shown in Figure 3-1. According to the previous data (Wen et al., 2006), AtUbc13A and AtUbc13B differ by only two amino acids, and both are conserved variations (Asp to Glu). Within BnUbc13s, BnUbc13A, B, I, J and L are identical to AtUbc13B; BnUbc13C and D are identical to each other but differ from AtUbc13B by 1 aa; BnUbc13E and K are identical to each other but differ from AtUbc13B by 1 aa; BnUbc13G, F and H differs from AtUbc13B by 1, 2 and 3 aa, respectively. All BnUbc13s are closer to AtUbc13B than to AtUbc13A (Figure 3-1).

```
AtUbc13A      MANSNLPRRIIKETQRLLSEPAPGISASPSEDNMRYFNVMILGPTQSPYEGGVFKLELFL      60
BnUbc13F      MANSNLPRRIIKETQRLLSEPAPGISASPSEENMRYFNVMVLGPTQSPYEGGVFKLELFL      60
BnUbc13H      MANSNLPRRIIKETQRLLSEPAPGISASPSEENMRYFNVMVLGPSQSPYEGGVFKLELFL      60
BnUbc13E      MANSNLPRRIIKETQRLLSEPAPGISASPSEENMRYFNVMVLGPSQSPYEGGVFKLELFL      60
BnUbc13K      MANSNLPRRIIKETQRLLSEPAPGISASPSEENMRYFNVMVLGPSQSPYEGGVFKLELFL      60
BnUbc13C      MANSNLPRRIIKETQRLLSEPAPGISASPSEENMRYFNVMILGPSQSPYEGGVFKLELFL      60
BnUbc13D      MANSNLPRRIIKETQRLLSEPAPGISASPSEENMRYFNVMILGPSQSPYEGGVFKLELFL      60
BnUbc13G      MANSNLPRRIIKETQRLLSEPAPGISASPSEENMRYFNVMILGPTQSPYEGGVFKLELFL      60
BnUbc13L      MANSNLPRRIIKETQRLLSEPAPGISASPSEENMRYFNVMILGPTQSPYEGGVFKLELFL      60
BnUbc13J      MANSNLPRRIIKETQRLLSEPAPGISASPSEENMRYFNVMILGPTQSPYEGGVFKLELFL      60
BnUbc13I      MANSNLPRRIIKETQRLLSEPAPGISASPSEENMRYFNVMILGPTQSPYEGGVFKLELFL      60
BnUbc13B      MANSNLPRRIIKETQRLLSEPAPGISASPSEENMRYFNVMILGPTQSPYEGGVFKLELFL      60
BnUbc13A      MANSNLPRRIIKETQRLLSEPAPGISASPSEENMRYFNVMILGPTQSPYEGGVFKLELFL      60
AtUbc13B      MANSNLPRRIIKETQRLLSEPAPGISASPSEENMRYFNVMILGPTQSPYEGGVFKLELFL      60
              *******************************:********:***:***************

AtUbc13A      PEEYPMAAPKVRFLTKIYHPNIDKLGRICLDILKDKWSPALQIRTVLLSIQALLSAPNPD      120
BnUbc13F      PEEYPMAVPKVRFLTKIYHPNIDKLGRICLDILKDKWSPALQIRTVLLSIQALLSAPNPD      120
BnUbc13H      PEEYPMSAPKVRFLTKIYHPNIDKLGRICLDILKDKWSPALQIRTVLLSIQALLSAPNPD      120
BnUbc13E      PEEYPMAAPKVRFLTKIYHPNIDKLGRICLDILKDKWSPALQIRTVLLSIQALLSAPNPD      120
BnUbc13K      PEEYPMAAPKVRFLTKIYHPNIDKLGRICLDILKDKWSPALQIRTVLLSIQALLSAPNPD      120
BnUbc13C      PEEYPMAAPKVRFLTKIYHPNIDKLGRICLDILKDKWSPALQIRTVLLSIQALLSAPNPD      120
BnUbc13D      PEEYPMAAPKVRFLTKIYHPNIDKLGRICLDILKDKWSPALQIRTVLLSIQALLSAPNPD      120
BnUbc13G      PEEYPMAAPKVRFLTKIYHPNIDKLGRICLDILKDKWSPALQIRTVLLSIQALLSAPNPD      120
BnUbc13L      PEEYPMAAPKVRFLTKIYHPNIDKLGRICLDILKDKWSPALQIRTVLLSIQALLSAPNPD      120
BnUbc13J      PEEYPMAAPKVRFLTKIYHPNIDKLGRICLDILKDKWSPALQIRTVLLSIQALLSAPNPD      120
BnUbc13I      PEEYPMAAPKVRFLTKIYHPNIDKLGRICLDILKDKWSPALQIRTVLLSIQALLSAPNPD      120
BnUbc13B      PEEYPMAAPKVRFLTKIYHPNIDKLGRICLDILKDKWSPALQIRTVLLSIQALLSAPNPD      120
BnUbc13A      PEEYPMAAPKVRFLTKIYHPNIDKLGRICLDILKDKWSPALQIRTVLLSIQALLSAPNPD      120
AtUbc13B      PEEYPMAAPKVRFLTKIYHPNIDKLGRICLDILKDKWSPALQIRTVLLSIQALLSAPNPD      120
              ******:.****************************************************

AtUbc13A      DPLSENIAKHWKSNEAEAVDTAKEWTRLYASGA      153
BnUbc13F      DPLSENIAKHWKSNEAEAVETAKEWTRLYASGA      153
BnUbc13H      DPLSENIAKHWKSNEAEAVETAKEWTRLYASGA      153
BnUbc13E      DPLSENIAKHWKSNEAEAVETAKEWTRLYASGA      153
BnUbc13K      DPLSENIAKHWKSNEAEAVETAKEWTRLYASGA      153
BnUbc13C      DPLSENIAKHWKSNEAEAVETAKEWTRLYASGA      153
BnUbc13D      DPLSENIAKHWKSNEAEAVETAKEWTRLYASGA      153
BnUbc13G      DPLSENIAKHWKSDEAEAVETAKEWTRLYASGA      153
BnUbc13L      DPLSENIAKHWKSNEAEAVETAKEWTRLYASGA      153
BnUbc13J      DPLSENIAKHWKSNEAEAVETAKEWTRLYASGA      153
BnUbc13I      DPLSENIAKHWKSNEAEAVETAKEWTRLYASGA      153
BnUbc13B      DPLSENIAKHWKSNEAEAVETAKEWTRLYASGA      153
BnUbc13A      DPLSENIAKHWKSNEAEAVETAKEWTRLYASGA      153
AtUbc13B      DPLSENIAKHWKSNEAEAVETAKEWTRLYASGA      153
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Figure 3-1: Sequence analysis of Ubc13s from *B. napus* and *A. thaliana* (Clustal Omega).

Amino acid sequence alignment of predicted *BnUBC13* gene products from *B. napus* and *Arabidopsis*. Identical residues shared by the majority of *Ubc13s* are highlighted.

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BnUBC13E        ATGGCCAACAGCAATCTTCCCCGAAGAATCATCAAGGAAACGCAACGTCTGCTCAGCGAA  60
BnUBC13K        ATGGCCAACAGCAATCTTCCCCGAAGAATCATCAAGGAAACGCAACGTCTGCTCAGCGAA  60
BnUBC13H        ATGGCCAACAGCAATCTGCCCCGAAGAATCATCAAGGAAACGCAACGTCTGCTCAGTGAA  60
BnUBC13D        ATGGCTAATAGCAATCTACCGCGAAGAATCATCAAGGAAACACAACGTCTACTTAGTGAA  60
BnUBC13F        ATGGCGAATAGCAATCTACCGCGAAGAATCATCAAGGAAACACAACGTCTACTTAGTGAA  60
BnUBC13B        ATGGCGAATAGTAATCTACCGCGAAGAATCATCAAGGAAACTCAACGGCTGCTTAGTGAA  60
BnUBC13J        ATGGCGAATAGTAATCTACCGCGAAGAATCATCAAGGAAACTCAACGGCTGCTTAGTGAA  60
BnUBC13C        ATGGCGAATAGTAATCTACCGCGAAGAATCATCAAGGAAACTCAACGCCTGCTTAGCGAA  60
BnUBC13A        ATGGCGAATAGTAATCTTCCTCGAAGAATCATCAAGGAAACTCAACGTCTTCTTAGTGAA  60
BnUBC13I        ATGGCGAATAGTAATCTTCCTCGAAGAATCATCAAGGAAACTCAACGTCTTCTTAGTGAA  60
BnUBC13L        ATGGCGAATAGTAATCTTCCTCGAAGAATCATCAAGGAAACTCAACGTCTTCTTAGTGAA  60
BnUBC13G        ATGGCGAATAGTAATCTTCCTCGAAGGATCATCAAGGAAACTCAACGTCTTCTTAGTGAA  60
                ***** ** ** ***** ** ***** ************** ***** ** ** ** ***

BnUBC13E        CCGGCTCCAGGTATAAGTGCATCTCCATCTGAGGAGAACATGAGGTACTTCAACGTTATG  120
BnUBC13K        CCGGCTCCAGGTATAAGTGCATCTCCATCTGAGGAGAACATGAGGTACTTCAACGTTATG  120
BnUBC13H        CCGGCTCCGGGTATAAGTGCATCTCCATCTGAGGAGAACATGAGATACTTCAACGTTATG  120
BnUBC13D        CCTGCTCCGGGGATAAGCGCGTCTCCGTCAGAGGAAAACATGCGTTATTTCAATGTCATG  120
BnUBC13F        CCTGCTCCGGGGATAAGTGCGTCTCCGTCAGAGGAAAACATGCGTTATTTCAATGTTATG  120
BnUBC13B        CCGGCTCCGGGGATAAGTGCGTCTCCGTCAGAGGAGAATATGCGATATTTCAATGTTATG  120
BnUBC13J        CCGGCTCCGGGGATAAGTGCGTCTCCGTCAGAGGAGAATATGCGATATTTCAATGTTATG  120
BnUBC13C        CCGGCTCCGGGGATAAGTGCGTCACCGTCAGAGGAGAATATGCGATATTTCAATGTTATG  120
BnUBC13A        CCCGCTCCGGGGATAAGTGCGTCTCCGTCAGAGGAAAATATGCGCTACTTCAATGTTATG  120
BnUBC13I        CCCGCTCCGGGGATAAGTGCGTCTCCGTCAGAGGAAAATATGCGCTACTTCAATGTTATG  120
BnUBC13L        CCCGCTCCGGGGATAAGTGCGTCTCCGTCAGAGGAAAATATGCGCTACTTCAATGTTATG  120
BnUBC13G        CCCGCTCCGGGGATAAGTGCGTCTCCCTCAGAGGAAAATATGCGCTACTTCAATGTTATG  120
                ** ***** ** ***** ** ** ** ** ***** ** *** * ** ***** ** ***

BnUBC13E        GTTCTTGGTCCTTCTCAATCACCTTATGAAGGAGGTGTTTTCAAGTTGGAGCTCTTTTTG  180
BnUBC13K        GTTCTTGGTCCTTCTCAATCACCTTATGAAGGAGGTGTTTTCAAGTTGGAGCTCTTTTTG  180
BnUBC13H        GTTCTTGGTCCTTCTCAATCACCTTATGAAGGAGGTGTTTTCAAGTTGGAGCTCTTTTTG  180
BnUBC13D        ATTCTTGGTCCTTCACAGTCTCCTTATGAAGGAGGAGTTTTCAAGTTGGAGCTCTTTTTG  180
BnUBC13F        GTTCTTGGTCCTACCCAGTCTCCTTATGAAGGAGGAGTTTTCAAGTTGGAGCTCTTTTTA  180
BnUBC13B        ATTCTTGGTCCTACCCAGTCTCCTTATGAAGGAGGAGTTTTCAAGTTGGAGCTCTTTTTG  180
BnUBC13J        ATTCTTGGTCCTACCCAGTCTCCTTATGAAGGAGGAGTTTTCAAGTTGGAGCTCTTTTTG  180
BnUBC13C        ATTCTTGGTCCTTCCCAGTCTCCTTATGAAGGAGGAGTTTTCAAGTTGGAGCTCTTTTTA  180
BnUBC13A        ATTCTTGGTCCTACCCAGTCTCCTTATGAAGGTGGAGTTTTCAAGTTGGAGCTCTTTTTA  180
BnUBC13I        ATTCTTGGTCCTACCCAGTCTCCTTATGAAGGTGGAGTTTTCAAGTTGGAGCTCTTTTTA  180
BnUBC13L        ATTCTTGGTCCTACCCAGTCTCCTTATGAAGGTGGAGTTTTCAAGTTGGAGCTCTTTTTA  180
BnUBC13G        ATTCTTGGTCCTACCCAGTCTCCTTATGAAGGTGGAGTTTTTAAGTTGGAGCTCTTTTTA  180
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BnUBC13E        CCTGAAGAGTACCCTATGGCAGCTCCCAAGGTTAGGTTTCTCACCAAGATATACCATCCT  240
BnUBC13K        CCTGAAGAGTACCCTATGGCAGCTCCCAAGGTTAGGTTTCTCACCAAGATATACCATCCT  240
BnUBC13H        CCTGAAGAGTACCCTATGTCAGCTCCCAAGGTTAGGTTTCTCACCAAGATATACCATCCT  240
BnUBC13D        CCTGAAGAATACCCTATGGCAGCTCCCAAAGTTAGGTTTCTCACCAAAATATACCATCCT  240
BnUBC13F        CCTGAAGAATACCCTATGGCTGTTCCCAAAGTTAGGTTTCTCACCAAGATATACCATCCT  240
BnUBC13B        CCTGAAGAGTATCCTATGGCAGCTCCCAAAGTTAGGTTTCTCACCAAGATTTACCATCCT  240
BnUBC13J        CCTGAAGAGTATCCTATGGCAGCTCCCAAAGTTAGGTTTCTCACCAAGATTTACCATCCT  240
BnUBC13C        CCTGAAGAGTATCCTATGGCAGCTCCCAAAGTTAGGTTTCTCACCAAGATTTACCATCCT  240
BnUBC13A        CCTGAAGAATATCCCATGGCAGCTCCCAAGGTTAGGTTTCTCACCAAGATATACCATCCT  240
BnUBC13I        CCTGAAGAATATCCCATGGCAGCTCCCAAGGTTAGGTTTCTCACCAAGATATACCATCCT  240
BnUBC13L        CCTGAAGAATATCCCATGGCAGCTCCCAAGGTTAGGTTTCTCACCAAGATATACCATCCT  240
BnUBC13G        CCTGAAGAATATCCCATGGCAGCTCCCAAGGTTAGGTTTCTCACCAAGATTTACCATCCT  240
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BnUBC13E      AATATTGACAAGCTGGGAAGAATCTGTCTTGATATCCTGAAAGACAAATGGAGCCCTGCT   300
BnUBC13K      AATATTGACAAGCTGGGAAGAATCTGTCTTGATATCCTGAAAGACAAATGGAGCCCTGCT   300
BnUBC13H      AACATTGACAAGCTTGGAAGAATCTGTCTTGATATCCTGAAAGACAAATGGAGCCCTGCT   300
BnUBC13D      AACATTGACAAGCTTGGAAGGATCTGCCTTGACATTCTGAAAGACAAATGGAGTCCTGCG   300
BnUBC13F      AACATTGACAAGCTTGGAAGGATCTGCCTTGACATACTGAAAGACAAATGGAGTCCTGCG   300
BnUBC13B      AACATTGACAAGCTTGGAAGAATTTGCCTTGACATATTGAAAGACAAGTGGAGTCCTGCG   300
BnUBC13J      AACATTGACAAGCTTGGAAGAATTTGCCTTGACATATTGAAAGACAAGTGGAGTCCTGCG   300
BnUBC13C      AACATTGACAAGCTTGGAAGAATTTGTCTTGACATACTGAAAGACAAATGGAGTCCTGCG   300
BnUBC13A      AACATTGACAAGCTTGGAAGAATCTGCCTTGACATACTGAAAGACAAGTGGAGTCCTGCA   300
BnUBC13I      AACATTGACAAGCTTGGAAGAATCTGCCTTGACATACTGAAAGACAAGTGGAGTCCTGCA   300
BnUBC13L      AACATTGACAAGCTTGGAAGAATCTGCCTTGACATACTGAAAGACAAGTGGAGTCCTGCA   300
BnUBC13G      AACATTGACAAGCTTGGAAGAATCTGCCTTGACATACTGAAAGACAAGTGGAGTCCTGCG   300
              ** *********** ***** ** ** ***** **  ********** ***** *****

BnUBC13E      CTACAAATACGAACAGTGCTCTTAAGTATTCAAGCTCTTCTGAGTGCACCAAACCCTGAT   360
BnUBC13K      CTACAAATACGAACAGTGCTCTTAAGTATTCAAGCTCTTCTGAGTGCACCAAACCCTGAT   360
BnUBC13H      CTACAAATACGAACAGTGCTCTTAAGTATTCAAGCTCTTCTGAGTGCACCAAACCCTGAT   360
BnUBC13D      CTACAGATAAGAACTGTGCTCTTGAGTATCCAAGCTCTTCTAAGTGCACCAAACCCTGAT   360
BnUBC13F      CTTCAAATAAGAACTGTGCTCTTGAGTATTCAAGCTCTTCTAAGTGCACCAAACCCGGAT   360
BnUBC13B      CTACAAATAAGAACTGTGCTCTTGAGTATTCAAGCTCTTCTAAGTGCACCAAACCCAGAT   360
BnUBC13J      CTACAAATAAGAACTGTGCTCTTGAGTATTCAAGCTCTTCTAAGTGCACCAAACCCAGAT   360
BnUBC13C      CTACAAATAAGAACTGTGCTCTTGAGTATTCAAGCTCTTCTAAGTGCACCAAACCCAGAT   360
BnUBC13A      CTACAAATAAGAACTGTGCTCTTGAGTATTCAAGCTCTTCTAAGTGCACCAAATCCAGAT   360
BnUBC13I      CTACAAATAAGAACTGTGCTCTTGAGTATTCAAGCTCTTCTAAGTGCACCAAATCCAGAT   360
BnUBC13L      CTACAAATAAGAACTGTGCTCTTGAGTATTCAAGCTCTTCTAAGTGCACCAAATCCAGAT   360
BnUBC13G      CTACAAATAAGAACTGTGCTCTTGAGTATTCAAGCTCTTCTAAGTGCACCAAACCCAGAT   360
              ** ** *** **** ******** ***** *********** *********** ** ***

BnUBC13E      GATCCATTGTCTGAGAACATCGCTAAGCATTGGAAGAGTAATGAGGCTGAAGCTGTGGAG   420
BnUBC13K      GATCCATTGTCTGAGAACATCGCTAAGCATTGGAAGAGTAATGAGGCTGAAGCTGTGGAG   420
BnUBC13H      GATCCATTGTCTGAGAACATCGCTAAGCATTGGAAGAGTAATGAGGCTGAAGCTGTGGAG   420
BnUBC13D      GATCCACTTTCTGAGAACATAGCTAAGCATTGGAAGAGTAATGAAGCTGAAGCTGTGGAG   420
BnUBC13F      GATCCACTGTCTGAGAACATAGCTAAGCATTGGAAGAGTAATGAAGCAGAAGCTGTTGAG   420
BnUBC13B      GATCCACTCTCTGAGAACATAGCTAAGCATTGGAAGAGTAATGAAGCTGAAGCTGTGGAG   420
BnUBC13J      GATCCACTCTCTGAGAACATAGCTAAGCATTGGAAGAGTAATGAAGCTGAAGCTGTGGAG   420
BnUBC13C      GATCCACTCTCTGAGAACATAGCAAAGCATTGGAAGAGTAATGAAGCTGAAGCCGTGGAG   420
BnUBC13A      GATCCCCTCTCTGAGAACATAGCTAAGCACTGGAAAAGCAATGAAGCAGAAGCTGTGGAG   420
BnUBC13I      GATCCCCTCTCTGAGAACATAGCTAAGCACTGGAAAAGCAATGAAGCAGAAGCTGTGGAG   420
BnUBC13L      GATCCCCTCTCTGAGAACATAGCTAAGCACTGGAAAAGCAATGAAGCAGAAGCTGTGGAG   420
BnUBC13G      GATCCCCTGTCTGAGAACATAGCTAAGCATTGGAAGAGTGATGAAGCAGAAGCTGTGGAG   420
              *****  * *********** ** ***** ***** **  **** ** ***** ** ***

BnUBC13E      ACAGCTAAAGAATGGACCCGTCTGTACGCAAGCGGCGCATGA   462
BnUBC13K      ACAGCTAAAGAATGGACCCGTCTGTACGCAAGCGGCGCATGA   462
BnUBC13H      ACAGCTAAAGAATGGACCCGTCTGTACGCAAGCGGCGCATAA   462
BnUBC13D      ACAGCTAAGGAGTGGACTCGTCTATATGCAAGTGGCGCTTGA   462
BnUBC13F      ACAGCTAAGGAGTGGACTCGTCTATATGCAAGTGGCGCTTGA   462
BnUBC13B      ACAGCTAAGGAGTGGACTCGTCTGTATGCAAGTGGCGCTTGA   462
BnUBC13J      ACAGCTAAGGAGTGGACTCGTCTGTATGCAAGTGGCGCTTGA   462
BnUBC13C      ACAGCTAAGGAGTGGACTCGTCTGTATGCAAGTGGCGCTTGA   462
BnUBC13A      ACAGCTAAGGAGTGGACTCGTCTATATGCAAGTGGCGCTTGA   462
BnUBC13I      ACAGCTAAGGAGTGGACTCGTCTATATGCAAGTGGCGCTTGA   462
BnUBC13L      ACAGCTAAGGAGTGGACTCGTCTATATGCAAGTGGCGCTTGA   462
BnUBC13G      ACAGCTAAGGAGTGGACTCGTCTATATGCAAGTGGCGCTTGA   462
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Figure 3-2: Sequence analysis of *UBC13* genes from *B. napus* (Clustal Omega).

3.2 Isolation of *B. napus UBC13* genes

The protein sequence of *Arabidopsis UBC13* were used to blast *B. napus* database,12 genes were identified to encode putative Ubc13s based on the high protein sequence identity and similarly with AtUbc13s. The gene ID, ORF length and percentage of amino acid identity with AtUbc13s are listed in Table 3-1.

Table 3-1: *Brassica napus UBC13* genes

Gene ID	Number of nucleotides	Location on individual chromosome	% Identity with AtUbc13B
BnaA06g11360D (A)	462	A06	100%
BnaC08g38130D (B)	462	C08	100%
BnaAnng13030D (C)	462	A (not known)	99.3%
BnaC08g17090D (D)	462	C08	99.3%
BnaA07g34450D (E)	462	A07	99.3%
BnaA08g23450D (F)	462	A08	98.7%
BnaC05g12900D (G)	462	C05	99.3%
BnaC06g39290D (H)	462	C06	98%
BnaC02g25260D (I)	462	C02	100%
BnaA07g38410D (J)	462	A07	100%
BnaC06g20310D (K)	462	C06	99.3%
BnaA02g19070D (L)	462	A02	100%

To clone these *BnUBC13* genes, primers were designed and synthesized for amplifying the open reading frames of these genes. To clone these genes into the yeast two hybrid vector pGBT9, the forward primers were designed to contain *Bam*H1, and reverse primers carry *Sal*1 restriction sites. Since BnUbc13 A, B, I, J, and L sequences are identical to AtUbc13B, which have been previously characterized (Wen et al., 2006), I, J, L genes were not cloned for further analysis. BnUbc13K was also not cloned as the sequence was similar to BnUbc13E. Although BnUbc13A and B sequences are identical to AtUbc13B, they were cloned to consider as additional controls.

3.3 Phylogenetic analysis of *BnUBC13* and *UBC13*s from other plant species

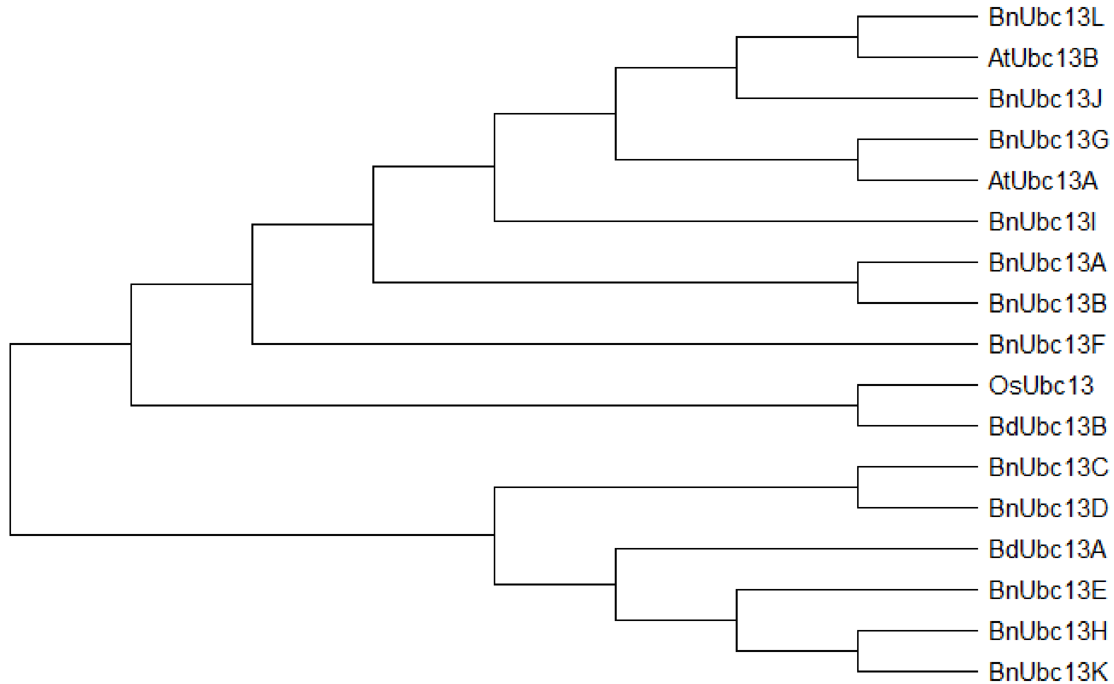


Figure 3-3: Phylogenetic analyses of hypothetical Ubc13 family proteins from different organisms.

The similarity clustering was conducted by using MEGA version X. High similarity is indicated by the short branch length between any two sequences. The prefixes for different species and the source of sequences (GenBank accession No) are: At, *A. thaliana* (AtUbc13A= AEE36165.1; AtUbc13B=NP564011); Os, *Oryza sativa (*NP001043834); Bd, *Brachypodium distachyon* (BdUbc13A=NP003567909; BdUbc13B=003569545).

3.4 Protein-protein interaction of *B. napus* Ubc13s with *A. thaliana* Uev1D

Ubc13 mediated Lys63-linked polyubiquitination requires Uev as a cofactor to function. Since BnUbc13s are plant proteins, AtUev1D was selected to observe the BnUbc13-AtUev1 interactions.

3.4.1 Physical interactions of BnUbc13s with AtUev1D revealed by yeast two-hybrid assay

Yeast two hybrid system (Fields and Song, 1989) was employed to analyze the protein-protein interaction of BnUbc13 and AtUev1D as the function of Lys63-linked ubiquitination by Ubc13 requires a Uev as a cofactor. Gal4 DNA-binding domain ($Gal4_{BD}$) vector pGBT9E was used to clone the ORFs of BnUbc13s and ORF of AtUev1D was cloned Gal4 activation domain ($Gal4_{AD}$) vector pGAD424E. As shown in Figure 3-4, expression of $Gal4_{BD}$-BnUbc13 with $Gal4_{AD}$-AtUev1D in yeast cells led to simultaneous induction of the endogenous P_{GAL1}-*HIS3* and P_{GAL2}-*ADE2* reporter genes, indicating that BnUbc13 interacts with AtUev1D. The strength of interaction appears to be the same. All BnUbc13s gave positive results with AtUev1D under high stringency (SD-Ade for 3 d) but, vectors alone with any of the BnUbc13s gave no interaction under the same condition. All of the above interactions are robust and deemed strong, as none of the negative controls reveals positive interactions under low stringency. Indeed, these results seem to be specific between BnUbc13s and AtUev1D as none of the proteins alone showed any positive interaction. Therefore, the results from yeast two-hybrid assay indicate that all BnUbc13s are able to physically interact with AtUev1D.

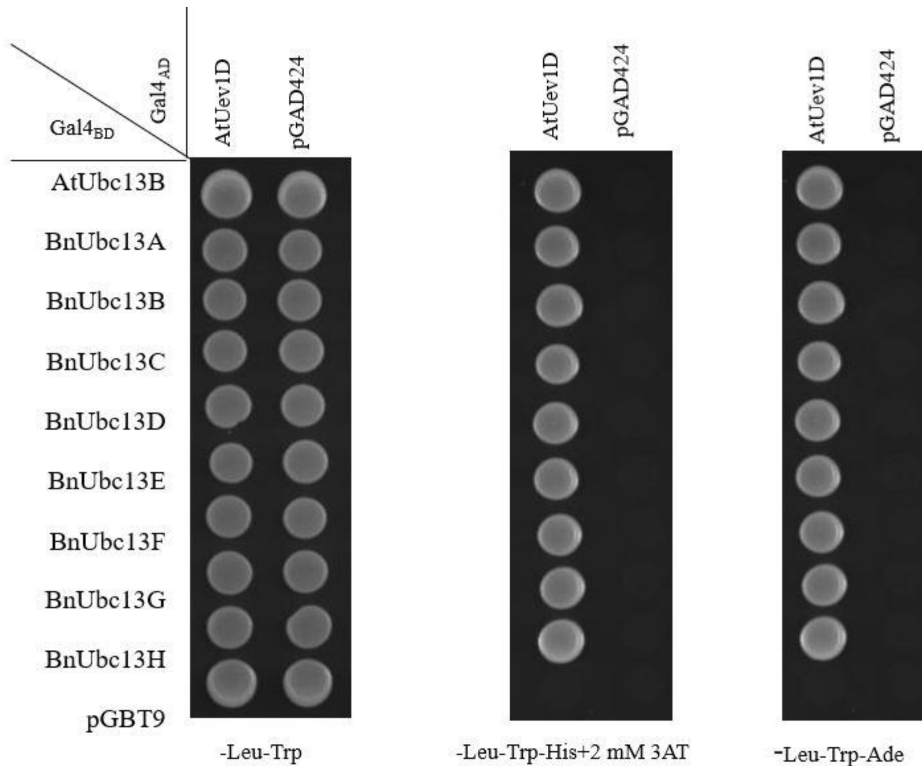
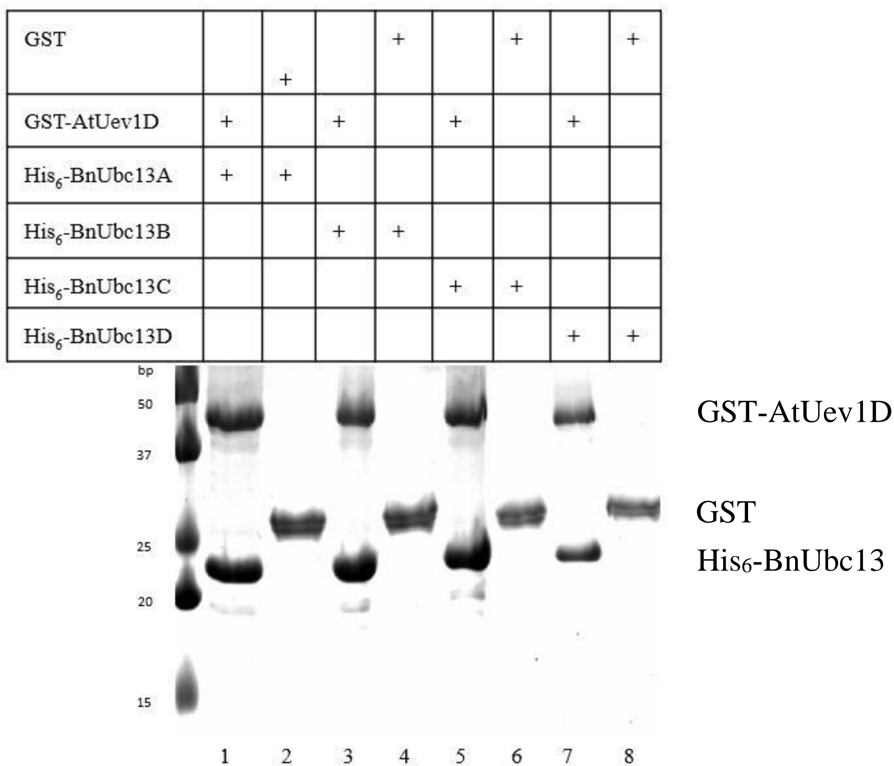


Figure 3-4: Interactions between BnUbc13s and AtUev1D in a yeast two-hybrid assay.The PJ69-4A transformants (five independent colonies from each transformation) carrying one $Gal4_{AD}$ (from pGAD424E) and one $Gal4_{BD}$ (from pGBT9E) construct were replicated on plates of SD-Trp-Leu (control) and SD-Trp-Leu-His plus various concentrations of 3-AT, and on SD-Trp-Leu-Ade, followed by incubation for 3 days at 30 ^{o}C.

3.4.2 Ubc13 and Uev interaction determined by a GST pulldown assay

The physical interaction between BnUbc13 and AtUev1D was further confirmed independently by a GST-affinity pull-down assay, as shown in Figure 3-5. In this experiment, bacterial cells were transformed with both His tagged BnUbc13s, and GST tagged AtUev1D and the produced proteins in bacterial cells was co-purified by adding to a column containing GST beads using the GST buffer. After incubation, washing and elution, GST-AtUev1D, but not GST alone, was found to be co-eluted only with all His_6-BnUbc13s. Hence, AtUev1D is able to form a stable heterodimer with all BnUbc13s. Target protein yields were monitored before and after IPTG induction as shown in Figure 3-6, which served as “inputs” for the pulldown assay as shown in Figure 3-5.

A

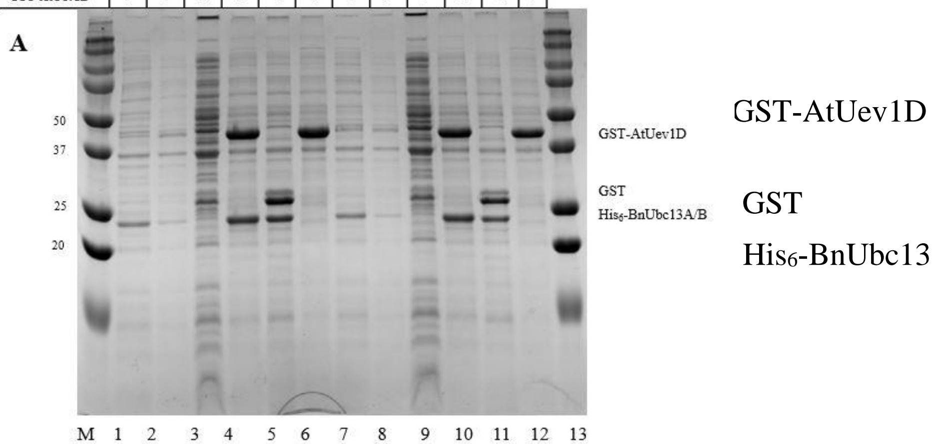


	1	2	3	4	5	6	7	8
GST		+		+		+		+
GST-AtUev1D	+		+		+		+	
His_6-BnUbc13A	+	+						
His_6-BnUbc13B			+	+				
His_6-BnUbc13C					+	+		
His_6-BnUbc13D							+	+

Figure 3-5: BnUbc13s binds AtUev1D in a GST pull-down assay.

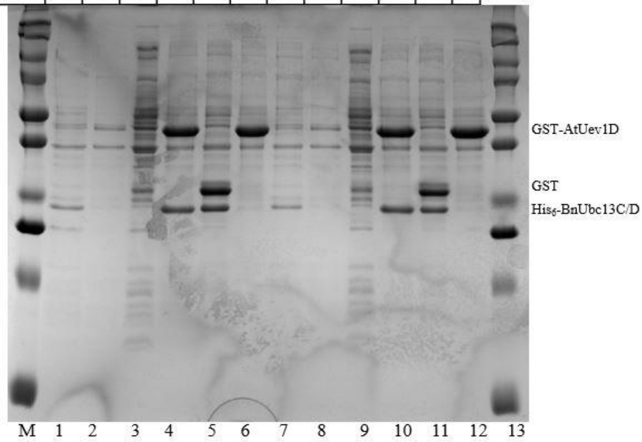
Co-purified GST-AtUev1D and His-BnUbc13s were added to microspin columns. After incubation and washing, the columns were eluted with reduced glutathione and subjected to SDS-PAGE gel analysis. (**A**) Lanes 1 to 8 show the purified GST-AtUev1D with His_6-BnUbc13A, GST alone with His_6-BnUbc13A, GST-AtUev1D with His_6-BnUbc13B, GST alone with His_6-BnUbc13B, GST-AtUev1D with His_6-BnUbc13C, GST alone with His_6-BnUbc13C, GST-AtUev1D with His_6-BnUbc13D, GST alone with His_6-BnUbc13D. (**B**) Lanes 1 to 8 show the purified GST-AtUev1D with His_6-BnUbc13E, GST alone with His_6-BnUbc13E, GST-AtUev1D with His_6-BnUbc13F, GST alone with His_6-BnUbc13F, GST-AtUev1D with His_6-BnUbc13G, GST alone with His_6-BnUbc13G, GST-AtUev1D with His_6-BnUbc13H, GST alone with His_6-BnUbc13H.

	Before IPTG induction			After IPTG induction			Before IPTG induction			After IPTG induction		
GST-AtUev1D His_6-BnUbc13A	+	-	-	+	-	-	-	-	-	-	-	-
GST His_6-BnUbc13A	-	+	-	-	+	-	-	-	-	-	-	-
GST-AtUev1D	-	-	+	-	-	+	-	-	-	-	-	-
GST-AtUev1D His_6-BnUbc13B	-	-	-	-	-	-	+	-	-	+	-	-
GST His_6-BnUbc13B	-	-	-	-	-	-	-	+	-	-	+	-
GST-AtUev1D	-	-	-	-	-	-	-	-	+	-	-	+



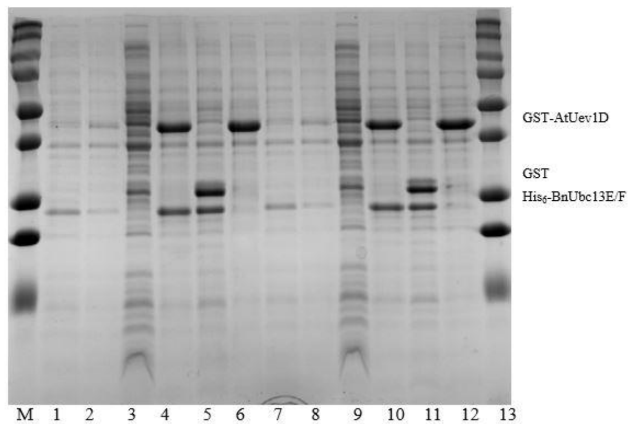
	Before IPTG induction			After IPTG induction			Before IPTG induction			After IPTG induction		
GST-AtUev1D His$_6$-BnUbc13C	+	-	-	+	-	-	-	-	-	-	-	-
GST His$_6$-BnUbc13C	-	+	-	-	+	-	-	-	-	-	-	-
GST-AtUev1D	-	-	+	-	-	+	-	-	-	-	-	-
GST-AtUev1D His$_6$-BnUbc13D	-	-	-	-	-	-	+	-	-	+	-	-
GST His$_6$-BnUbc13D	-	-	-	-	-	-	-	+	-	-	+	-
GST-AtUev1D	-	-	-	-	-	-	-	-	+	-	-	+

B



	Before IPTG induction			After IPTG induction			Before IPTG induction			After IPTG induction		
GST-AtUev1D His$_6$-BnUbc13E	+	-	-	+	-	-	-	-	-	-	-	-
GST His$_6$-BnUbc13E	-	+	-	-	+	-	-	-	-	-	-	-
GST-AtUev1D	-	-	+	-	-	+	-	-	-	-	-	-
GST-AtUev1D His$_6$-BnUbc13F	-	-	-	-	-	-	+	-	-	+	-	-
GST His$_6$-BnUbc13F	-	-	-	-	-	-	-	+	-	-	+	-
GST-AtUev1D	-	-	-	-	-	-	-	-	+	-	-	+

C



	Before IPTG induction			After IPTG induction			Before IPTG induction			After IPTG induction		
GST-AtUev1D His6-BnUbc13G	+	-	-	+	-	-	-	-	-	-	-	-
GST His6-BnUbc13G	-	+	-	-	+	-	-	-	-	-	-	-
GST-AtUev1D	-	-	+	-	-	+	-	-	-	-	-	-
GST-AtUev1D His6-BnUbc13H	-	-	-	-	-	-	+	-	-	+	-	-
GST His6-BnUbc13H	-	-	-	-	-	-	-	+	-	-	+	-
GST-AtUev1D	-	-	-	-	-	-	-	-	+	-	-	+

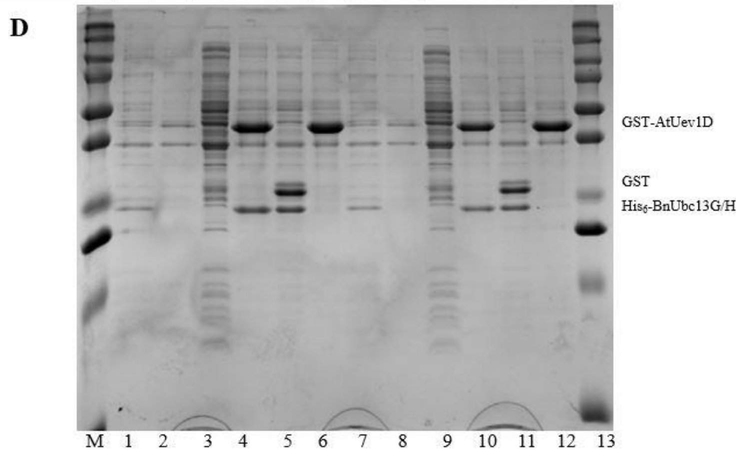


Figure 3-6: Expression of BnUbc13s and AtUev1D before and after IPTG induction. Bacterial cells were induced by 0.2 mM IPTG and followed by incubation at 37 °C for 6 hours. (**A**) BnUbc13A (lanes 1-6) and BnUbc13B (lanes 7-12). (**B**) BnUbc13C (lanes 1-6) and BnUbc13D (lanes 7-12). (**C**) BnUbc13E (lanes 1-6) and BnUbc13F (lanes 7-12). (**D**) BnUbc13G (lanes 1-6) and BnUbc13H (lanes 7-12). Lanes 1-3 and 7-9 contain cell extracts before induction, and lanes 4-6 and 10-12 contain cell extracts after IPTG induction.

3.5 Functional complementation of yeast *ubc13* null mutants by *BnUBC13s*

3.5.1 *BnUBC13* rescued yeast *ubc13* mutant from killing by DNA-damaging agents

3.5.1.1 *BnUBC13*s complemented the yeast *ubc13* error-free DDT defect

Budding yeast *UBC13* function in the error-free DDT pathway (Broomfield et al., 1998; Brusky et al., 2000). Two types of experiments were carried out to check whether BnUbc13s have

the same functional properties as yeast Ubc13. Gradient plate assay and serial dilution assays were performed to determine whether *BnUBC13s* could functionally complement the error-free damage tolerance defect in the yeast *ubc13* null mutant. The yeast *ubc13* mutant displays an increased sensitivity to a variety of DNA-damaging agents such as UV, 4NQO (4-Nitroquinoline 1-oxide), MMS (methyl methanesulfonate) (Brusky et al., 2000), which can be assessed by a serial dilution assay. Expression of *BnUBC13*s from the yeast two-hybrid plasmid rescued the *ubc13* mutant from killing by MMS (alkylation damage), 4-nitroquinoline 1- oxide (4NQO, bulky lesions) and UV irradiation to a level comparable to wild type cells; in contrast, the pGBT9 vector alone did not confer any DNA damage resistance to the *ubc13* mutant.

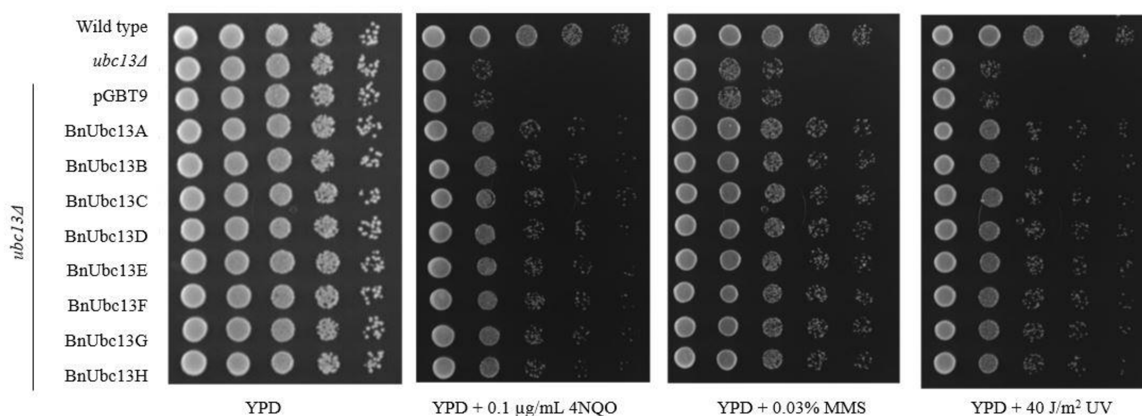


Figure 3-7: Functional complementation of the yeast *ubc13* null mutant by *BnUBC13s.* HK578-10D (wild type); WXY904 (*ubc13*Δ) and WXY9 (*ubc13*Δ) transformants were grown overnight and printed onto YPD, YPD + 0.1 µg/mL 4NQO and YPD + 0.03% MMS plates, and YPD plate followed by 40 J/m^2 UV irradiation. The plates were incubated at 30 ºC for 2 days before being photographed. Several transformants of each treatment were tested with the same result, and only one is shown here.

3.5.1.2 *BnUBC13*s rescued the *ubc13 rev3* double mutant

According to previous studies, the error-free DDT pathway requires yeast *UBC13* (Brusky et al., 2000) and Lys63-linked polyubiquitination either *in vitro* (Hofmann and Pickart, 2001) or *in vivo* (Hoege et al., 2002). To determine whether the *BnUBC13*s also play critical roles in error-

free DDT, we took advantage of the previous observation that yeast mutants defective in both error-prone and error-free DDT branches become extremely sensitive to certain types of DNA damage like induced by a DNA damaging agent like MMS (Broomfield et al., 1998; Xiao et al., 1999). In order to find out whether *BnUBC13*s have the same function as yeast *UBC13,* gradient plate assay was carried out to determine whether *BnUBC13*s could functionally complement the error-free damage tolerance defect in yeast *ubc13* mull mutant.

As shown in Figure 3-8, in an extremely low MMS concentration (0.001%) when neither *ubc13* nor *rev3* single mutant display apparent sensitivity, the *ubc13 rev3* double mutant did not grow at all in a gradient plate assay, indicating their strong synergistic interaction. When transformed with *BnUBC13* genes*,* the double mutant restored growth in the MMS gradient plates, inicating that *BnUBC13s* can rescue the severe double mutant phenotype and that *BnUBC13*s function in the error-free pathway.

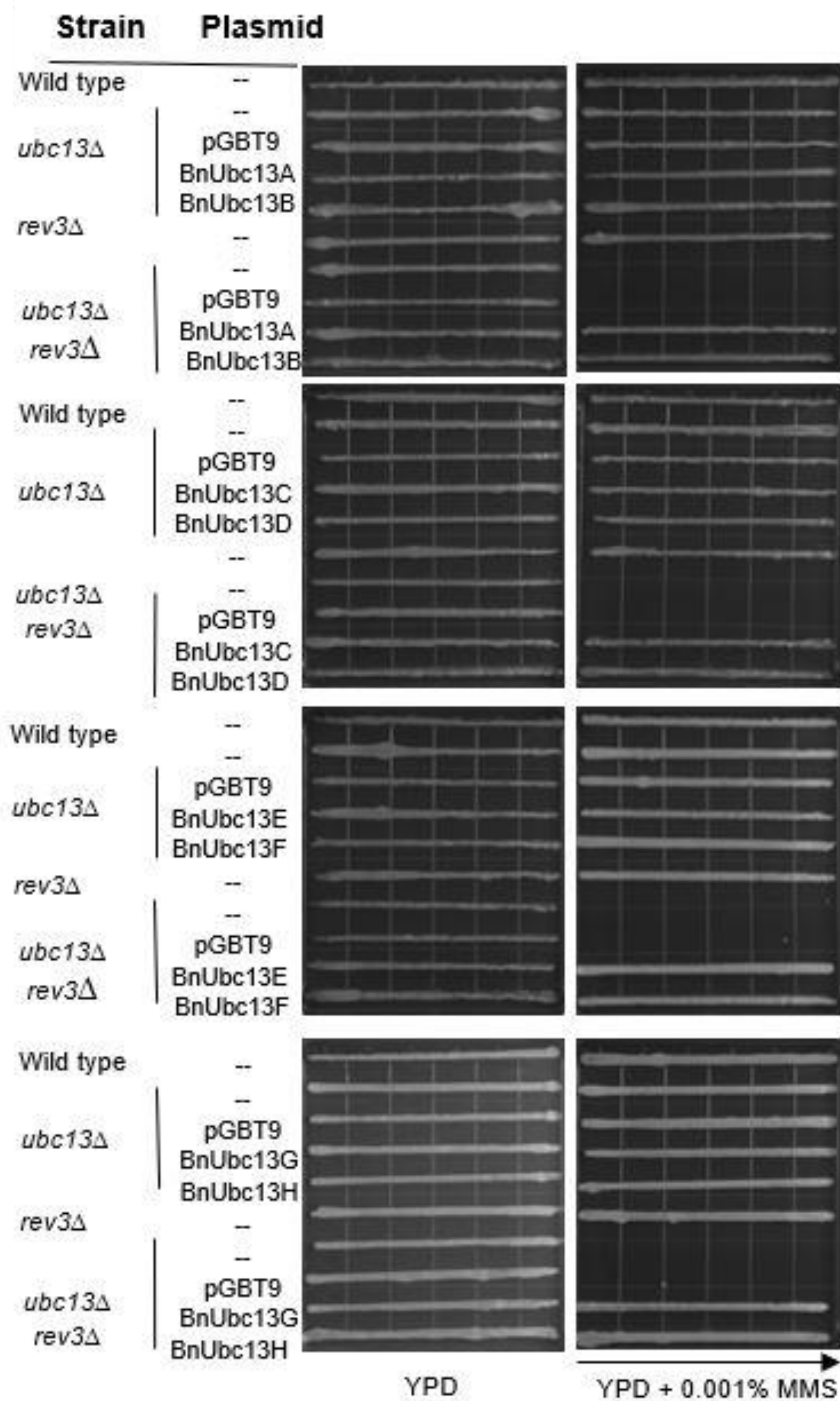

Strain
Plasmid
Wild type
ubc13Δ
rev3Δ
ubc13Δ
rev3Δ
pGBT9
BnUbc13A
BnUbc13B
BnUbc13C
BnUbc13D
BnUbc13E
BnUbc13F
BnUbc13G
BnUbc13H
YPD
YPD + 0.001% MMS

Figure 3-8: Functional complementation of the yeast *ubc13* mutant and *ubc13 rev3* double mutant by *BnUBC13*. Yeast strain HK578-10D (wild type), *ubc13*Δ (WXY904), *ubc13*Δ transformants, *rev3Δ* (WXY1233), *ubc13Δ rev3Δ* (WXY921) and *ubc13Δ rev3Δ* transformants were grown on the YPD plate and YPD + 0.001% MMS gradient plate to check whether *BnUBC13*s can complement the yeast *ubc13*Δ mutant. The plates were incubated at 30 ºC for 2 days before the photographs. The arrow indicates higher MMS concentrations. Several transformants of each treatment were tested with the same result, and only one is shown here.

3.5.1.3 Dual rescue of yeast *ubc13 mms2* by *BnUBC13*s and *AtUEV1D*

Figure 3-9 shows another experiment to check whether BnUbc13 and AtUev1D are dually functional in yeast cells. In budding yeast cells, Ubc13 and a Uev called Mms2 have physical interaction (Hofmann and Pickart, 1999) and targets for error-free DDT (Broomfield et al., 1998; Brusky et al., 2000). In order to assess in vivo complex formation and and functions between *Arabidopsis* Uev1D and BnUbc13s, yeast *ubc13 mms2* double mutant was created and co-transformed it with *AtUEV1D* and *BnUBC13*s. The *ubc13Δ mms2Δ* double mutant was employed to find out when both proteins are from plants, whether plant-plant proteins interact similarly in yeast cells. When the double mutant cells were transformed with only *BnUBC13* or *AtUEV1D*, the transformed cells did not display enhanced resistance to MMS implying that both Ubc13 and Uev are required for successful DDT function. When both *BnUBC13*s and *AtUEV1D* are present in the cell, it completely restored the MMS resistance to the wild-type level. According to Figure 3-9, BnUbc13 can replace yeast Ubc13, while AtUev1D was employed as the second protein to confirm the plant protein interaction. The experiment was carried out to confirm plant proteins BnUbc13 and AtUev1D interact with each other and promote error-free DT in yeast cells. Results from the above experiment reveals that *AtUEV1D* and *BnUBC13*s can jointly complement *ubc13*Δ and *mms2*Δ in yeast, indicating that BnUbc13 must be able to bind AtUev1D in yeast cells to form a functional E2 complex and promote Lys63-linked polyubiquitination, which is a highly conserved process within eukaryotes.

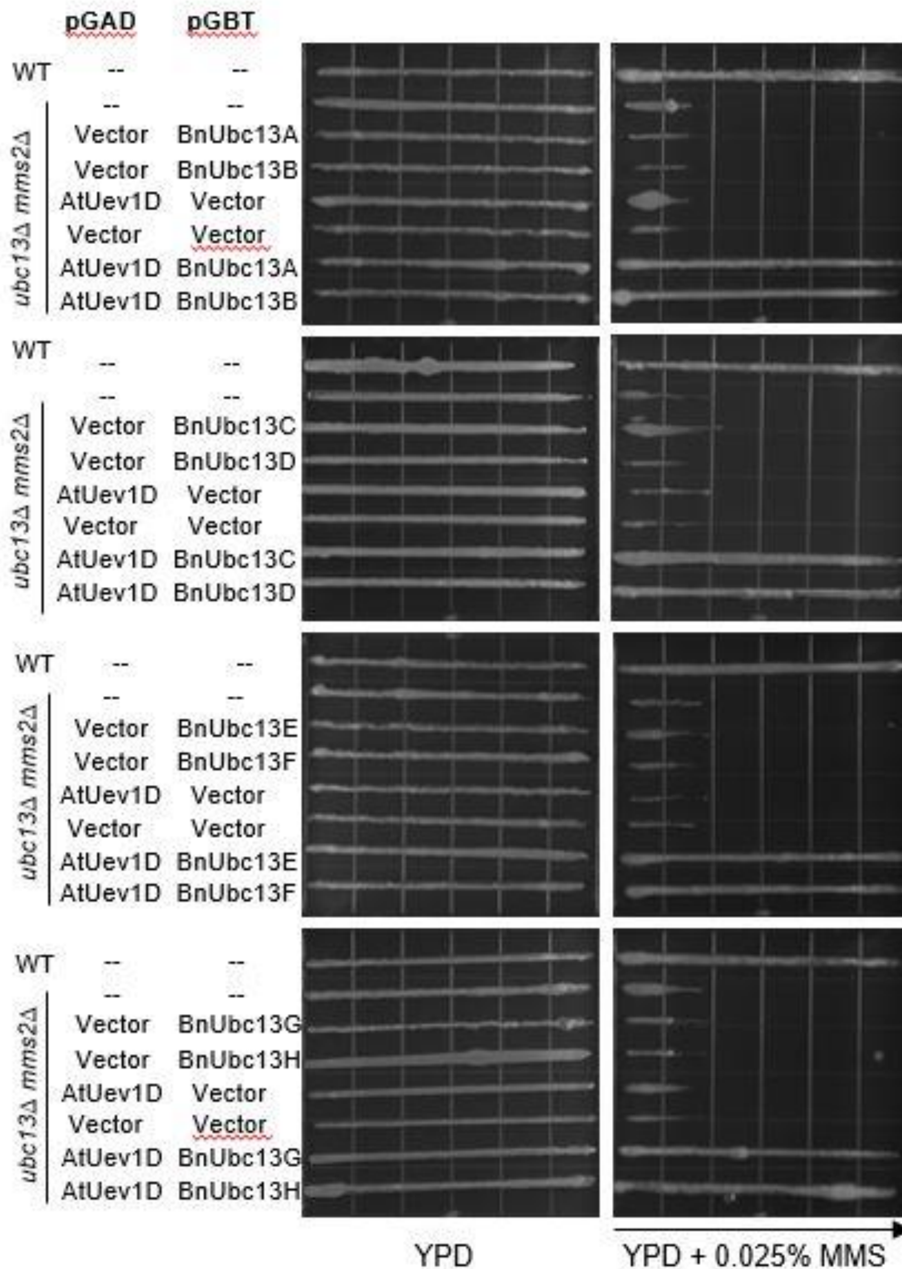


Figure 3-9: Functional complementation of the yeast *ubc13 mms2* double mutant by *BnUBC13*s and *AtUev1D*. Yeast strain HK578-10D (wild type), *ubc13Δ mms2Δ* (WXY955) and *ubc13Δ mms2Δ* transformants were grown on the YPD plate and YPD + 0.025% MMS gradient plate. The plates were incubated at 30 ºC for 2 days before the photographs. The arrow indicates higher MMS concentrations. Several transformants of each treatment were tested with the same result, and only one is shown here.

3.5.2 Protection of *ubc13* cells from spontaneous mutagenesis by *BnUBC13* genes

Yeast *UBC13* is a member of the error-free DDT pathway and plays an important role in protecting yeast cells from mutagenesis and cell death caused by DNA-damaging agents (Broomfield et al., 1998; Brusky et al., 2000). Therefore, a spontaneous mutagenesis assay was performed to determine whether *BnUBC13s* could functionally complement the error-free PRR defect in the yeast. DBY747 cells with *ubc13*Δ carry a *trp1-289* amber mutation that can be reverted to Trp+ by nucleotide substitutions (Xiao and Samson, 1993). One of the most astonishing phenotypes of a yeast *mms2* (Broomfield et al., 1998) or *ubc13* (Brusky et al., 2000) mutant is its ability to increase spontaneous mutagenesis massively, indicating that these genes play an important role in protecting cells from genome instability. In this experiment it was apparent that inactivation of *UBC13* causes nearly 27-fold increase in spontaneous mutation rate compared with wild-type yeast strain. In contrast, when *ubc13*Δs cells were transformed with *BnUBC13*s, the spontaneous mutation rate dropped to near wild-type levels. These results suggest that BnUbc13s are able to replace the Ubc13 DDT function in yeast. In addition, it can be assumed that BnUbc13s can work with Mms2 in yeast to promote Lys63 polyubiquitination. The large increase in spontaneous mutation rate supports a notion that *UBC13* plays a vital role in maintaining host genome stability.

Table 3-2: Spontaneous mutation rates of *S. cerevisiae ubc13* mutants.

Strain[a]	Key Alleles	Rate (x 10^{-8})[b]	Fold increase[c]
DBY747	Wild type	17.07 ±0.55	1
WXY849	*ubc13Δ*	459.45 ±7.34	26.91
WXY849/BnUbc13A	*ubc13Δ/BnUBC13A*	27.28 ±1.99	1.59
WXY849/BnUbc13B	*uubc13Δ/BnUBC13B*	28.05 ±4.66	1.64
WXY849/BnUbc13C	*ubc13Δ/BnUBC13C*	30.83 ±1.70	1.80
WXY849/BnUbc13D	*ubc13Δ/BnUBC13D*	29.38 ±2.64	1.72
WXY849/BnUbc13E	*ubc13Δ/BnUBC13E*	30.59 ±2.66	1.79
WXY849/BnUbc13F	*ubc13Δ/BnUBC13F*	31.24 ±5.71	1.83
WXY849/BnUbc13G	*ubc13Δ/BnUBC13G*	28.07 ±5.07	1.64
WXY849/BnUbc13H	*ubc13Δ/BnUBC13H*	31.98 ±3.17	1.87

[a] All strains are isogenic derivatives of DBY747.

[b] Spontaneous mutation rates are the average of three independent experiments with standard deviations.

[c] Relative to the wild type of mutation rate.

3.6 BnUbc13 mediates Lys63-linked polyubiquitination with AtUev1 *in vitro*

Extensive studies have identified that Ubc13 is the only known E2 capable of catalyzing the Lys63-linked polyubiquitination (Hofmann and Pickart, 1999); however, Ubc13 alone is insufficient and Uev is a compulsory cofactor to promote Lys63-linked Ub chain assembly. In addition, the Ubc13-Uev complex catalyzes poly-Ub chain assembly only through Ub-K63 but not Ub-K48 linkage (Hofmann and Pickart, 1999, 2001; McKenna et al., 2001). AtUbc13A and AtUbc13B promote Lys63-linked polyubiquitination with any of the four AtUev1s (Wen et al., 2008). BnUbc13 and AtUev1D proteins were purified for an *in vitro* ubiquitination assay to identify the catalytic activity of this E2 complex. BnUbc13 alone, AtUev1D alone, BnUbc13 + AtUev1D with Ub-K63R mutation did not form poly-Ub chains and, as a result no additional bands were identified on the membrane. When both BnUbc13s and AtUev1D were combined with wild Ub in the same reaction, di-Ub, tri-Ub and even longer Ub chains were readily formed. In contrast, the reaction mixture containing BnUbc13, AtUev1D and Ub-K63R completely abolished the formation of poly-Ub chains. The obtained results demonstrate that BnUbc13 and AtUev1D can form free poly-Ub chains and that these chains are Lys63-linked (Figure 3-10A, lanes 1, 5, 9 and Figure 3-10B, lanes 4, 8). The very bottom band of the western blot was deemed to be mono-Ub, whose intensity did not appear to account for the total Ub input. This might be due to the small size of Ub that may have run out from the gel. Another possibility may be due to protein transfer from gel to the membrane. Alternatively, the antibodies may have a higher affinity for poly-Ub chains than mono-Ub.

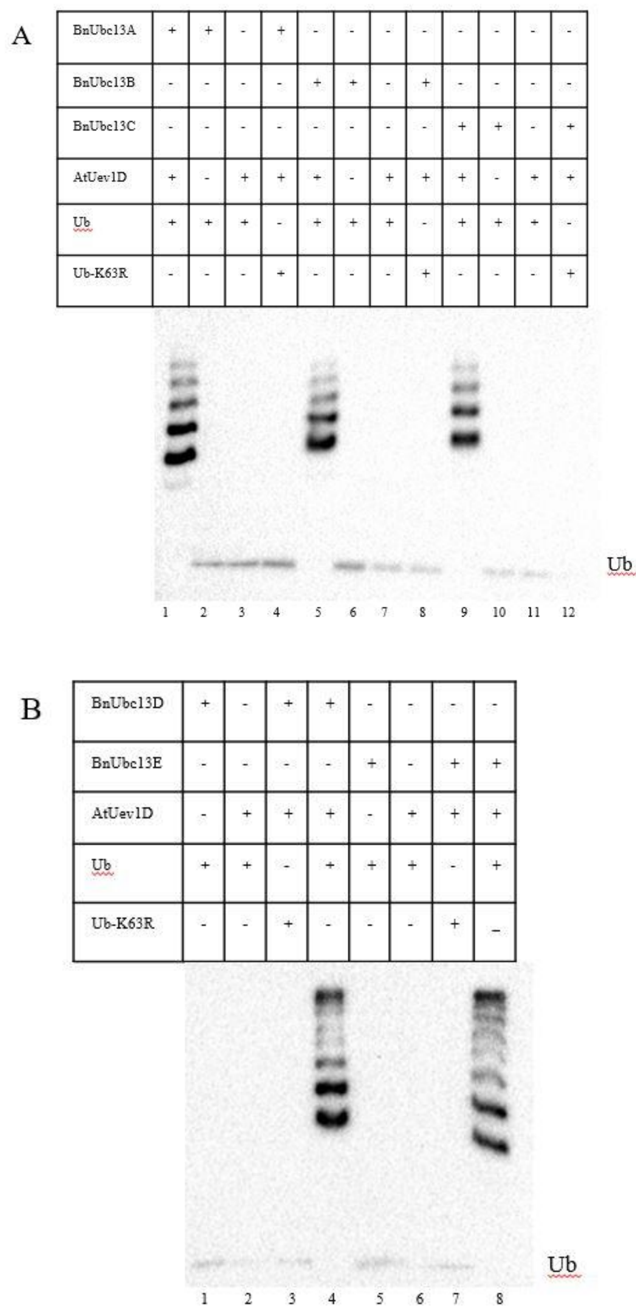


Figure 3-10: *In vitro* Ub conjugation assay using purified proteins of *Brassica napus* Ubc13s and *Arabidopsis thaliana* Uev1D. (**A**) Ub conjugation by BnUbc13A, B, C and AtUev1D. (**B**) Ub conjugation by BnUbc13D, E and AtUev1D. An *in vitro* Ub conjugation assay was performed using purified proteins as indicated. Assay samples were subjected to SDS-PAGE and western blot was performed using anti-Ub antibody and poly-Ub formation was monitored.

3.7 Screening of canola *Bnubc13* mutant lines after genome editing

One sgRNA was designed to target five *BnUBC13* genes and constructed into a pYLCRISPR/Cas9$_{ubi}$-H binary vector. Thirty selected plants were used for the analysis using *B. napus* cv. Westar (DH12075) as a reference. PCR reactions were proceeded for five target *BnUBC1*3 genes and the PCR products were confirmed by agarose gel electrophoresis based on the anticipated product size and sequences around the target site were subsequently determined. As summarized in Table 3-3 and illustrated in Figures 3-11 ~3-15, the analyzed plants contained insertion, deletion and substitution mutations in all five targeted *BnUBC13* genes. The purpose of the screen was to obtain genome edited plants, particularly homozygous mutations in the targeted genes. It was found that over half (17/30) lines carried *BnUBC13K* homozygous mutations and only 10% (3/30) carried homozygous *BnUBC13H* mutations, whereas about 1/3 lines carried homozygous mutations in other three *BnUBC13* genes. However, more than half (16/30) lines carried heterozygous *BnUBC13H* mutations (Table 3-3), indicating that all five *BnUBC13* genes were efficiently targeted. Another striking difference was that the *BnUBC13I* gene was mainly mutated by nucleotide insertions (8/10) but no base substitution was found. In contrast, *BnUBC13H* was mainly mutated by base substitution (18/21) but not by insertion (Table 3-3).

Table 3-3: Results of gene knocked out mutants showing the type of mutation

Gene	Type of Mutation		Plant lines carrying the mutations		
	Homozygous Mutation	Heterozygous Mutation	Insertion	Deletion	Substitution
BnaA02g1970D/BnUBC13L	10	5	14, 21, 22, 23, 28, 29	26	1, 7, 8, 9, 10, 11, 12, 24
BnaC02g2560D/BnUBC13I	10	0	6, 7, 17, 19, 22, 23, 26, 29	1, 5	-
BnaA07g38410D/BnUBC13J	9	3	3, 7, 27	5, 26, 30	1, 4, 6, 9, 19, 22
BnaC06g20310D/BnUBC13K	17	2	5, 7, 9, 12, 14, 17, 21, 24, 27, 28, 29, 30	1, 23, 26	6, 19, 20, 21

BnaC06g39290D/BnUBC13H	3	16	-	3	5, 6, 7, 11, 13, 14, 15, 17, 18, 19, 20, 21, 23, 24, 25, 26, 27, 28

Table 3-4 indicates that 28 out of 30 regenerated plant lines contained at least one *BnUBC13* mutations, out of which six lines (1, 5, 6, 19, 22 and 23) contained mutations in four *BnUBC13* genes, and two lines (7 and 26) contained mutations in all five *BnUBC13* genes. Line 26 contained four homozygous mutations and one heterozygous mutation.

Table 3-4: Results of gene knocked out mutants

Line	***BnUBC13L***	***BnUBC13I***	***BnUBC13J***	***BnUBC13K***	***BnUBC13H***
1	Heterozygous (C-to-G)	Homozygous (-A)	Heterozygous (A-to-C)	Homozygous (-A)	NA
2	NA	NE	NE	NE	NA
3	NA	NE	Homozygous (+C)	NE	Homozygous (-A)
4	NA	NE	Heterozygous (T-to-G)	NE	NA
5	NA	Homozygous (-A)	Homozygous (-A)	Homozygous (+A)	Heterozygous (G-to-C)
6	NE	Homozygous (+A)	Heterozygous	Heterozygous (C-to-G)	Heterozygous (T-to-C)
7	Heterozygous (C-to-T)	Homozygous (+A)	Homozygous (+T)	Homozygous (+T)	Heterozygous (C-to-A)
8	Heterozygous (T-to-A)	NE	NE	NE	NA
9	Heterozygous (C-to-T)	NE	Homozygous (C-to-T)	Homozygous (+A)	NA
10	Heterozygous (A-to-T)	NE	NE	NE	NA
11	Homozygous (+T)	NE	NE	NE	Homozygous (A-to-G)
12	Homozygous (A-to-C)	NE	NE	Homozygous (+A)	NA
13	NE	NE	NE	NE	Heterozygous (T-to-C)
14	Homozygous (+A)	NE	NE	Homozygous (+A)	Homozygous (T-to-C)
15	NA	NE	NE	NE	Heterozygous (T-to-C)
16	NA	NE	NE	NE	NA
17	NA	Homozygous (+G)	NE	Homozygous (+C)	Heterozygous (C-to-A)
18	NA	NE	NE	NE	Heterozygous (A-to-T)
19	NA	Homozygous (+T)	Homozygous (G-to-T)	Homozygous (A-to-C)	Heterozygous (A-to-T)
20	NA	NE	NE	Homozygous (A-to-C)	Heterozygous (A-to-T)
21	Homozygous (+C)	NE	NE	Homozygous (+A)	Heterozygous (A-to-T)
22	Homozygous (+A)	Homozygous (+A)	Homozygous (+A)	Heterozygous (G-to-A)	NA
23	Homozygous (+T)	Homozygous (+T)	NE	Homozygous (-A)	Heterozygous (T-to-C)
24	Homozygous (A-to-C, C-to-T)	NE	NE	Homozygous (+G)	Heterozygous
25	NE	NE	NE	NE	Heterozygous (A-to-T)
26	Homozygous (-A)	Homozygous (-A)	Homozygous (-A)	Homozygous (-A)	Heterozygous (T-to-C)
27	NA	NE	Homozygous (+A)	Homozygous (+C)	Heterozygous (T-to-C)
28	Homozygous (+A)	NE	NE	Homozygous (+C)	Heterozygous (T-to-C)
29	Homozygous (+A)	Homozygous (+A)	NE	Homozygous (+A)	NA
30	NA	NE	Homozygous (-A)	Homozygous (+C)	NE

NA: Not Applicable **NE: Not Edited**

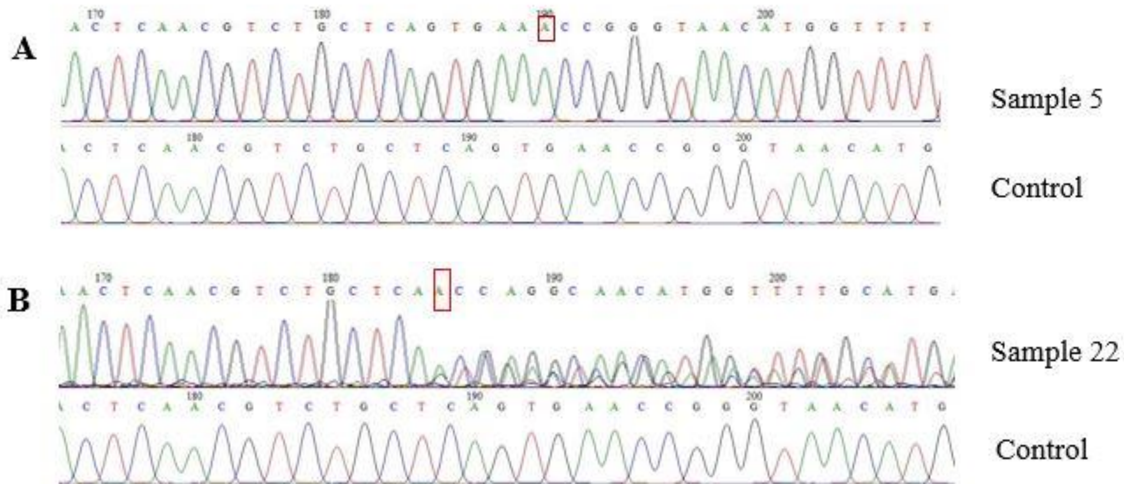


Figure 3-11: Sample identification of *BnUBC13K* mutations. (A) Homozygous mutation in line 5. Wild type sequence is given in the lower panel as a reference. In comparison, both alleles in line 5 contained a +A insertion (boxed). (B) Heterozygous mutation in line 22. Wild type sequence is given in the lower panel as a reference. The mutant allele contained a +A insertion (boxed).

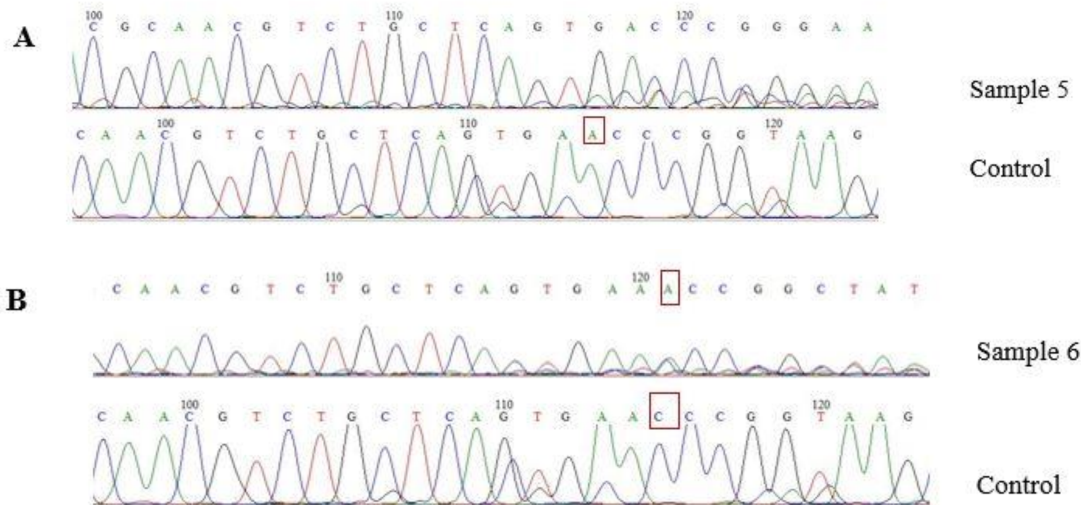


Figure 3-12: Sample identification of *BnUBC13J* mutations. (A) Homozygous mutation in line 5. Wild type sequence is given in the lower panel as a reference. In comparison, both alleles in line 5 contained a -A deletion (boxed). (B) Heterozygous mutation in line 6. Wild type sequence is given in the lower panel as a reference. The mutant allele contained A substituted by C (boxed).

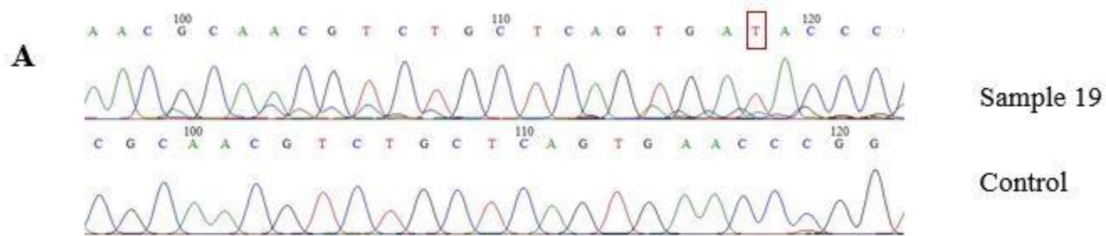


Figure 3-13: Sample identification of *BnUBC13I* mutations. (A) Homozygous mutation in line 19. Wild type sequence is given in the lower panel as a reference. In comparison, both alleles in line 19 contained a +A insertion (boxed).

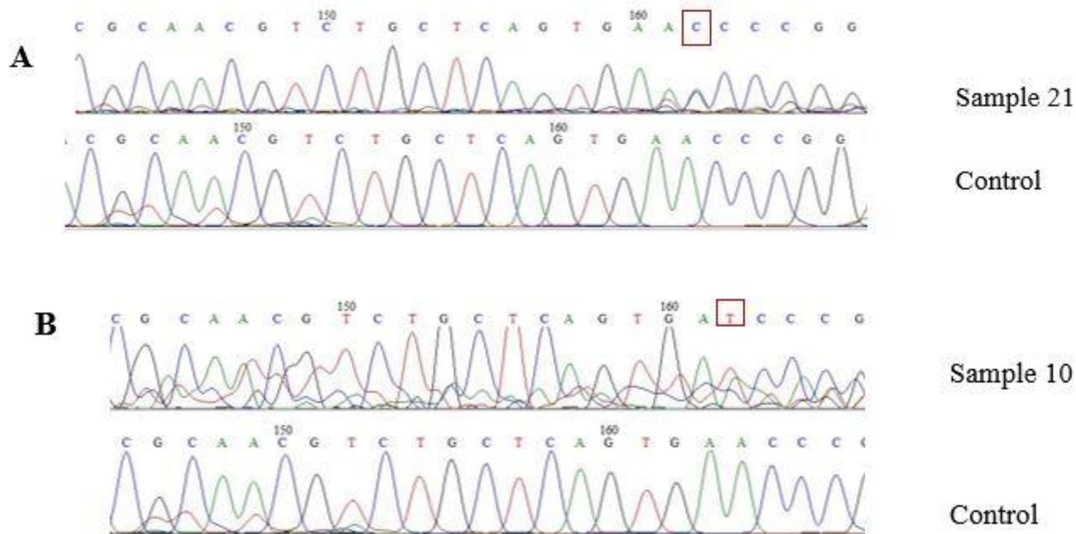


Figure 3-14: Sample identification of *BnUBC13L* mutations. (A) Homozygous mutation in line 21. Wild type sequence is given in the lower panel as a reference. In comparison, both alleles in line 21 contained a +C insertion (boxed). (B) Heterozygous mutation in line 10. Wild type

sequence is given in the lower panel as a reference. The mutant allele contained a A substituted by T (boxed).

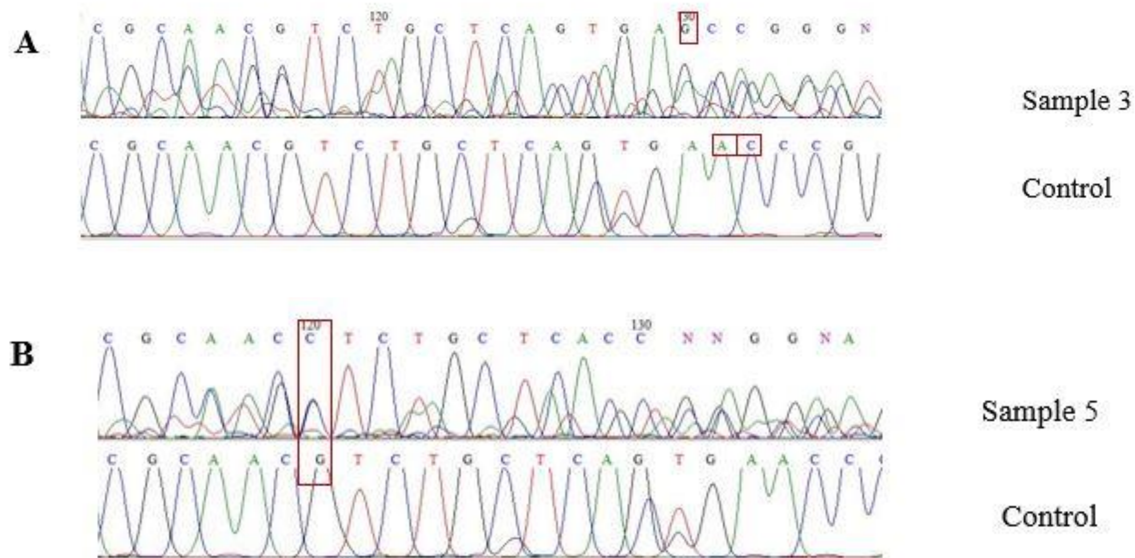


Figure 3-15: Sample identification of *BnUBC13H* mutations. (A) Homozygous mutation in line 3. Wild type sequence is given in the lower panel as a reference. In comparison, both alleles in line 3 contained a -A deletion (boxed) and C got converted to G. (B) Heterozygous mutation in line 5. Wild type sequence is given in the lower panel as a reference. The mutant allele contained a C substituted by G (boxed).

3.8 Analysis of *BnUBC13* expression from the RNA-seq data

3.8.1 Transcriptional analysis of *BnUBC13* genes in response to the *P. brassica* infection

Three commercial canola cultivar lines 15, 16 and 20 supplied by Crop Protection Services, Nutrien, Saskatoon, Saskatchewan, Canada were used for the inoculation and assessment of transcriptomic responses against infection by LG2 of *P. brassicae*. All three cultivars contain similar genetic background, and the only difference is that each line carries different combinations of *CR* (Clubroot Resistant) genes. Line 16 carries the *Rcr1* gene in Chromosome A03; Line 20 carries the *Crr1a* gene in Chromosome A08 and Line 15 carries both *CR* genes. Line 16 was susceptible and Line 15 and 20 were moderately resistant to the *P. brassicae* LG2 infection.

When a 1.5-fold change at the transcript level was considered significant, all *BnUBC13* genes in Line 16 showed significant decrease at transcription levels after inoculation, while other

two lines showed very little variation except for *BnUBC13L* in Line 15 (1.6-fold) (Table 3-5). According to the RNA seq data, all *BnUBC13* genes showed reduced transcript levels by more than twofold in Line 16 after pathogen inoculation. The order of the relative expression varies as follows: *BnUBC13K* (2-fold) < *H/D* (2.5-fold) < *I* (2.7-fold) < *B* (2.8-fold) < *F* (2.9-fold) < *A/J* (3.4-fold) < *L* (3.7-fold) < *G* (3.8-fold) < *C* (4-fold) < *E* (4.4-fold) (Figure 3-17).

Table 3-5: RNA-seq analysis of *BnUBC13* genes in three different canola lines

Gene	**Line CPS#15**		**Line CPS#16**		**Line CPS#20**	
	Before Inoculation	After Inoculation	Before Inoculation	After Inoculation	Before Inoculation	After Inoculation
	Avg total	Avg total	Avg total	Avg total	Avg total	Avg total
BnaA06g11360D (A)	1044	978	3201	928.3333	1274.333	1117.333
BnaC08g38130D (B)	955	852.6667	2074	743.3333	619.6667	462.6667
BnaAnng13030D (C)	831.6667	822.6667	2795.667	685.6667	1406	1112.667
BnaC08g17090D (D)	594.3333	749	1171	470.6667	416	475
BnaA07g34450D (E)	702.3333	791.3333	1519	342.6667	861	836.6667
BnaA08g23450D (F)	380.3333	463	1550.667	539.6667	611.6667	583.3333
BnaC05g12900D (G)	878.3333	859	2749.667	728	1206.667	961.6667
BnaC06g39290D (H)	843	1018.667	2798	1133	706.6667	549.6667
BnaC02g25260D (I)	615.6667	778.3333	1556	578	654	544.3333
BnaA07g38410D (J)	65	83.66667	252.3333	74	99.33333	87
BnaC06g20310D (K)	137	220.6667	391	200.3333	148.6667	168.6667
BnaA02g19070D (L)	514.6667	753.6667	1363	366.6667	751	663.3333

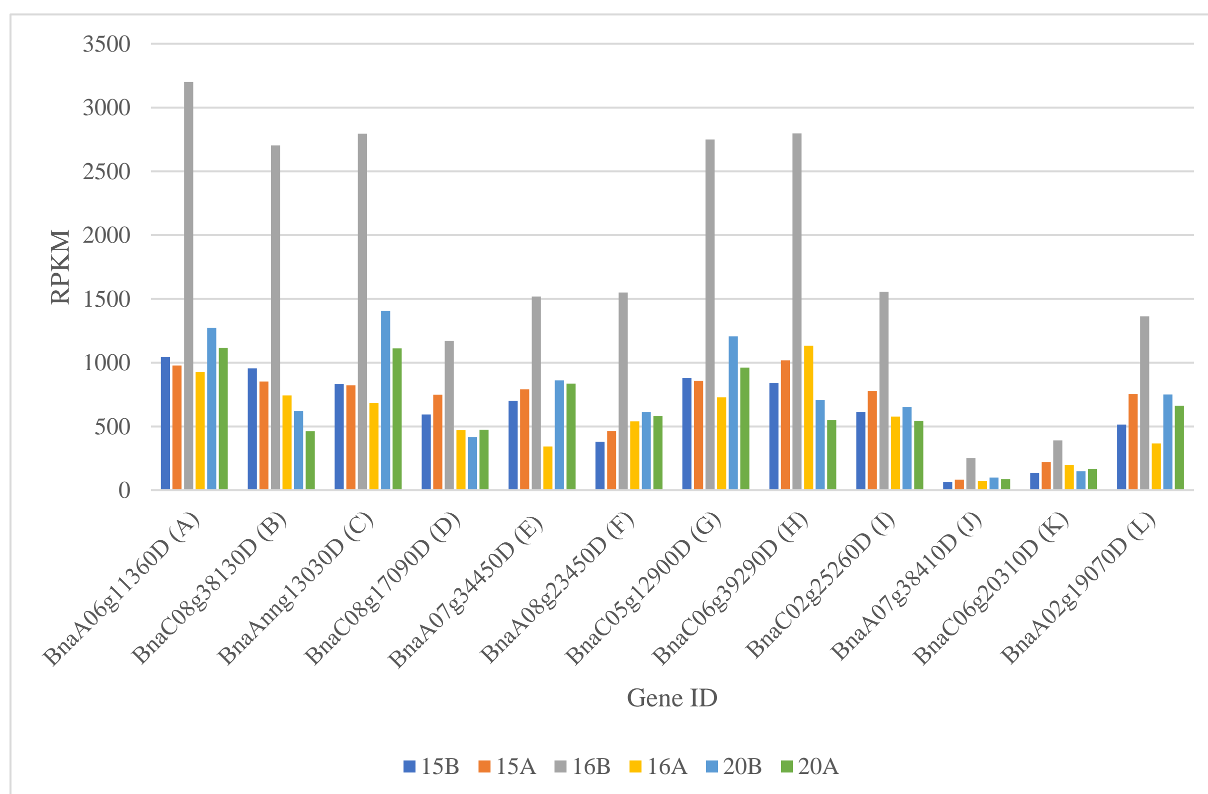


Figure 3-16: Comparison of RNA seq data based on before and after clubroot inoculation on 12 *BnUBC13* genes in cultivars 15,16 and 20.

Table 3-6: Fold change of *BnUBC13* transcripts in canola lines carrying *CR* genes.

Gene ID	Cultivar	Before Inoculation	After Inoculation	Fold change
BnaA06g11360D (A)	15	1.00	0.94	0.94
	16	3.07	0.89	0.29
	20	1.22	1.07	0.87
BnaC08g38130D (B)	15	1.00	0.89	0.90
	16	2.17	0.78	0.36
	20	0.65	0.48	0.79
BnaAnng13030D (C)	15	1.00	0.99	1.13
	16	3.36	0.82	0.23

	20	1.69	1.33	0.97
BnaC08g17090D (D)	15	1.00	1.26	1.22
	16	1.97	0.79	0.35
	20	0.70	0.80	0.95
BnaA07g34450D (E)	15	1.00	1.13	1.29
	16	2.16	0.49	0.29
	20	1.23	1.19	0.88
BnaA08g23450D (F)	15	1.00	1.22	1.46
	16	4.08	1.42	0.27
	20	1.61	1.53	0.88
BnaC05g12900D (G)	15	1.00	0.98	0.89
	16	3.13	0.83	0.36
	20	1.37	1.09	0.75
BnaC06g39290D (H)	15	1.00	1.12	1.26
	16	3.32	1.34	0.40
	20	0.84	0.65	1.14
BnaC02g25260D (I)	15	1.00	1.26	0.98
	16	2.53	0.94	0.26
	20	1.06	0.88	0.80
BnaA07g38410D (J)	15	1.00	1.29	1.21
	16	3.88	1.14	0.40
	20	1.53	1.34	0.77
BnaC06g20310D (K)	15	1.00	1.61	1.26
	16	2.85	1.46	0.37
	20	1.09	1.23	0.83
BnaA02g19070D (L)	15	1.00	1.46	1.61
	16	2.65	0.71	0.51
	20	1.46	1.29	1.13

Before and after inoculation data is displayed, all relative to cultivar 15 before inoculation sample.

3.8.2 Validation of RNA seq data through qRT-PCR

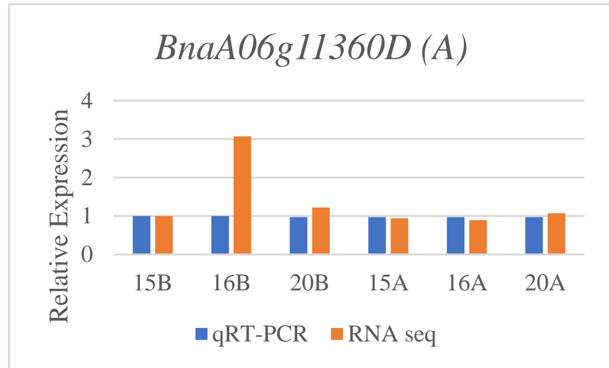
To independently assess the expression profile of *BnUBC13* genes in response to the clubroot infection, qRT-PCR experiments were performed with the same three commercial canola cultivar lines 15, 16 and 20, and the overall reliability of the data was analyzed. The qRT-PCR results obtained from this study (Table 3-6) do not clearly reflect similar trends with the RNA seq data (Table 3-5), although the fluctuation remained not very drastic. This data indicates that all *BnUBC13* genes behave like a housekeeping gene without changing the transcript levels even under stress conditions where clubroot pathogen is inoculated. Our hypothesis predicts that the resistant variety like 15 have reduced *BnUBC13* expression levels compared to the susceptible variety 16 as it increases the susceptibility when *UBC13* is expressed high. This was observed in qRT-PCR results for *BnUBC13C*, *D*, *F*, *G* and *H* genes, although the difference does not appear to be dramatic in most cases. Overall, the results are consistent with a previous report considering *UBC13* as housekeeping genes (Zang et al., 2012).

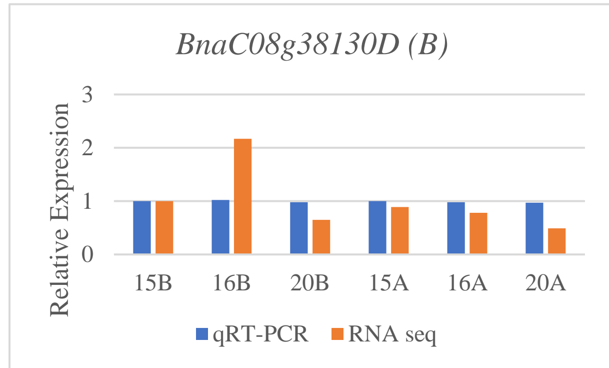
Table 3-7: Relative *BnUBC13* gene expression before and after pathogen inoculation in three different cultivars.

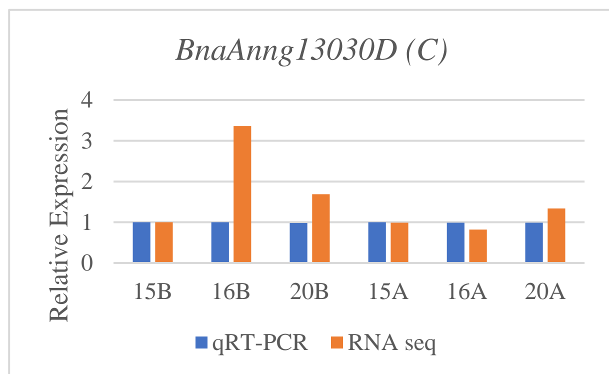
Gene ID	Cultivar	Before Inoculation	After Inoculation
BnUBC13A (A06g11360D)	15	1.00	0.96
	16	1.00	0.97
	20	0.97	0.97
BnUBC13B (C08g38130D)	15	1.00	0.99
	16	1.01	0.98
	20	0.98	0.97
BnUBC13C (Anng13030D)	15	1.00	1.00
	16	1.00	0.99
	20	0.98	0.99
BnUBC13D (C08g17090D)	15	1.00	0.99
	16	0.99	1.04
	20	0.99	1.01

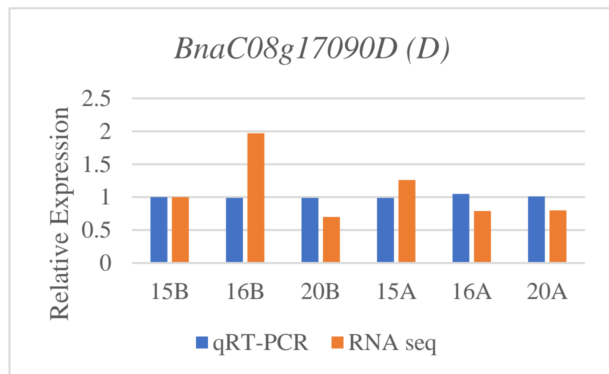
BnUBC13E (A07g34450D)	15	1.00	0.97
	16	0.99	0.94
	20	0.87	0.92
BnUBC13F (A08g23450D)	15	1.00	0.95
	16	1.00	0.95
	20	0.96	0.94
BnUBC13G (C05g12900D)	15	1.00	0.99
	16	0.99	0.99
	20	0.99	0.98
BnUBC13H (C06g39290D)	15	1.00	1.00
	16	0.99	1.00
	20	0.98	1.00
BnUBC13I (C02g25260D)	15	1.00	1.04
	16	1.05	1.05
	20	1.04	1.03
BnUBC13J (A07g38410D)	15	1.00	0.99
	16	1.01	0.99
	20	1.00	0.99
BnUBC13K (C06g20310D)	15	1.00	1.06
	16	1.11	1.03
	20	1.11	1.10
BnUBC13L (A02g19070D)	15	1.00	1.14
	16	1.08	1.14
	20	1.18	1.17

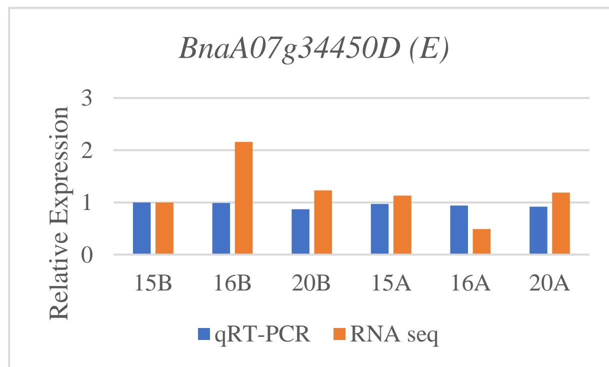
Before and after inoculation data is displayed, all relative to cultivar 15 before inoculation sample.

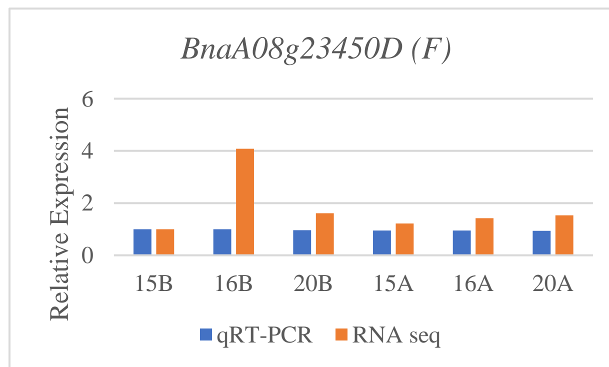

BnaA06g11360D (A)
Relative Expression
4
3
2
1
0
15B
16B
20B
15A
16A
20A
qRT-PCR
RNA seq


BnaC08g38130D (B)
Relative Expression
3
2
1
0
15B
16B
20B
15A
16A
20A
qRT-PCR
RNA seq


BnaAnng13030D (C)
Relative Expression
4
3
2
1
0
15B
16B
20B
15A
16A
20A
qRT-PCR
RNA seq


BnaC08g17090D (D)
Relative Expression
2.5
2
1.5
1
0.5
0
15B
16B
20B
15A
16A
20A
qRT-PCR
RNA seq


BnaA07g34450D (E)
Relative Expression
3
2
1
0
15B
16B
20B
15A
16A
20A
qRT-PCR
RNA seq


BnaA08g23450D (F)
Relative Expression
6
4
2
0
15B
16B
20B
15A
16A
20A
qRT-PCR
RNA seq

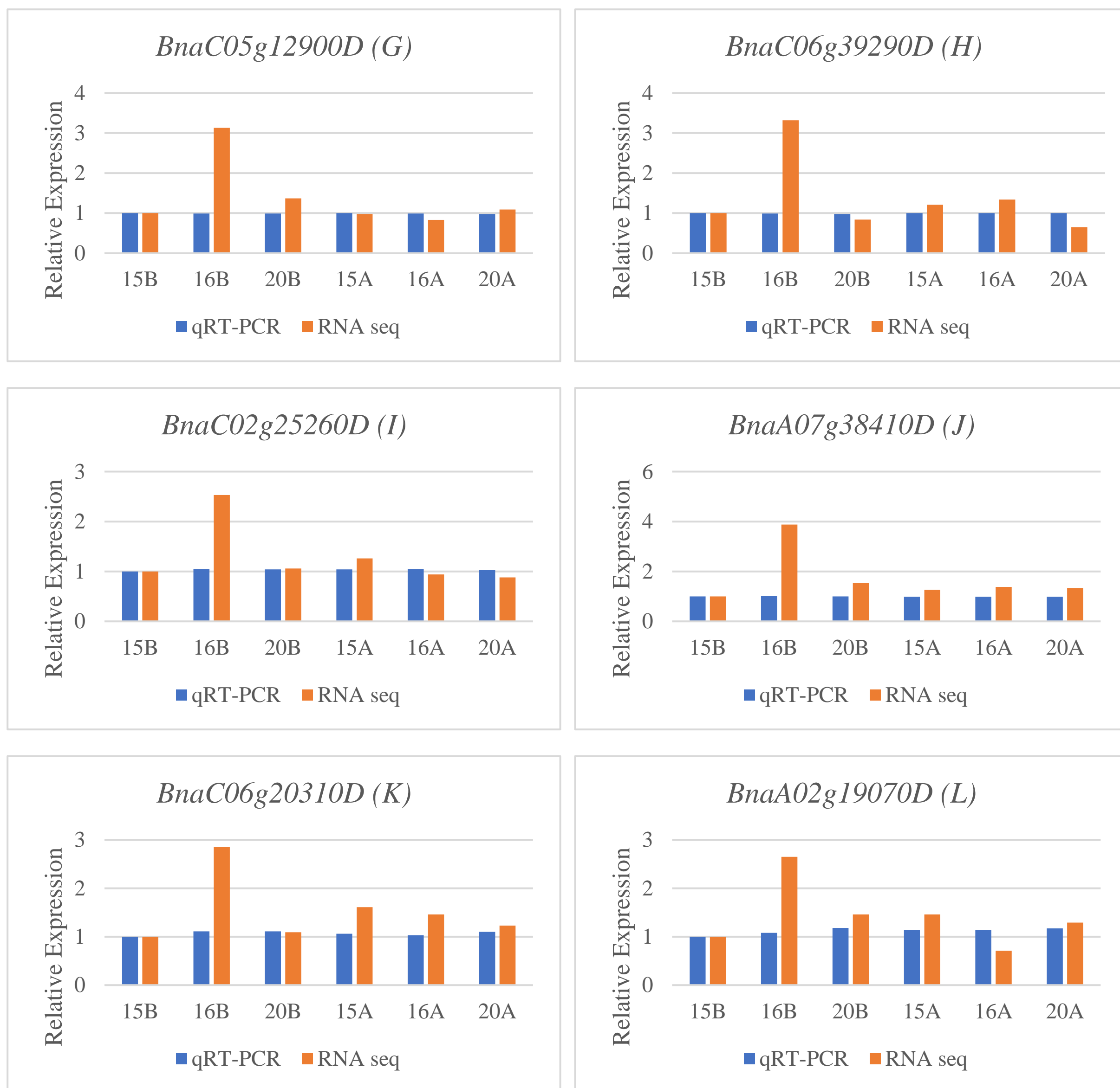


Figure 3-17: Relative expression profile of 12 *BnUBC13* genes by RNA-seq and qRT-PCR analyses. Orange bars indicate the relative expression of RNA-seq data, while blue bars indicate the relative expression of qRT-PCR relative to line 15 before inoculation. B, before inoculation; A, after inoculation.

CHAPTER FOUR: DISCUSSION AND FUTURE DIRECTIONS

4.1 Discussion

4.1.1 *B. napus* Ubc13s promotes Lys63-linked polyubiquitination

Ubc13 is the only known enzyme to catalyze Lys63-linked polyubiquitination. As a result, Ubc13 has become unique among E2/Ubcs. In mammalian cells, DNA damage response and NF-κB activation are two well-characterized pathways in which Ubc13-mediated Lys63-linked polyubiquitination plays critical roles. Other pathways related to stress response (Arnason and Ellison, 1994), mitochondrial inheritance (Fisk and Yaffe, 1999), plasma membrane protein endocytosis (Galan and Haguenauer-Tsapis, 1997), ribosome function (Spence et al., 2000), innate immunity (Deng et al., 2000a; Zhou et al., 2004a) and cell-cycle checkpoints (Bothos et al., 2003) may also involve Lys63-linked polyubiquitination. The unique feature of Ubc13 compared to other Ubcs is its ability to form a stable complex with a Uev, which is homologous to Ubcs but lacks the active Cys residue (Broomfield et al., 1998; Sancho et al., 1998). In plants, Ubc13 plays roles in regulating apical dominance (Yin et al., 2007), root development, auxin response (Wen et al., 2014), iron metabolism (Li and Schmidt, 2010) and immunity (Wang et al., 2019; Yao et al., 2021). In addition, Ubc13 may work with Uev1D to promote Lys63-linked polyubiquitination and DNA damage response (Wen et al., 2008). One of the goals of this study is to investigate and unveil exact cellular functions of Ubc13-mediated Lys63 linked polyubiquitination of *Brassica napus*. It was found that Ubc13 alone can form thiolester bond with Ub, but Lys63-linked ubiquitination requires Uev as a cofactor (Hofmann and Pickart, 1999; McKenna et al., 2001). In this study, we examined the physical interaction of BnUbc13s and AtUev1D by three different assays: the yeast two hybrid assay, GST pulldown assay and Lys63-linked polyubiquitination assay. The yeast two hybrid assay results suggest that all BnUbc13s can form stable heterodimer with AtUev1D. The GST pulldown assay further confirmed that BnUbc13s can directly form stable complexes with AtUev1D. An *in vitro* ubiquitination was employed to confirm the Lys63-poly-Ub chain formation from BnUbc13s and AtUev1D physical interaction. The experiment reveals that poly-Ub molecules are formed when both BnUbc13 and AtUev1D are present, whereas neither BnUbc13s nor AtUev1D alone could generate free poly Ub chains. In addition, it was confirmed that the poly-Ub chain formation was via Lys63 linkage, since poly-Ub conjugates were not detected when using the K63R mutant Ub.

Overall, the current study with BnUbc13 shows that *B. napus* Ubc13s can form a stable complex with Uev1D and promotes Ubc13-mediated Lys63-linked polyubiquitination. Although none of the assays are quantitative, our data suggest the BnUbc13-AtUev1D interaction is strong.

4.1.2 BnUbc13s are involved in DNA damage tolerance

Lys48-linked polyubiquitination is considered as conventional proteosome-mediated ubiquitination that targets proteins for degradation whereas Lys63-linked polyubiquitination is considered to be a fundamentally different processes involved in signal transduction (Pickart, 2001). The target of the current study is to find out cellular activities of Ubc13-mediated Lys63-linked polyubiquitination in *B. napus* and functionally characterize them.

Ubc13-mediated Lys63-linked polyubiquitination is utilized by plants as a means of gene regulation in one or more cellular pathways. It shows that Ubc13-mediated DNA damage tolerance function is conserved from yeast to mammals (Andersen et al., 2005) and hence likely from *Arabidopsis* to *Brassica*. This study demonstrated that *BnUBC13* genes were able to functionally replace the corresponding yeast *UBC13* for the error-free DDT function, including resistance to killing by different DNA damaging agents like MMS, 4NQO and UV and suppression of spontaneous mutagenesis. Furthermore, both yeast *UBC13* and *MMS2* genes can be replaced by the corresponding *BnUBC13s* and *AtUEV1D*, indicating that similar functions are inherited in plants**.** Although these results were demonstrated in yeast cells, it is believed that *BnUBC13* may behave in the same manner in its own host. However, desired *in vivo* studies require appropriate assays in plants that are currently unavailable.

4.1.3 BnUbc13 and clubroot disease

Data presented in this report indicate that *Brassica napus* contains 12 highly conserved *UBC13* genes encoding nearly identical proteins. Ubiquitination in general and Ubc13-mediated polyubiquitination in particular appear to be primarily involved in environmental stress responses. Plants are continuously threatened by diverse microbial pathogens in addition to diverse environmental conditions. To survive, plants have evolved unique and sophisticated defense mechanisms that act simultaneously against multiple external stresses. In yeast, *UBC13* expression is DNA damage inducible (Brusky et al., 2000). In mammalian cells, the Ubc13-Uev complex

functions in TRAF6-mediated stress response pathways following activation by proinflammatory cytokines (Deng et al., 2000a; Wang et al., 2001b), as well as bacterial and viral infections (Zhou et al., 2004b). In *Arabidopsis*, *UBC13* differentially regulate two programmed cell death (PCD) pathways in response to low temperature stress and pathogen invasion, as spontaneous cell death lesions and hypersensitivity to low temperature stress was observed in the *ubc13* knockout mutant (Wang et al., 2019). It was found that all 12 *BnUBC13* transcript levels remain relatively constant under clubroot infection in each cultivar line regardless of resistant or susceptible cultivars. This observation is not surprising, as *UBC13* has been deemed a housekeeping gene in rice (Zang et al., 2012). In contrast, the *AtUEV1* gene expression fluctuates dramatically (Wen et al., 2008), suggesting that plant UEV1 gene products may serve as regulatory subunits.

Preliminary studies in our laboratory indicate that *UBC13* is a clubroot susceptibility gene in *Arabidopsis*. Hence, our overall objective in this study was to create canola mutants defective in the *BnUBC13* function to assess their response to the *P. brassicae* infection. A CRISPR-Cas9 construct was made to simultaneously target five *BnUBC13* genes. This study screened 30 regenerated canola lines at the targeted loci. It was revealed that 28/30 lines contained at least one *BnUBC13* mutation, indicating that the targeting efficiency was very high. As expected, the majority of the mutations were single nucleotide insertion or deletion, resulting in null mutant alleles. More excitingly, detailed sequence analyses revealed the majority were homozygous mutations and that all five target genes were efficiently targeted. Strikingly, two generated lines contained mutations in all five target genes, one of which had four homozygous mutations and one heterozygous mutation. The excellent spectrum of mutation distribution allowed us to directly test these selected lines for their response to the clubroot pathogen *P. brassica* infection.

In summary, genome edition by the current CRISPR-Cas9 technology turned out to be extremely effective, and the products in this round of proof-of-principle can be directly applied to functional testing.

4.2. Conclusions

The present study describes the isolation and functional characterization of *Brassica napus UBC13 (BnUBC13*) genes. Eight selected *BnUBC13* genes *BnUBC13A*, *BnUBC13B, BnUBC13C, BnUBC13D, BnUBC13E, BnUBC13F, BnUBC13G* and *BnUBC13H* were cloned and functionally

characterized. When all the observations and results are put together it provides data to suggest BnUbc13s-AtUev1D complex formation and involvement in promoting Lys63-linked polyubiquitination and possible roles in the DNA damage response. One conclusion drawn from this study is the high-degree Ubc13 conservation from *Arabidopsis thaliana* to *Brassica napus*. A reasonable prediction is the involvement of plant Ubc13-Uev1 in the error-free DNA tolerance in plants. In addition, it demonstrates that BnUbc13 and AtUev1D form a stable complex and mediate Lys63-linked polyubiquitination *in vitro*. Furthermore, bioinformatic analysis of RNA sequence data and our experimental data suggest that *BnUBC13*s are housekeeping genes. Finally, sequence analysis of CRISPR-Cas9 edited canola plants demonstrates that the genome editing strategy was extremely successful and can simultaneously targeting multiple genes.

4.3. Future directions

A parallel study should be carried out with *Brassica napus* Uevs. Due to the large number of *BnUBC13* genes to be cloned and characterized, studies with *BnUEV1* genes were not studied. It is predicted that Uev1s are also highly conserved between *B. napus* and *Arabidopsis* but may not be as high as BnUbc13s. Furthermore, *BnUEV1* gene expression may be highly variable, and their functions are diverse, as observed in *Arabidopsis* (Wang et al., 2020).

To obtain homozygous mutations for all five genes. With the success in the initial targeting, this task becomes simple. For example, if Line 26 is allowed to be self-pollinated, only the heterozygous *BnUBC13H* alleles will segregate and ¼ progenies are expected to be homozygous mutations for all five genes. This line, along with other homozygous genome edited plants, will be inoculated with *Plasmodiophora brassica*, the clubroot pathogen to test their response to the disease. If the plants become resistant, other agricultural properties will be examined. If they are still not as resistance to the clubroot disease as anticipated, the remaining *BnUBC13* genes will be targeted and characterized.

Cascade of enzymatic steps are involved in ubiquitination including Ub, E1, E2, and E3 (Hochstrasser, 1996). The only enzyme that has the ability to catalyze Lys63-linked poly-Ub in yeast and mammalian cells, acting as a novel signal in DNA repair (Hofmann and Pickart, 1999) and NF-kB activation (Deng et al., 2000b) is Ubc13. The current study shows that BnUbc13 with

AtUev forms a complex promoting Lys63 poly-Ub chain, functioning DNA repair pathway. Therefore, it will be helpful to identify cognate E3s and the ubiquitination substrate(s) involved in the clubroot disease. This will provide important directions to better understand functions of the plant Ubc13-Uev complex.

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