CLONING OF *WUB3a* GENE (DROUGHT STRESS TOLERANT GENE) ISOLATED FROM *Triticum aestivum*

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Abiotic stress is one of the important problems affected on plant production by causing physiological and biochemical changes like generating ROS (reactive oxygen species) casing damage in compartments of cellular under biotic and environmental stress decreasing electron transport and photosynthesis Tian *et al*. (2014) ubiquitination one of the important processes of post-translational modification of proteins in eukaryotes, It plays a crucial role regulating different biological process in plant, (Dye and Schulman, 2007) Ubiquitin is a 76-amino acid long protein linked to lysine residues in target protein (Hershko *et al*., 2000). The ubiquitination process involved three enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3). The roles in plants are involved in transcription-dependent resistance to drought stress and high temperature (Kim and Kim, 2013; Liu *et al*., 2014). Due to the strong influence of ubiquitination on the ultimate fate of the substrate, this modification regulates a wide variety of biological processes, including but not limited to immune and stress responses, homeostasis, photosynthesis, DNA and hormone synthesis, embryogenesis regulation, transcription, and signal transduction (Kwon *et al*., 2006; Sadanandom *et al*., 2012; Yates and Sadanandom, 2013; Jiang and Chen, 2012 and Welchman *et al*., 2005). E2 the ubiquitin-conjugating enzyme plays role in *Arabidopsis thaliana* flowering (Xu *et al*., 2009), responses to the pathogen, and low-temperature stress (Wang *et al*., 2019) response to drought stress (Ahn *et al*., 2018) plant immunity in *Nicotiana benthamiana* (Zhou and Zeng, 2017), and immune signaling (Zhou and Zeng, 2018), in root development by affecting auxin signaling (Wen *et al*., 2014) the stability of thylakoid membrane protein complexes, leaf photosynthesis, under high-temperature stress, Tian *et al*., (2014), plant development and reproduction. (Linden and Callis, 2020 and Khan *et al*., 2018), also overexpression of the *Ta-Ub2* gene in transgenic tobacco improved salt and drought tolerance (Krieger *et al*., 2011 and Guo *et al*., 2008). The current study aimed to clone of *wub3a* gene for transformation in tobacco plants to study the expression of the gene under biotic and environmental stress.

MATERIAL AND METHOD

The *WUB3* gene was isolated from the cDNA of the double haploid 4 (DH4) wheat *Triticum aestivum* was kindly provided by Dr. Tarek Hewezi from Laboratoire de Biotechnologie et d'Amélioration des Plantes (BAP), Castanet Tolosan, France under 30% PEG drought stress from the root after 48 h of treatment according to Ibrahim, (2017), using a specific primer for the *WUB3* gene 234 bp table (1) and procured from Bioron GmbH (Germany) Amplified cDNA of DH4 wheat genotype by using Taq-DNA polymerase (Takara Taq R050A) kit, The PCR program consisted of a 3min incubation at 94°C followed by 35 cycles of 94°C /30s, (58ºC) /30 s and 72ºC/1 min, with a final extension step of 72ºC/7 min, the PCR product was separated by 1.2% agarose gel electrophoresis using TAE buffer with 0.004% red safe dye.

PCR fragments were clean up using Wizard® SV gel and PCR clean-up system according to Hengen (1997), the fragments of PCR product 234bp, were sequenced on an applied biosystems automatic sequencer (ABI PRISM®1200 DNA Sequencer, Bioron GmbH, Germany). The sequence was compared with sequences of representatives of the most related *Triticum aestivum* species and deposited in the GenBank under accession no. MW344069 and sequencing genome databases by using the BLAST program. The 3d structure of the translated protein was designed using https://swissmodel.expasy.org/interactive/0natNJ/models.

Cleavage the cDNA and binary vector (pBi121).

Wub3 gene and the binary vector (pBi121) pBi121 provided from Dr. tarek hewezi) were digested according to instructions provided by the manufacturer (Promega) with two restriction enzymes XbaI for forward and salI for reverse.

1. ***E Coli* (DH5α) competent cells preparation and transformation.**

E. coli competent cells were prepared according to Dagert and Ehrlich (1979).

2. **Detection of *WUB3* gene in transformed bacteria *E. coli* using colony PCR.**

The transformed bacteria with the *wub3* gene were amplified by using primers as shown in Table (1) and Taq-DNA polymerase according to the instruction of the manufacturer (Takara Taq R050A) kit, The PCR consisted of a 3min incubation at 94°C followed by 35 cycles of 94ºC /30s, (58ºC) /30 s and 72 ºC/1 min, with a final extension step of 72ºC/7 min. the PCR product was separated by 1.2% agarose gel electrophoresis using TAE buffer with 0.004% red safe dye.

RESULTS AND DISCUSSION

The isolated *WUB3* gene from the cDNA DH4 wheat *Triticum aestivum* un-

der drought stress 30% PEG from the root after 48 h of treatment as shown in Fig. (1).

The nucleotide sequence analysis of the *wub3* (ubiquitin) gene

The nucleotide sequence analysis of the obtained result was deposited at GenBank under accession no. MW344069 the sequence length of *WUB3* gene is 234bp with complete open reading frame., gives 97% nucleotide identity with accession number XM_044562939.1, XM_044562938.1, XM_040392135.1 and XM_040392134.1, 96% accession number AY862401, 95 % to 4 genes sequences with accession numbers XM_044555799.1, XM_044552162.1, AK446944.1, and AK446236.1 while accession number AK453662.1 was 94% as shown in Fig. (2 and 3). The gene coding sequence contains one domain of 76-amino acids that belong to the ubiquitin E2 enzyme ubiquitin family protein.

The translated protein sequence analysis of ubiquitin-protein (*wub3*) gene

The obtained ORF sequence coding to 77 amino acids with molecular weight ~8.66KDa, isoelectric point ~ pH 7.54 gave 100% protein identity to protein id AAX55761.1, WP_203595744.1, ABR25913.1, WP_220545033.1 and XP_044326075.1 while KAG0512820.1 and PWA47355were 98% identity. Fig. (4) and Fig. (5).

The results in Table (2) show a difference in nucleotide sequence compared with the original gene at the nucleotide no.39, 42, 45, 84, 129, 135, 156, 159, and 168, while the amino acid sequence is not affected, meaning that the substitution of the nucleotide result is the alternative codon of the amino acid.

The structure of the E2 ubiquitin enzyme protein

The protein consists of 77 amino acids sequence, structured in 5 beta strands and 3 helixes, Beta strand1 (1-7), Beta strand2 (12-18), helix1 (23-34), helix2 (38-40), Beta strand3 (42,45), Beta strand4 (47-49), helix3(57-59), Beta strand5 (66-70) and two cross-links, the first one 48-Glycyl lysine isopeptide (Lys-Gly) (interchain with G-Cter in ubiquitin) the second was 76-Glycyl lysine isopeptide (Gly-Lys) (interchain with K- in acceptor proteins) according to Unipart analysis https://www.uniprot.org/blast/ as shown in Fig. (6).

Binary vector construction and transformation

The cDNA of the full-length *WUB3* gene was cloned into the binary vector (pBi121) by xbaI and salI restriction sites. The plasmid pBi121 carries the neomycin phosphotransferase (NPTII) gene and the β-glucuronidase (GUS) gene. The neomycin phosphotransferase (NPTII) gene is under the control of the nopaline synthase (nos) promoter and the terminator from nopaline synthase (nos) which provides a polyadenylation signal. The neomycin phosphotransferase

(NPTII) gene confers kanamycin resistance. The β-glucuronidase (GUS) activity is under the control of the cauliflower mosaic virus 35S promoter and the terminator from nopaline synthase (nos) provides polyadenylation as shown in Fig. (7).

The recombinant plasmid was introduced in *E. coli* (DHα) and positive cells were selected on an LB media containing 5 mg/ml kanamycin as shown in Fig. (8). The transformed bacteria were conformed with colony PCR (Fig. 9), lane 3-8: random colonies selection of transformed *WUB3*.

The ubiquitin (Ub) gene is coding to 76-amino acid globular protein, Ub protein is induced by different stresses in plants and animals (Guo *et al*., 2004), The ubiquitin Plays an important role in removing misfolded or damaged proteins and in controlling the regulatory proteins during environmental stress (Vierstra, 2003; Stone, 2014). The ubiquitin conjugation process requires three types of ubiquitin enzymes: E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin-protein ligase) (Zientara-Rytter and Subramani, 2019). Generally, ubiquitination plays a role in the regulation of plant development and tolerance to environmental stress in dicot and monocot plants. In transgenic plants expression of the Ta-Ub2 gene improves tolerance to environmental stress by regulating genes expression and increasing antioxidant enzymes (Kang *et al*., 2016). E2 enzymes have been found in Caenorhabditis elegans (Jones *et al*., 2001), 14 in Saccharomyces cerevisiae (Michelle *et al*., 2009), 48 in rice (Bae and Kim, 2014), 75 in maize (Jue *et al*., 2015), 72 in banana (Dong *et al*., 2016), 43 in *Vitis vinifera* (Gao *et al*., 2017), 34 in *Carica papaya* (Jue *et al*., 2017), 59 in tomato (Sharma and Bhatt, 2017), 40 in *Dimocarpus longan* Lour, (Jue *et al*., 2018) and 57 in potato (Liu *et al*., 2019). Overexpression of Ub conjugates transgenic tobacco improvement photosynthesis under high temperatures up to 45ºc harmonious with the increasing the synthesis of the ATPase in the thylakoid membrane and improving the efficiency of (PSII) photosystem II, enhancing antioxidant enzyme activity and D1protein in transgenic plans under high temperature, on the other hand, they found decreasing in ROS (reactive oxygen species) accumulation and (carbonylation and malondialdehyde) MDA levels Tian *et al*., (2014)

Zhan *et al*. (2020) studied the lysine-ubiquitinated K^{ub} proteins function and found that 25% of the K^{ub} proteins in tobacco were positioned in the chloroplast and their results showed that several lysine-ubiquitinated K^{ub} proteins were identified in photosystems I and II and electron transport (K^{ub}) and suggested that K^{ub} may play a critical role in carbon fixation in photosynthetic organisms. In all eukaryotes the ubiquitin-proteasome system the UPS and autophagy pathways also play crucial roles in regulating a wide range of cytological and physiological processes by selectively removing regulatory pro-

teins after they are no longer needed, such as those involved in plant development and stress response (Xu. and Xue, 2019) in soybean hairy roots. Lin, *et al*., (2021) studied the infection of *Phytophthora sojae* and founded that the pathogenic secrete effectors into host cells to manipulate host immunity and benefit infection such as an RxLR effector, Avr1d, promoted. The Avr1d inhibits the E3 ligase activity of GmPUB13 so Avr1d considers competing with E2 ubiquitin-conjugating enzymes for GmPUB13 binding and repressing the GmPUB13 E3 ligase activity thus causing the inhibition of the host immunity and the incidence the infection. While Chen, *et al*., (2020) analyzed E2 family proteins of soybean and found 91 E2 proteins respond to drought such as the transgenic soybean with E2 member, GmUBC9, and affect the time of flowering through interaction with the E3-like protein HUB2 and regulation of. H2Bub1. On the other hand, Sun *et al*., (2020) reported that E2 conjugases ubiquitin carrier proteins (UBC1 and UBC2) regulate PROTEIN KINASE 4 (MPK4) and MYB42-mediated SALT OVERLY SENSITIVE 2 (SOS2) expression to salt stress in Arabidopsis. Also, many of the genes encoding the putative E2 enzymes in rice and potato genomes are induced by stresses such as salt and cold as well as by abscisic acid (ABA) (Zhiguo, *et al*., 2015 and Liu, *et al.*, 2019).

SUMMARY

The (ubiquitin-conjugating enzymes) E2s plays important role in response to various stresses in the plant, found in eukaryotes. The wub3a drought-tolerant gene coding to the E2 ubiquitin enzyme was isolated from the cDNA of double haploid 4 (DH4) genotype (*triticum aestivum*) under drought stress 30% PE and sequenced with 234bp complete open reading frame. The gene was deposited at GenBank under accession no. MW344069 and cloned in pBi121 expression vector and transformed in *E. coli* DH5α for preparing to transformation in the plant. The results illustrated that there is a different nucleotide sequence in the *wub3a* gene and the other sequence in GenBank while the translated protein was the same sequence. The gene requires more studies in the future.

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Table (1): The specific primers designed for the Wub3 gene (drought-tolerant gene) and sequences.

Gene name	Protein	Primer sequence (5′ – 3′)		Ms (bp)	annealing
Wub3	upiqitin	F	ATGCAGATTTTTGTGAAAACCCTCAC	234	56ºC
		R	TTACTGACCACCACGGAGGC		
		F	TATATCTAGAATGCAGATTTTT-GTGAAAACCCTCAC		58ºC
		R	TATAGTCGACTTACTGACCACCACGGAGGC		

Table (2): The effect of changing nucleotide sequence on translated protein sequence of full length *wub3* gene compared with the original gene.

N. A. no.	WUB3 Original gene AY862401.1	WUB3A A. no. MW344069.1	Original Protein id. AAX55761.1	Protein id QVV41478.1
39	T	C	No change	
42	C	T		
45	T	G		
84	T	C		
129	T	G		
135	C	T		
156	T	C		
159	T	C		
168	A	G		

*** N. A. No. = Nucleotide number, * A. No. = Accession number**

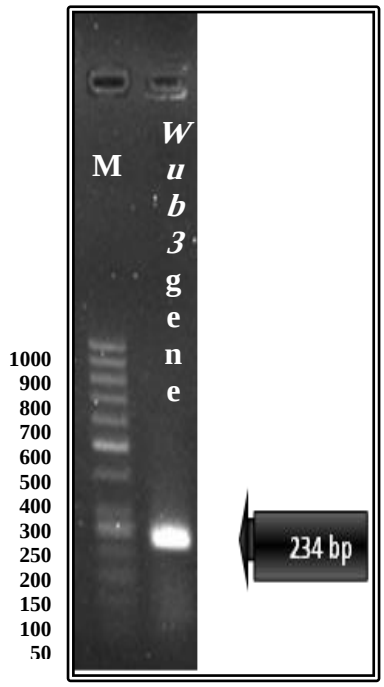
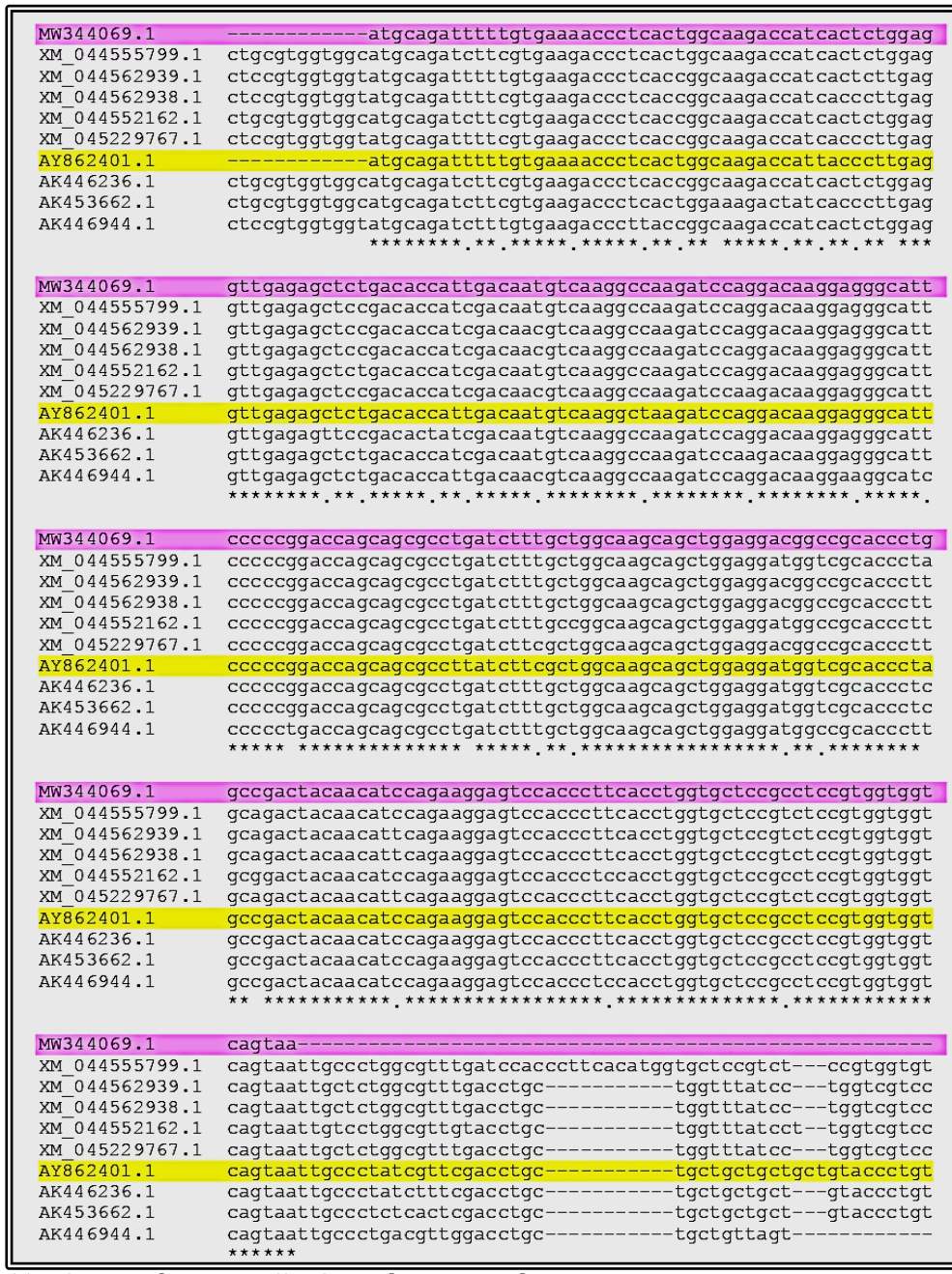


Fig. (1): *Wub3* gene isolated from DH4 wheat *Triticum aestivum* under drought stress 30 % PEG after 48h of treatment.



(*) = Conserved sequence, (.) = Somewhat conserved.

Fig. (2): The full length nucleotide sequence alignment of *wub3* gene was submitted with accession no. MW344069.1 in GenBank that isolated from wheat *Triticum aestivum* 234bp open reading frame that gives 97% nucleotide identity with accession number XM_044562939.1, XM_044562938.1, XM_040392135.1 and XM_040392134.1 , 96% accession number AY862401, 95 % to 4 genes sequences with accession numbers XM_044555799.1, XM_044552162.1.

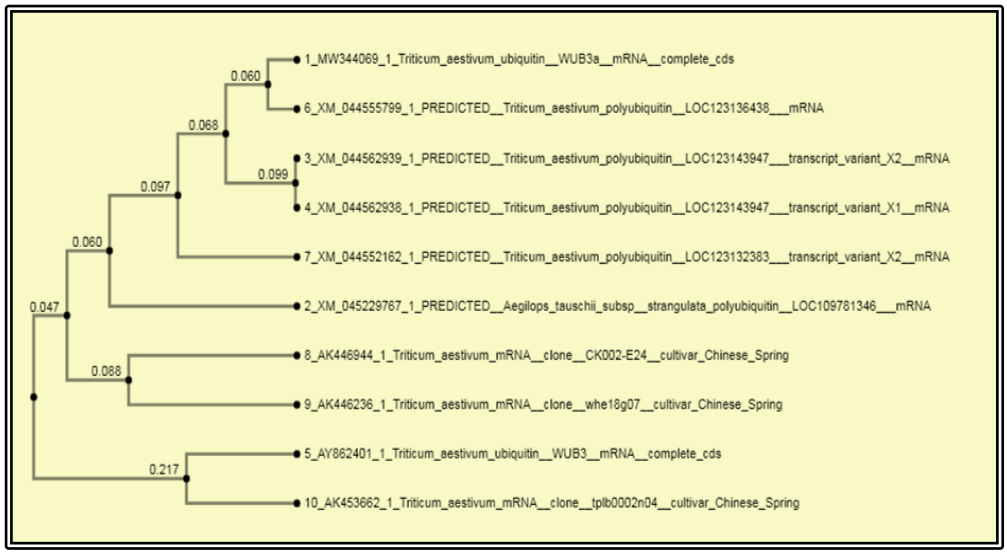


Fig (3): The phylogenetic tree of *wub3a* sequence accession no.MW344069 and the other DNA sequence in GenBank.

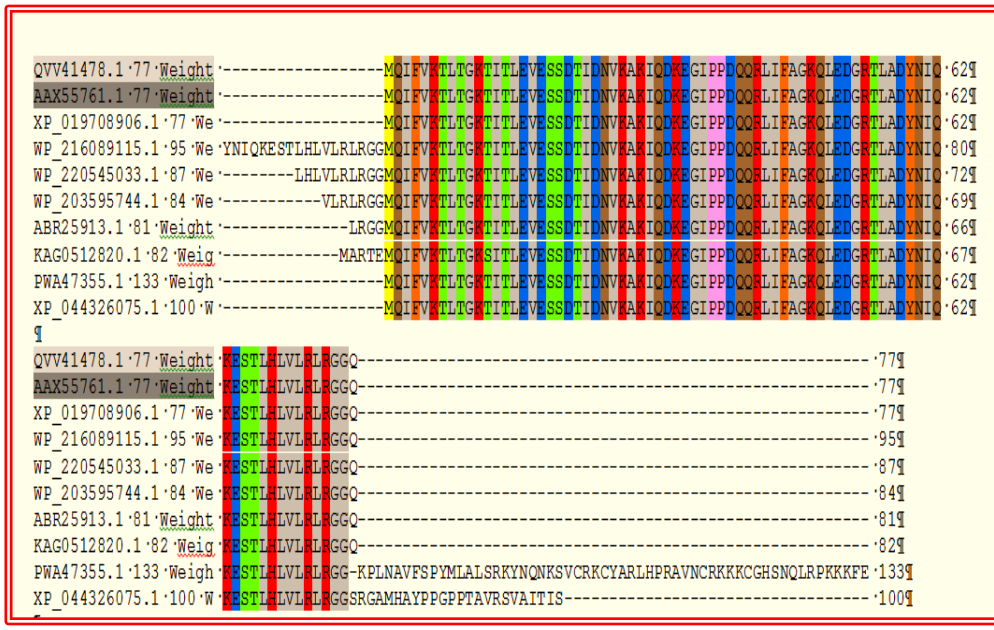


Fig (4): The multi-alignment sequence of wub3a ubiquitin accession no. QVV41478 and the other protein sequence in GenBank.

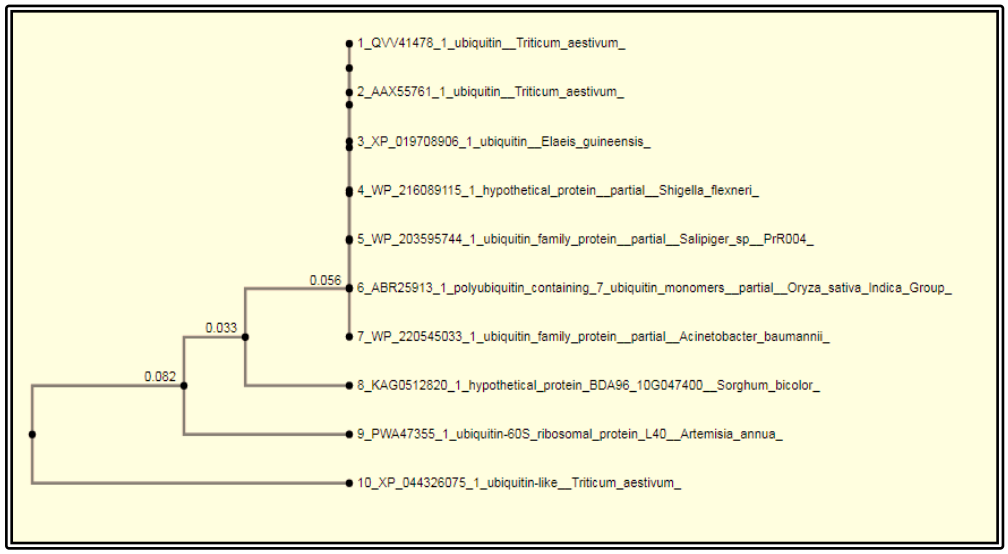


Fig. (5): The phylogenetic tree of wub3a ubiquitin accession no. QVV41478 and the other protein sequence in GenBank.

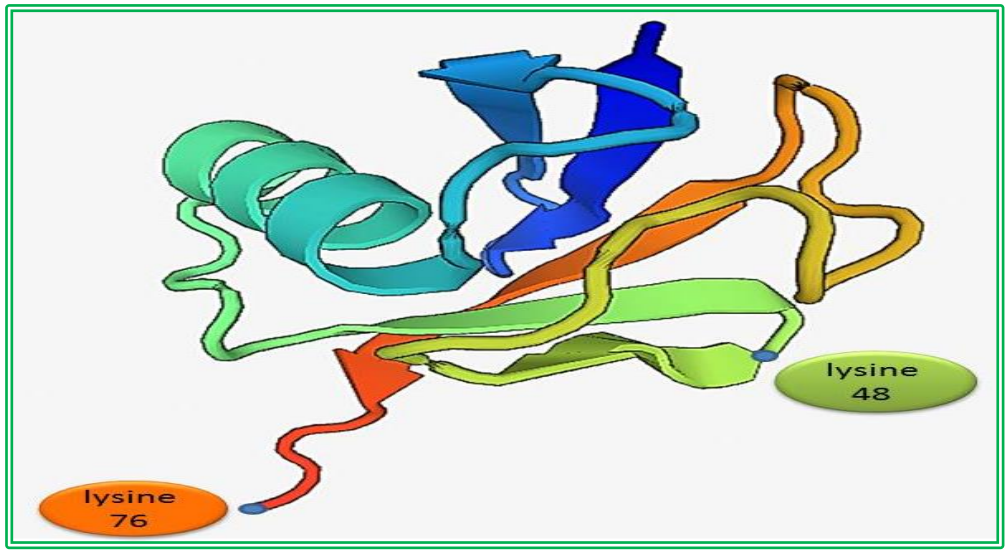


Fig. (6): The 3D structure translated protein of E2 ubiquitin enzyme.

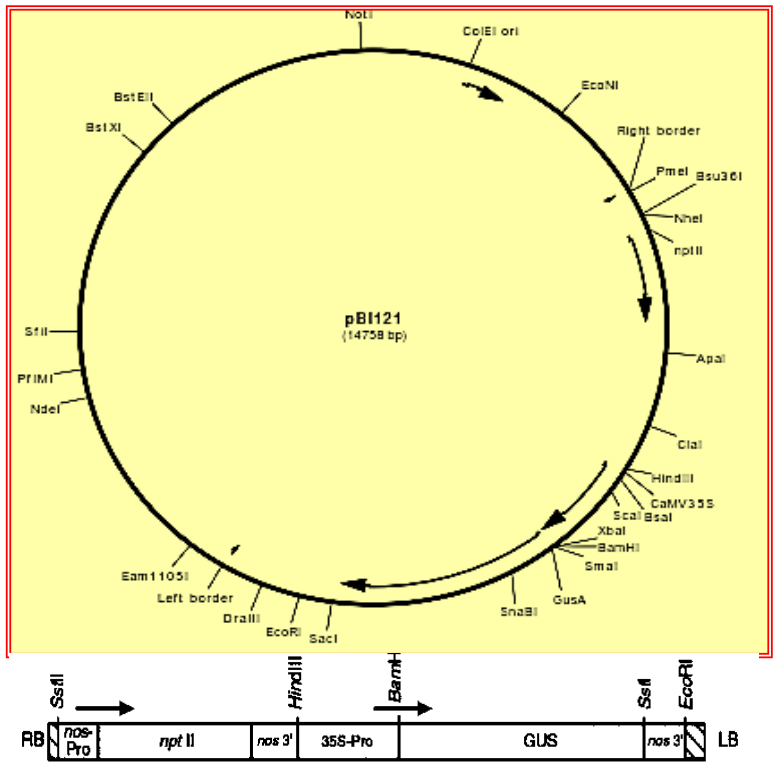


Fig. (7): PMap of the T-DNA region of binary vector pBI121 cassette.

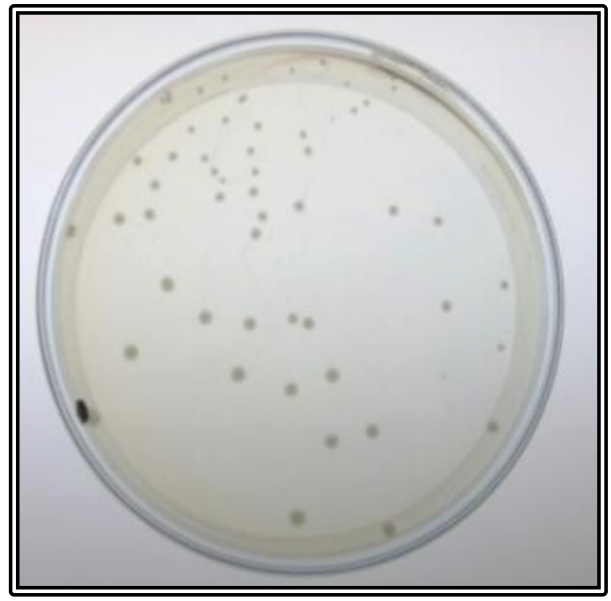


Fig. (8): Transformed *E. coli* (DHα) cells grown on LB agar plates containing kanamycin for the *wub3* gene

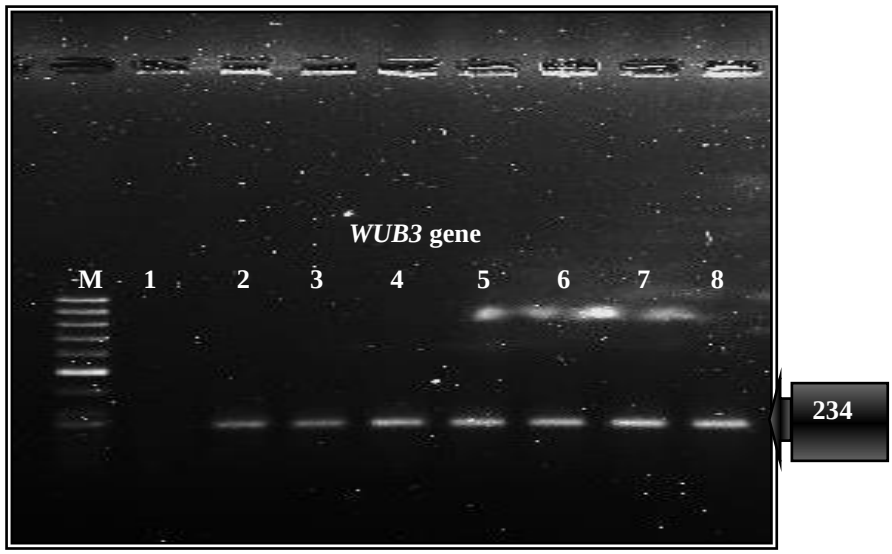


Fig. (9): Confirmation of cloning *WUB3* gene in *E coli* by colony PCR technique, M gene Rular 100 bp DNA ladder (fermentase), laine1: *E Coli* with pbi121 only without *WUB3* gene (negative control), lane 2: positive control (pbi121 plus *WUB3*), lane 3-8: random colonies selection of transformed cells.