تعیین ساختار پروتئین و ارزیابی سطح بیان ژن *Tup1* قارچ *Zymoseptoria tritici*

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چکیده

مقدمه: قارچ *Zymoseptoria tritici* بیمارگر دیمورفی است که تغییر در الگوی رشد آن نقشی اساسی در ایجاد بیماری دارد. به همین جهت، پروتئین Tup1، که یک سرکوبگر رونویسی عمومی و تنظیم کننده شناخته شده ای برای تغییر شکل است، کاندیدای واجد شرایط برای مطالعه مقدماتی در این بیمارگر است.

مواد و روش‌ها: با استفاده از Tup1p *Saccharomyces cerevisiae* بعنوان مدل، دومین‌های عملکردی پروتئین با استفاده از نرم افزار InterPro (Pfam) ترسیم شد. سپس ساختار سوم Zt.Tup1p توسط نرم افزار InterPro (Pfam)نشان داده شد. سرانجام ، میزان بیان *Zt.Tup* در شرایط *in vitro* و *in vivo* توسط Real-Time RT-PCR ارزیابی شد.

نتایج: پروتئین Zt.Tup1p دارای ساختارهای عمومی موجود در در سایر پروتئین های یوکاریوتی شبه Tup، شامل دومین N–ترمینال، منطقه میانی محافظت نشده و یک دومین C– ترمینال با هفت تکرار WD است. همچنین نتایج نشان داد که دو المنت غنی از گلوتامین (Q1, Q2) موجود در *S. cerevisiae*، در ساختار Zt.Tup1p وجود ندارد. نتایج مشخص کردند که بیان ژن *Zt.tup1* در مرحله میسلیومی افزایش می‌یابد. بعلاوه افزایش بیان ژن در روزهای ۱۴ و ۲۴ بعد از تلقیح به گیاه نیز مشاهده شد.

بحث و نتیجه‌گیری: وجود هفت تکرار WD محافظت شده در Zt.Tup1p و سایر قارچ های مرتبط نشان می‌دهد که این دومین‌های عملکردی نقش مهمی در زندگی این میکروارگانیسم‌ها دارند. با توجه به داده‌های مطالعه حاضر، بیان بالای ژن Zt.tup1 در مرحله میسلیوم نشان می‌دهد که این پروتئین ممکن است نقش مهمی در توسعه میسلیوم و تشکیل کونیدی داشته باشد.

علاوه بر این، افزایش بیان در زمان نکروتروفی که با توسعه پیکنیدی مشخص می شود، نشانگر این است که ژن ممکن است نقشی اساسی در رشد و بیماری‌زایی *Z. tritici* داشته باشد. مطالعه حاضر را می‌توان بعنوان شروع یک نظریه پردازی جدید و جامع برای شفاف سازی عملکردهای دقیق این ژن در طول رشد و بیماری زایی در نظر گرفت.

واژه‌های کلیدی: بیان ژن، بیماری‌زایی، تکرارهای WD، Tup1، *Zymoseptoria tritici*

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Determining the Protein Structure and Evaluating the Gene Expression Level of *Tup1* from *Zymoseptoria tritici*

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Abstract

Introduction: *Zymoseptoria tritici* is a dimorphic pathogenic fungus in which the switching growth pattern plays a crucial role in the development of diseases. Therefore, Tup1 protein, the global transcriptional repressor which is a well-known regulator of dimorphism might be a qualified candidate for preliminary study in the pathogen.

Materials and methods: Using *Saccharomyces cerevisiae* Tup1p as a model, the functional domains of the protein were plotted using InterPro (Pfam) software. Then, *Zt*.Tup1p tertiary structure was represented by Accelrys DS visualize software. Finally, the *in vitro* and *in vivo* levels of expression of *Zt.Tup* were evaluated by the Real-Time RT-PCR method.

Results: *Zt*.Tup1p included public structures available in the other Tup1-like eukaryotic proteins, with an N-terminal domain, a poorly conserved middle region, and a C-terminal domain with seven WD repeats. Also, the results showed that the two glutamine-rich elements (Q1 and Q2) contrary to what was identified in the *S. cerevisiae,* do not exist in *Zt*.Tup1p structure. The results indicated that the expression level of *Zt.tup1* up-regulated during mycelial stage. In addition, *Zt*.Tup1p was partly up-regulated in 14th and 24th post-inoculations.

Discussion and conclusion: The presence of seven conserved WD repeats in the C terminal domain of *Zt*.Tup1p and the other related fungi indicated that such functional domains play an important role in the life of these microorganisms. According to the results of the present study, the up-regulation of *Zt.tup1* gene during the mycelial stage suggests that it may play a major role in the mycelium development than in conidia formation. Furthermore, its up-regulation during necrotrophy which is characterized by the development of pycnidia shows that the gene may play a key role in the growth and pathogenicity of *Z. tritici*. The present study can be considered as promising new leads for starting a new comprehensive survey for clarifying the detailed functions of this gene during the development and pathogenicity.

Key words: Expression level, Pathogenicity, Tup1, WD repeats, *Zymoseptoria tritici*

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Introduction

In the fungi kingdom, dimorphism refers to the capacity to change the morphology between yeast-like growth and filamentous states (1). Growth in the yeast state refers to the production of two independent cells by mitotic divisions, budding or fission. In the filamentous state, two different models were described. In the first model, the pseudohyphal model, cells following cytokinesis become elongated. They, however, fail to separate and remain joined as chains of cells. In the second model, true hyphae with long continuous tubes are produced, then nuclei separate in the tubes. Increasing evidence has shown that pathogenic fungi use this ability to manage growth between saprophytic and pathogenic forms (2). This morphological switch occurs in response to many environmental stimuli and the role of two cAMP and MAPK signaling pathways in regulating dimorphism has been verified (1, 3, 4).

Tup1, the global transcriptional repressor is a well-known regulator that controls dimorphism and has conserved status in evolutionarily distant organisms (5). In *Saccharomyces cerevisiae*, the model yeast, the mechanism of action, and the structure of Tup1p were studied comprehensively. In this yeast, Tup1p associates with Ssn6 (Cyc8) and forms the Ssn6-Tup1 transcriptional co-repressor complex consisting of four monomers of Tup1 and one monomer of Ssn6. Ssn6p is a protein with tetratricopeptide repeat (TPR) motifs that mediates protein-protein interactions (6). None of these proteins have a DNA binding region and their role was played by interaction with specific DNA-binding transcription factors (7). Tup1–Ssn6 is required for the transcriptional repression of more than 300 genes under standard growth conditions (8). A wide variety of gene families contain at least nine groups: glucose repressible genes (9), mating type regulated genes (10), Nucleo DNA damage-inducible genes (11), oxygen-regulated genes (12), Osmo stress-inducible genes (13), a fatty acid regulated gene (14), flocculation-related genes (15), sporulation-related genes (16), and a meiosis-related gene (17) which can be regulated by this complex.

Several studies showed that three functional domains with diverse impose on transcription regulation were defined for Tup1 protein in budding yeast. The first domain, N- terminal is a region with 72 amino acids (aa), the C-terminal has seven WD repeats (340–713 aa), and the middle region is between two parts. The N-terminal is involved in the interaction with Ssn6 and the formation of the co-repressor complex (18). It also mediates oligomerization (19). The middle region is involved in repression as well as interaction with the N-terminal of histones. This region is located at 73–385 and amino acids 120–316, and has a high affinity for binding to H3 and H4 histones (20) and provides molecular evidence for direct interaction between the Ssn6-Tup1 complex and chromatin organization. It was pointed out that in the middle region, the Tup1 histone-binding domain corresponded to the repression domain (21). According to another study (22), there are two independent repression domains, one in 73–200 aa and the other within 288–389 aa as suggested. Some studies suggested that Tup1p has two repression domains, one in the N-terminal which contains the Ssn6-association region, and the other in the C-terminal, in a region overlapping with the first WD repeats (7). Therefore, the histone binding region overlaps with two repression domains located in N- and C-terminals. The WD40 repeats domain, located in the C-terminal region, forms a highly conserved protein-protein interaction domain (20). Each WD repeat is a 40-amino-acid motif that possesses a highly-conserved tryptophan-aspartate or WD sequence. This motif was found in proteins and it often physically interacts with other proteins and

functions as a scaffold in multimeric complexes construction (23). Proteins with WD repeats are involved in a wide variety of processes, including gene repression, signal transduction, secretion, RNA splicing, and progression through the cell cycle (24). According to this description, Lamas-Maceriras et al. (25) identified four functionally important domains in Tup1 protein in *S. cerevisiae*. In animal pathogenic fungi, the function of Tup1p as one of the best-known regulators of dimorphism was investigated but its role in most plant pathogenic fungi is unknown (26).

Zymoseptoria tritici is one of the most important fungal pathogens in wheat (27). This fungus causes septoria tritici blotch (STB), a destructive disease of wheat (28). *Z. tritici* is a dimorphic pathogen with two filamentous and yeast-like states. This switch in growth pattern plays an important role in the entry of the pathogen into the infection cycle (29). The identification and functional analysis of effectors can produce precise knowledge for disease management techniques (30). Therefore, various studies were dedicated to reveal molecular aspects of dimorphism and pathogenicity in Z. *tritici* with an emphasis on effectors discovery. Now, the critical role of different components of two cAMP and MAPK signaling pathways in this field has been verified (31-33) and these investigations are still ongoing (34, 35).

In the present study, the sequence and structure of Tup1p of Z. *tritici* as an eligible candidate were studied. The survey was conducted by studying Tup1p from *S. cerevisiae* as the model organism and some dimorphic, plant pathogenic fungi. The presence of identified domains and the amount of their conservation were examined and the secondary structure of the protein was predicted. Finally, the best tertiary structure was drawn and presented and the expression profile of Tup1p in *Z. tritici* was examined in conidial and mycelial stages at different time points of the infection cycle in the greenhouse assay.

Materials and Methods

Species and Accession Numbers: Tup1p sequences of four plant pathogenic and dimorphic filamentous fungal species including *Z. tritici* IPO323 (Tup1, XP_003850038), *Verticillium dahlia* (rco-1, EGY14823), *Ustilago maydis* (Tup1, EAK84267), and *Claviceps pupurea* (rco-1, AAB63195) and three dimorphic yeast species including *S. cerevisiae* (Tup1p, AAA35182), *Candida albicans* (Tup1, AAB63195), and *S. pombe* (Tup1, AAB81475) were used for multiple alignment analysis. Tup1 sequence in *U. maydis* was provided from MIPS *U. maydis* Database (http://mips.gsf.de/genre/proj/ustilago/). *V. dahlia* Tup1 sequence was obtained from *Verticillium* group database (http://www.broadinstitute.org/). *C. albicans* and *S. cerevisiae* Tup1 sequences were provided from CGD (http://www.candidagenome) and SGD (http://www.yeastgenome.org/), respectively. The rest of the Tup1 sequences were obtained from the NCBI (http://www.ncbi.nlm.nih.gov/) (Table1).

Table 1- The Studied Microorganisms and the Accession Number of Their Tup1

Microorganism	Accession number	Ref.
Z. tritici IPO323	Tup1, XP_003850038	http://www.ncbi.nlm.nih.gov/
Verticillium dahlia	rco-1, EGY14823	http://www.broadinstitute.org/
Ustilago maydis	Tup1, EAK84267	http://mips.gsf.de/genre/proj/ustilago/
Claviceps pupurea	rco-1, AAB63195	http://www.ncbi.nlm.nih.gov/
S. cerevisiae	Tup1p, AAA35182	http://www.yeastgenome.org/
Candida albicans	Tup1, AAB63195	http://www.candidagenome
S. pombe	Tup1, AAB81475	http://www.ncbi.nlm.nih.gov/

Sequence Analysis: The physicochemical characteristics of *Z. tritici* Tup1p were defined using a primary sequence and with the help of the ProtParam tool (http://web.expasy.org/protparam/).

Sequence Alignment, Phylogenetic Analysis, and Domain Structure: The detailed study of the Tup1 protein sequences in these fungi was performed using ClustalW2 for multiple sequence alignments and the InterProScan sequence search tool from the European bioinformatics institute (http://www.ebi.ac.uk/) was applied for domain structure analysis. The schematic diagram of the obtained domains using Pfam was presented after the analysis by keeping proportions of each domain according to the total length of the proteins. The phylogenetic analysis of full-length protein sequences was performed using MEGA5 software with the neighbor-joining method using default setting (36).

Prediction of Secondary and Tertiary Structure: Chou and Fasman secondary structure prediction (CFFSSP) server at http://www.biogem.org/tool/chou-fasman/, SOPMA software at http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html, GOR software at http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html, and the program JPred 3 (http://www.compbio.dundee.ac.uk/wwwjpred/) were employed to predict the secondary structure.

I-TASSER software at http://zhanglab.ccmb.med.umich.edu/I-TASSER/, LOMETS software at http://zhanglab.ccmb.med.umich.edu/LOMETS/, MUSTER software at http://zhanglab.ccmb.med.umich.edu/MUSTER/, and PHYRE http://www.sbg.bio.ic.ac.uk/~phyre/ software were employed for tertiary structure prediction. The profile of energy minimization was computed by Swiss-PdbViewer software. The AMPAGE database at http://mordred.bioc.cam.ac.uk/~rapper/rampage.php and Swiss-PdbViewer software were used for the detection of the structural stability of the proteins by Ramachandran plot.

The Expression Level of *Zt.Tup1*: To study the expression profile of *Zt.Tup1* in conidial and mycelium phases, IPO323 was inoculated on yeast glucose broth and maintained in shaker-incubator at 15 and 25 $^{\circ C}$, respectively, for five days at a speed of 125 rpm. Then, the samples were freeze-dried and used for RNA extraction. For greenhouse experiments, the susceptible wheat cultivar, Kavir, was used. Spore suspension of IPO323 was used for the inoculation of ten-day-old plants by using previously-used protocols (37). At 0, 4, 14, and 24 days after inoculation, three biological replications of the infected leaves were collected for RNA extraction. The total RNA isolation was done with RNX-Plus kit (Cinagene, Iran). Reverse transcription was performed using the RevertAid Reverse Transcriptase kit (Thermo Scientific, Germany) by the oligo-dT primer. For transcript quantification, the following primer pairs were designed, respectively: for *Tup1* and *β-tubulin* using the Primer3Plus online software (http://www. bioin forma tics.nl/prime rs3pl us): forward 5′-CACCTCCACAAGAGCAACAA -3′, reverse 5′- CTTGTCCTCCGCTCCTGTAG -3′ and Forward 5′-ACCATCTCCGGCGAACAT -3′, and reverse 5′-GGAATTCTCGACCAGCTGG -3′. The expression analyses of the *Tup1* gene were carried out using SYBER Green qPCR Master mix (YTA, Iran) with Corbett RG-6000 Real-time PCR machine (Corbett Life Science, QIAGEN, Germany). Initially, the expression level of each sample was normalized with the *β-tubulin* gene and then the comparative Ct method was used to calculate the expression levels (38).

Results

Based on the results of the ProtParam tool, the atomic composition of *Z. tritici* Tup1p contains 9021 atoms comprising carbon (2860), hydrogen (4422), nitrogen (846), oxygen (871), and sulfur (22). Also, $C_{2860}H_{4422}N_{846}O_{871}S_{22}$ has been retrieved as *Zt*.Tup1 molecular formula. The retrieved primary sequence of *Z. tritici* Tup1p has 603 amino acids, 65.299 kD molecular weight, and 6.44 theoretical pI. A majority of amino acids are present in the sequence including glycine (11.3%), proline (9.6%), alanine (7.6%), and glutamine (7.6%). Cysteine exhibited a minimum frequency (1.2%) of residue. The sequence included about 60 negatively-charged residues (Asp + Glu) and 54 positively-charged residues (Arg + Lys). The aliphatic index and the instability index were calculated as 65.52 and 43.98, respectively. This tool graded the protein as unstable and the grand average of hydropathicity (GRAVY) was -0.579.

The result of multiple sequence alignments and domain structure analyses of Tup1 proteins from these fungi are shown in Fig. 1 (and Sup 1). The three main and functionally-identified domains of Tup1p structure in *S. cerevisiae* as a model yeast (18) are shown in Fig. 1A (and Sup 1): (i) N-terminal region contains 72 amino acids and is involved in complex formation with Ssn6 and formation of the oligomeric state of Tup1p; (ii) middle region (residues 73–385) contains different parts: two repression domains (R1 and R2) that have the ability to interact with other factors, two glutamine regions (Q1 and Q2) and one ST-rich domain; and (iii) C-terminal region with seven WD repeats that are involved in the interaction with other proteins and it is shown that the first WD repeat partially overlaps with R2 repression domain.

Based on *S. cerevisiae*, these domains include the Tup_N domain in the amino-terminal region, seven WD40 domain repeats in the carboxyl-terminal region, and a poorly-conserved middle region.

Tup1p sequence similarity in *Z. tritici* and *S. cerevisiae* was 39.66 % (Sup 2). Among the sequences examined, Tup1p sequence of C. *purpurea* has the highest similarity with *Z. tritici* Tup1p (60.76%).

The phylogenetic analysis of full-length protein sequences showed that the sequences sort into two clades, one of them includes plant pathogenic species and the other includes yeast species (Fig. 2). The topology of the clades showed the closest relationship between the *V. dahlia* and *C. purpurea* sequences in the first clade and *S. cerevisiae* and *C. albicans* in the second clade.

The secondary structural analysis of the protein was done. The random coil is the most frequent (62.02%), followed by an extended strand (21.56%) and finally Alpha helix as being the least frequent (16.42%) (Table 2). The dominance of the coiled regions indicates the high level of conservation and stability of the protein structure (39).

Since the factor exerts its numerous effects through complex and multiple interactions, it was necessary to perform further investigations to show the presence of the characterized domains and motifs. The1–72 residues in the N-terminal region of Tup1p are required for the organization of both Tup1-Ssn6 complex and oligomeric status of Tup1 (22). In *Zt*.Tup1p, a partly-conserved helix structure is formed in the secondary structure at positions 1-72 (these residues are indicated with h in Sup 3.). Two glutamine-rich elements (Q1 and Q2) described in *Sc*.Tup1 p (25) were not present in *Zt*.Tup1p. Therefore, the region 1–72 of *Zt*.Tup1p did not conserve in the major part of the sequence. Also, the sequence covering positions 96–116 lacked the α-helical structure and the Q1-rich element. This indicated the structural difference in this part of the sequence which included a repression-associated

region that has not been previously reported. Considering that *Zt.*Tup1 N-terminus did not have a high similarity with *Sc.*Tup1p, we tried to compare the sequence among filamentous fungi species used in this study (Sup 3).

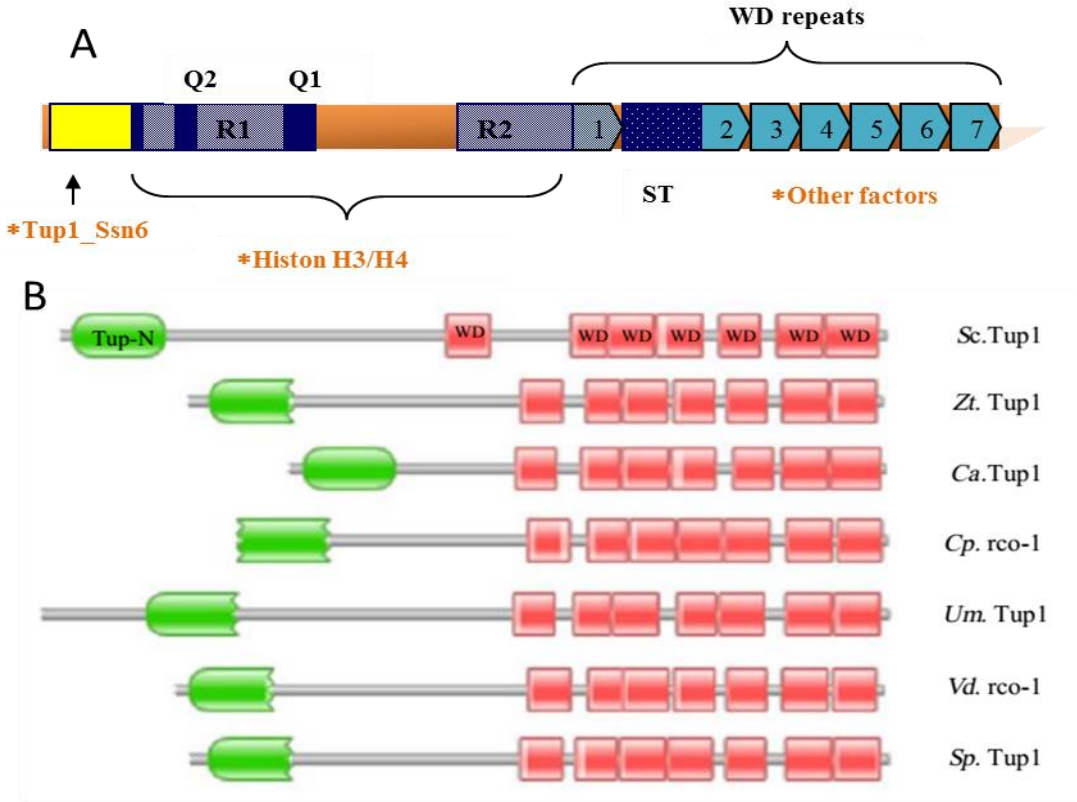

Fig. 1- A) The characterized functional domains of Tup1p in *Saccharomyces cerevisiae* are shown in this diagram (adapted by Lamas-Maceiras et al.(25)). B) The comparison of the conserved domains of Tup1 proteins among different fungi species. The conserved structure of Tup1 proteins in *S. cerevisiae* (*Sc.*Tup1), *Zymoseptoria tritici* (*Zt.*Tup1), *Candia albicans* (*Ca.*Tup1), *Saccharomyces pombe* (*Sp.*Tup1), *and Ustilago maydis* (*Um.*Tup1), *Verticillium dahlia* (*Vd*. rco-1), and *Claviceps purpurea* (*Cp.* rco-1). The domains were depicted with the comparison to functionally characterized-domains of *S. cerevisiae* using InterPro (Pfam) software. All domains described for *Sc.*Tup1 are available in the rest and include the N-terminal Tup_N domain (green square), seven WD40 domains in the C-terminal region (red squares), and a less-conserved middle region.

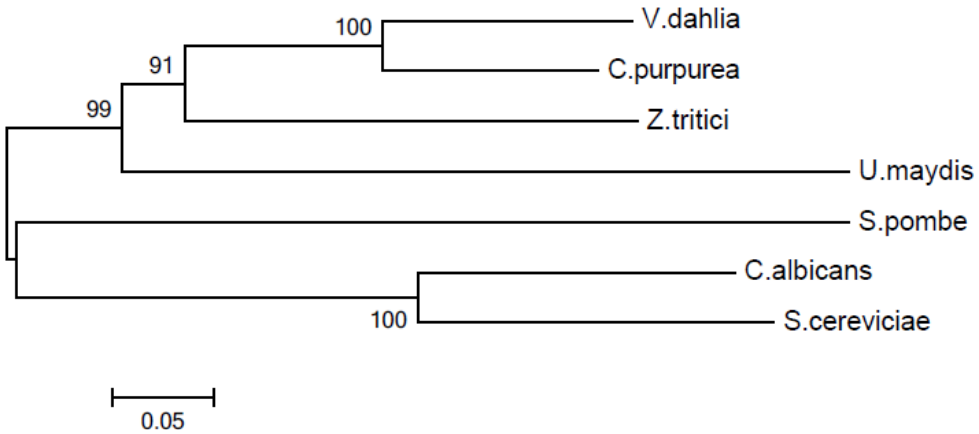

Fig. 2- Dendrogram of Tup1p protein in different species, based on ClustalW alignment of full-length translated protein sequences

Table 2- Secondary Structure Elements of the *Zt*.Tup1p

Structural elements	Number of residues	Percentage of residues
Alpha helix (Hh)	99	16.42%
310 helix (Gg)	0	0.00%
Pi helix (Ii)	0	0.00%
Beta bridge (Bb)	0	0.00%
Extended strand (Ee)	130	21.56%
Beta turn (Tt)	0	0.00%
Bend region (Ss)	0	0.00%
Random coil (Cc)	374	62.02%
Ambiguous states (?)	0	0.00%
Other states	0	0.00%

The results indicated that the secondary structure of *Um*.Tup1p had a significant difference with the rest of the sequences. On the other hand, *Zt*.Tup1p, *Cp*. Rcor-1 and *Vd*. Rcor-1 showed a high similarity in this region. The multi-alignment analysis Tup1p homologous in these fungi revealed that they did not have R1 and R2 regions with the same sequences which were observed in *Sc*.Tup1p. ST region and also Q1 and Q2 sequences did not exist but *Vd*. Rcor-1 had one Q-rich region in 168-183 (Sup. 1).

The carboxy-terminal region of Tup1 protein is an important fragment in protein-protein interaction. Tup1p appertains to the WD family of eukaryotic proteins specified by having a conserved region with seven repeats named WD40 motifs. This repeat is a degenerate one known as a beta-transducin repeat. WD40 is a variable region in both length and composition with approximately 40 amino acids and contains a core region with a uniform length. It was suggested that proteins containing WD40-repeats have a variety of functions such as signal transduction, transcription regulation, cell cycle control, autophagy, and apoptosis (40). The common function of all WD40-repeat units is to provide a rigid scaffold for protein interactions and to form multi-protein complexes. The results showed high conservation in the amino-acid sequences of this region. Also *Zt*.Tup1p, *Cp*.Rcor-1 and *Vd*.Rcor-1 C-terminus comparison showed that all three sequences had a high similarity and conservation in seven WD repeats (Fig. 1 and Sup. 1).

All the generated tertiary structures by I-TASSER, LOMETS, MUSTER of the *Zt*.Tup1 protein models were subjected to a series of tests for assaying their internal consistency and reliability. The result of the structural stability test of the obtained best tertiary structure by Ramachandran plot is shown in Fig. 3.

According to this test, 94.7% of the residues are located in a favored region. Also, 4.3% and 1% of the residues are located in allowed and outlier regions, respectively. *Zt*.Tup1p tertiary structures were depicted by the Accelrys Ds visualize software and is shown in Fig. 4.

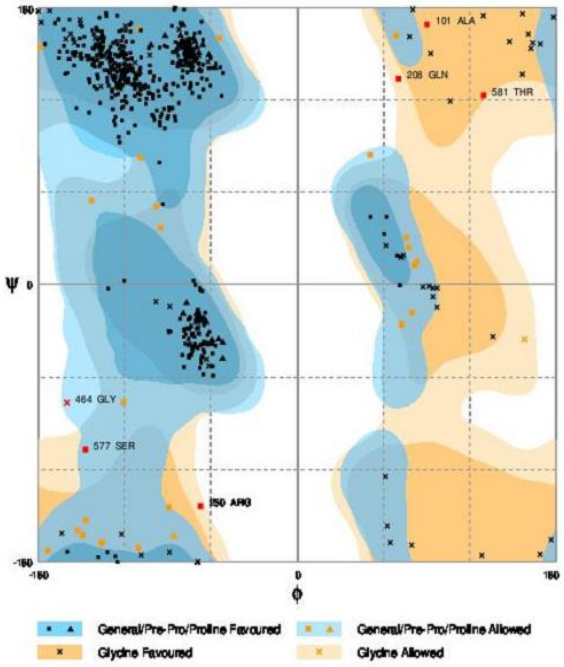

Fig. 3- Ramachandran plot of the best tertiary structure of *Zt*.Tup1p

Evaluating *Zt.Tup1* transcriptional level, higher expression was observed in the mycelia stage in comparison with the conidial stage (Fig. 5). *In planta*, the expression profile corresponded remarkably well with the early stage of infection or biotrophic phase (4 dpi) and also the necrotrophic phase (>8 dpi, Fig. 5).

Fig. 4- *Zt.*Tup1p tertiary structure depicted by the Accelrys DS visualizer software

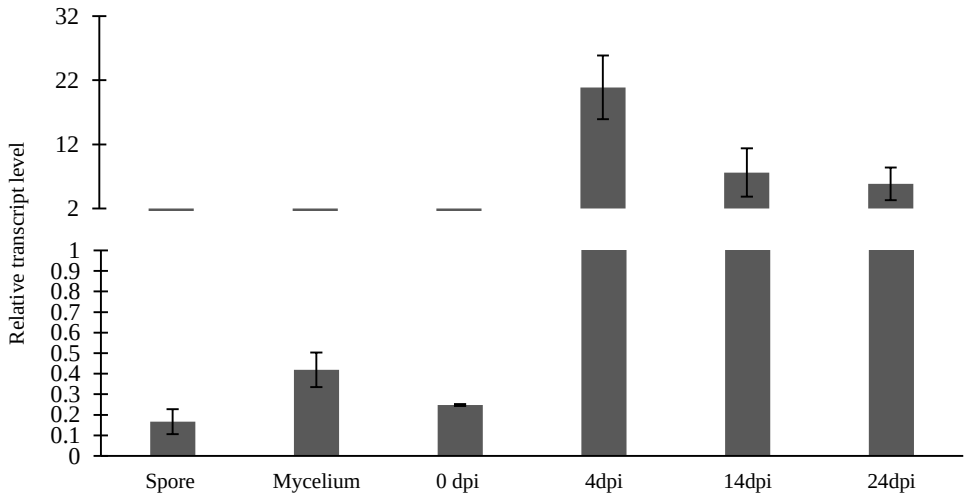

Fig. 5- Transcript levels of *Zt.Tup1* in conidial and mycelium phases and during infection or biotrophic phases as detected by RT-qPCR. Error bars represent the standard error (N = 3). Normalization of reads was done with respect to the *β-tubulin* as a reference gene.

Discussion and Conclusion

In *Z. tritici* as a phytopathogenic ascomycetea, the switch from yeast-like growth to a filament phenotype plays a critical role in the infection cycle of the pathogen. This process occurs in response to different environmental stimuli and is highly controlled by complex genetic pathways (41, 42).

The *Zt*.Tup1p and Tup1p of other filamentous fungi studied in this paper contained most of the identified functional domains of *Sc*.Tup1p. Also, the complete sequence comparison showed that they had a different degree of sequence similarity. In other words, they include public structures available in the other Tup1-like eukaryotic proteins, with an N-terminal domain, a poorly-conserved middle region, and a C-terminal domain with seven WD repeats. Overall, despite the differences in the sequence, the structure of functional domains was preserved in all studied proteins. These results indicate that these functional domains have been preserved during the evolution due to their critical importance (18).

The amino acid residues of 72 N-terminal are involved in a complex formation with Ssn6. It was suggested that Ssn6 may play the role of an adapter between Tup1p and DNA-bound proteins (43). The formation of two α-helical segments in the first 72 amino acids of *Zt*.Tup1p was confirmed showing that the Ssn6 interaction domain is conserved. Considering that in the *Zt*.Tup1p sequence, Q1 region (residues 96–116) is not conserved, Q2 (residues 173–194) is not present, the α-helix predicted in this segment is not formed in the *Zt*.Tup1p structure. Furthermore, contrary to what was identified in *S. cerevisiae*, the N-terminal domain comparison between *Zt*.Tup1p and the other filamentous fungi showed that most of them do not have two glutamine-rich elements (Q1 and Q2). Probably, in pathogenic filamentous fungi, this sequence does not have virulence effects, but confirmation of this hypothesis needs further research.

Although the R1 repression domain was not conserved among these filamentous fungi, R2 was partly conserved. Lamas-Maceiras et al. suggested that this difference could have several effects on the structure and function of the protein since the amino acid sequence (residues 96–116) may have other roles or such changes may create a new capability in repression responses and ultimately may be ineffective in protein function (25).

The crystal structure of the Tup1p WD domain has been characterized. This structure confirmed that the seven repeated units create a propeller-like figure with seven blades in which each blade is made up of four β strands (43, 44). The study showed that WD repeats in C-terminus are available and highly conserved in *Zt*.Tup1p and other filamentous fungi. WD domain is responsible for transcriptional repression and protein-protein interactions. The ST sequences of the middle region (residues 365–385) between WD1 and WD2 are not present in the filamentous fungi studied in this research. Zhang et al. found that the deletion of the ST region in *S. cerevisiae* Tup1p did not show loss of repression. They suggested that this region may be unique to yeasts Tup1p and is not present in other higher eukaryotic homologs nor is important for repression (18).

The phylogenetic analysis of full-length protein sequences showed that the sequences are divided into two clades: one includes plant pathogenic species and the other one includes yeast species. The topology of the clades showed the closest relationship between the *V. dahlia* and *C. purpurea* sequences in the first clade as well as *S. cerevisiae* and *C. albicans* in the second clade. Considering these results, it seems that the filamentous fungal and yeast species in evolutionary terms have two separate paths. During the course of evolution, mutations occurred for better fitness and these changes led to the separation of filamentous fungi and yeasts in phylogenetic studies.

As mentioned earlier, Tup1 protein is known for its role in dimorphism. In the present study, *Zt.Tup1* expression was

examined in two stages of the fungal life cycle that are associated with transformation.

The expression of the gene was evaluated in both conidial and mycelia stages as well as different post-inoculation time points during the pathogenic interaction with wheat. According to the results of the present study, the up-regulation during the mycelial stage suggests that *Zt*.Tup1p may play a major role during mycelium development. During the stage of infection, low expression was found in the first-time point. Subsequently, the expression was strongly induced (about 20-fold) during biotrophy at 4 dpi and then constantly decreased until 24 dpi. In addition, *Zt.Tup1* was partially up-regulated during necrotrophy at 14 and 24 dpi. These findings show that *Zt*.Tup1p may play a key role in the pycnidium development.

Overall, the expression profiling suggested that *Tup1* may play a fundamental role in *Z. tritici* growth and development. Contrary to Chen et al.'s study on *Magnaporthe oryzae*, the authors of the present study observed a higher transcript level for *Tup1* in the mycelia stage (45). However, it was proved that *Mo.Tup1* is essential for vegetative growth and correct hyphal branching. Even though the role of Tup1p in fungal dimorphism seems conserved, the control pathways associated with this process frequently differed among fungi (26). Roldán et al. stated that the role of Tup1p in the regulation of morphological changes in many plant pathogenic fungi is partly unknown (46).

In summary, the present study provided basic information on the structure and function of the *Zt*.Tupl p. It is expected that these results could be useful in the development of new pathogen management strategies and could be an initial step for further research on the role of this protein in the mechanisms of pathogenicity in *Z. tritici*.

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```
Sp.Tup1       ------------------------------------------------------------  0
Sc.Tup1       ------------------------------------------------------------  0
Ca.Tup1       ------------------------------------------------------------  0
Um.Tup1       MYSHRSIVPSAGSQGPPSGPPPPGPGGAGPSQAAAAAAAAAAAAAAAQQAPHPGGPVAGGP  60
Zt.Tup1       ------------------------------------------------------------  0
Vd.Rco-1      ------------------------------------------------------------  0
Cp.Rco-1      ------------------------------------------------------------  0

Sp.Tup1       --------------------------------VNELLEAVKKEFEDICQKTKTVEAQKDDF  29
Sc.Tup1       --------------------MTASVSNTQNKLNELLDAIRQEFLQVSQEANTYRLQN-QK  39
Ca.Tup1       ------------MS------MYPQRTQHQQRLTELLDAIKTEFDYASNEASSFK-KV-QE  40
Um.Tup1       GGPAPTGPPGAAPGPPGAAPPAGAPHTGSARLADLLDFVRHEFDLLGNDTAQFKA-Q-RD  118
Zt.Tup1       --------------MYNAHRGMAPGPQPGSRLADLLEQVRAEFDAQVGR---------SS  37
Vd.Rco-1      -------------------MGGAAPPVNQSRLEELLEQIRTEFSSQSRT---------TE  32
Cp.Rco-1      ------------MSMYSHRGMGAVPLGNSGRLNELLDQIRAEFETQLRQ---------TE  39
                                          : :**: :: **

Sp.Tup1       EYKAMISAQINEMALMKQTVMDLEMQQSKVKDRYEEEITSLKAQLEARRKEIASGVVPQS  89
Sc.Tup1       DYDFKMNQQLAEMQQIRNTVYELELTHRKMKDAYEAEIKHLKLGLEQRDHQIASLTVQQQ  99
Ca.Tup1       DYDSKYQQQAAEMQQIRQTVYDLELAHRKIKEAYEEEILRLKNELDTRDRQMKNGFQQQQ  100
Um.Tup1       EMEHRVTSQVSEVNMMQTHFYELEKRHTQIIQQYEEEVKRLRSILDSRGLSSEH-GAASA  177
Zt.Tup1       DHEHQLHNQIQEMDVIKSKIYQLETTHVAMKNKYEEEIARLRHELEQRGGPSQ------G  91
Vd.Rco-1      AYEHQIQAQVSEMQHVREKVFNMEQMHLNLKQKYEEELAHLRRQLEISRGGNAPPGMNSG  92
Cp.Rco-1      GFEHQISAQVSEMQLVREKVYAMEQTHMTLKQKYEEEISMLRHQLENSRKGGPQPGMP-G  98
                .     *  *:  ::  .  :*  :  : : ** *:  *:  *:

Sp.Tup1       SKTKHGRNSVSFGKYGNAGPFNSDNSSKPLILNNGSSGGTPKNLRSPAIDSDGTVLAPIQ  149
Sc.Tup1       QQQQQQQQVQQHLQQ-QQQQLAAASASVPVA------QQPPA-TTS-------ATATPAA  144
Ca.Tup1       QQQQQQQQQQ---QQ-QQQQIVAPPAA------------PPA------------------  126
Um.Tup1       T--------GPMSGP-PHLPASATSGPLPIA----SAGGPPP-PGGPGGAGSAAMFGPNG  223
Zt.Tup1       P--------HGASSS-QPAPPAIGH--------------------GPANLFQGIMAGGAG  122
Vd.Rco-1      P--------PPHNAP-SQQPPSIPS--------------------G-NGLFNGIMAGGGQ  122
Cp.Rco-1      P--------PQHAGP-SQQPPSIAP--------------------G-NGLFSGIMTGSNQ  128

Sp.Tup1       TSNVDLGSQYYSSPHVRPAVGATMAGSAMRTFPSNLPLG----------------------  188
Sc.Tup1       --NTTTGSPSAFPV---QASRPNLVGSQLPTT----TLPVVSS------NAQQQLPQQQL  189
Ca.Tup1       ------------------------------------------------------------  126
Um.Tup1       --NGALGG----------------PGSEYGRGPNGFDREREPPRGPTPGGKDS--KRMRV  263
Zt.Tup1       --GPGLAP-----PPQEQQGQPGMPGHMQGQMPPSLNAPPGPPHNP---FAYG-------  165
Vd.Rco-1      ---GGLAPP---PPPQDQQMGP--QQHQMPQGPPGLPAPPPPPPPQSQPPHFQQQQQQQQ  174
Cp.Rco-1      ---AGLAPPQQHPPPQEQQMGP--Q-HQMAQGPPGLPVPPPHPN----------------  166

Sp.Tup1       ------------------------------------------------------------  188
Sc.Tup1       QQQQLQQQQPPPQVSVA--PLSNTAINGSPTSKETTT-----LPSVKAPESTLKETEPEN  242
Ca.Tup1       ---------PPT-------P---------------------------------------  130
Um.Tup1       EGPASHYSGPPSPGPERERDWIKKEERGDRGPPPRADEKWDRDQRVGGGAH---------  314
Zt.Tup1       -----QLQGP---------P--AANGYGSQQPPQPTASPGPGKPRLGGPPGLRGPATPQQ  209
Vd.Rco-1      QQQQQQQQQPPQQAPYPQGP--VPGGIG-AQPPQSTASPGPGRRGIGRPPGGVGPATPQI  231
Cp.Rco-1      ------AQQPPYQGGYPQGP--VSNGMG-PQPPQSTASPGPGRRGVGRPPNVGGPATPQI  217
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Sp.Tup1       ------------------HPPPPSDSANSSVT-----PIAAPLVVN-------GKV----  214
Sc.Tup1       NNTSKINDTGSATTA---TTT-----TATETEIKPKEEDATPASLHQDHYLVPYNQRANH  294
Ca.Tup1       ------------------------------------VTSLSVIDKSQYIVNPTQRANH  152
Um.Tup1       ------LGPGPVQSPHGAPPLPPSNLYNRDMHRDARDRDSRPSEVASA--DSPGAAAAAA  366
Zt.Tup1       HPAS--TYPGSPQ---VARPTPPPNRD---------------VQIAT---D--FQYTPAQ  244
Vd.Rco-1      NTPI--PFPGATQSPQVSHPTPDHARM----------------------G--NPHPPPP  264
Cp.Rco-1      NTPV--PYSGNAQSPQVSHPTPDHGRM----------------------G--GPR----  246

Sp.Tup1       -SGNPPYPAEI--IPTSNVPNREEKDWTVTSNVPNKEPPISVQLLHTLEHTSVICYVRFS  271
Sc.Tup1       SKPIPPFLLDLDSQSVPDALKKQTNDYYILYN-PALPREIDVELHKSLDHTSVVCCVKFS  353
Ca.Tup1       VKEIPPFLQDLDIAKANPEFKKQHLEYYVLYN-PAFSKDLDIDMVHSLDHSSVVCCVRFS  211
Um.Tup1       GNVTLASLSDMDPDEIPKELKKEGPDWLAIFN-PKVKRTLDVNLVHTFLHESVVCCVRFS  425
Zt.Tup1       LEQIGNQLSEYDVDKLPPHLKRQGDDWFAVFN-PRVHRKLDVELVHSLPHQSVVCCVRFS  303
Vd.Rco-1      APPVGNALGDLDIDRLPSSAKKSGYDWFVVYN-ERVPRMIDVDLVHTLGHESVVCCVRFS  323
Cp.Rco-1      VPPVGNALAELDLDSVAPHHKKTGSDWYAIFN-PSVQRVLDVDLVHSLAHESVVCCVRFS  305
                        :          ::   ::    *       :.::: ::: * **:* *:**

Sp.Tup1       ADGKFLATGCNRAAMVFNVETGKLITLLQEESSK--------------------------  305
Sc.Tup1       NDGEYLATGCNKTTQVYRVSDGSLVARLSDDSAANNHRNSITENNTTTSTDNNTMTTTTT  413
Ca.Tup1       RDGKFIATGCNKTTQVFNVTTGELVAKLIDESSNENK------------------------  248
Um.Tup1       ADGKYLATGCNKSAQIFDTKTGAKTCVLTDQS-AN--------------------------  459
Zt.Tup1       HDGRFIATGCNRSAQIFDVNTGKQVCHLMDQS-TN--------------------------  337
Vd.Rco-1      HDGKYVATGCNRSAQIYDVQTGEKLCILQDET-AD--------------------------  357
Cp.Rco-1      YDGKYVATGCNRSAQIYDVQSGEKVCVLEDHNAQD--------------------------  340
               **.::*****::: :: .  *     * :..

Sp.Tup1       --------------------------REGDLYVRSVAFSPDGKYLATGVEDQQIRIWDIA  339
Sc.Tup1       TTITTTTAMTSAAELAKDVENLNTSSSPSSDLYIRSVCFSPDGKFLATGAEDRLIRIWDIR  473
Ca.Tup1       ----------------------DDNTTASGDLYIRSVCFSPDGKLLATGAEDKLIRIWDLS  287
Um.Tup1       -------------------------SKGDLYIRSVCFSPDGKCLATGAEDRQIRIWDIG  493
Zt.Tup1       -------------------------GDGDLYIRSVCFSPDGRYLATGAEDKIIRVWDIG  371
Vd.Rco-1      -------------------------VSGDLYIRSVCFSPDGKYLATGAEDKLIRT----  387
Cp.Rco-1      -------------------------MTADLYIRSVCFSPDGRYLATGAEDKLIRVWDIA  374
                                       .***:***.*****: ****.**: **

Sp.Tup1       QKRVYRLLTGHEQEIYSLDFSKDGKTLVSGSGDRTVCLWDVEAGEQKLILH---------  390
Sc.Tup1       NRKIVMILQGHEQDIYSLDYFPSGDKLVSGSGDRTVRIWDLRTGQCSLTLS---------  524
Ca.Tup1       TKRIIKILRGHEQDIYSLDFFPDGDRLVSGSGDRSVRIWDLRTSQCSLTLS---------  338
Um.Tup1       KKKVKHLFSGHKQEIYSLDYSKDGRIIASGSGDKTVRIWDVENGQLLHTLYTSPGLEHGP  553
Zt.Tup1       AKVIRHQFSGHDQDIYSLDFASDGRYIASGSGDRTIRIWDLQDNQCVLTLS---------  422
Vd.Rco-1      -RTIRNTFSGHEQDIYSLDFARDGRTIASGSGDRTVRLWDIEPGSNTLTLT---------  437
Cp.Rco-1      SRSIRNHFSGHEQDIYSLDFARDGRTIASGSGDRTVRLWDIETGSNTLTLT---------  425
               : :   : **.*:*****:  .*  :.*****::: :**:.  .    *

Sp.Tup1       TDDGVTTVMFSP-DGQFIAAGSLDKVIRIWTS-SGTLVEQLH-------GHEESVYSVAF  441
Sc.Tup1       IEDGVTTVAVSPGDGKYIAAGSLDRAVRVWDSETGFLVERLDSENESGTGHKDSVYSVVF  584
Ca.Tup1       IEDGVTTVAVSP-DGKLIAAGSLDRTVRVWDSTTGFLVERLDSGNENGNGHEDSVYSVAF  397
Um.Tup1       SEAGVTSVSISS-DNRLVAAGALDTLVRVWDAQTGKQLERLK-------SHKDSIYSVSF  605
Zt.Tup1       IEDGVTTVAMSP-NGRFVAAGSLDKSVRIWDTRSGVLVERTEG----EQGHKDSVYSVAF  477
Vd.Rco-1      IEDGVTTVAISP-DTKYVAAGSLDKSVRVWDIHQGYLLERLEG----PDGHKDSVYSVAF  492
Cp.Rco-1      IEDGVTTVAISP-DTQYVAAGSLDKSVRVWDIHSGFLVERLEG----PDGHKDSVYSVAF  480
               : ***:* .*  : : :***:**  :*:*    *  :*: .       .*::*:*** *

Sp.Tup1       SPDGKYLVSGSLDNTIKLWELQCVSNVA----PSMYKEGGICKQTFTGHKDFILSVTVSP  497
Sc.Tup1       TRDGQSVVSGSLDRSVKLWNLQNANN----KSDSKTPNSGTCEVTYIGHKDFVLSVATTQ  640
Ca.Tup1       SNNGEQIASGSLDRTVKLWHLEGKS-----------DKKSTCEVTYIGHKDFVLSVCCTP  446
Um.Tup1       APDGKSLVSGSLDKTLKLWDLTGTAKAVQENRAEEKGGHANCATTFVGHKDYVLSVSCSP  665
Zt.Tup1       SPDGEHLVSGSLDKTIRMWRLNPRAQYQPGS-LAPQARGGDCVRTFEGHKDFVLSVALTP  536
Vd.Rco-1      SPNGKDLVSGSLDKTIKMWELSTPR----GL-PNPGPKGGRCVKSFEGHRDFVLSVALTP  547
Cp.Rco-1      SPNGKDLVSGSLDRTIKMWELISPR----GG-QSAAPKGGKCVKTFDGHRDFVLSVALTP  535
              : :*: :.*****.::::* *                  . *  :: **:*::***  :
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```
Sp.Tup1         DGKWIISGSKDRTIQFWSPDSPHSQLTLQGHNNSVISVAVS------PNGHCFATGSGDL   551
Sc.Tup1         NDEYILSGSKDRGVLFWDKKSGNPLLMLQGHRNSVISVAVANGSSLGPEYNVFATGSGDC   700
Ca.Tup1         DNEYILSGSKDRGVIFWDQASGNPLLMLQGHRNSVISVAVSLNSK--GTEGIFATGSGDC   504
Um.Tup1         DGQWVASGSKDRGVQFWDPKTAQAQFVLQGHKNSVIAINLS------PAGGLLATGSGDF   719
Zt.Tup1         DGAWVMSGSKDRGVQFWDPVTGDAQLMLQGHKNSVISVAPS------PMGTLFATGSGDM   590
Vd.Rco-1        DAAWVMSGSKDRGVQFWDPRTGATQLMLQGHKNSVISVAPS------PTGGYFATGSGDM   601
Cp.Rco-1        DANWVLSGSKDRGVQFWDPRTGSTQLMLQGHKNSVISVAPS------PQGGYFATGSGDM   589
                :  :: ****** : **.  :    : ****.****::  :           :******

Sp.Tup1         RARIWSYEDL---       561
Sc.Tup1         KKARIWKYKKIAPN      713
Ca.Tup1         KARIWKWTKK---       514
Um.Tup1         NARIWSYDRISN-       731
Zt.Tup1         KARIWRYAPYGGP       603
Vd.Rco-1        RARIWSYRSLQ--       612
Cp.Rco-1        KARIWSYRPY---       599
                .**** :
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Sup.1- Results of alignment of Tup1 protein sequences from *Saccharomyces pombe* (*Sp.* Tup1), *Saccharomyces cerevisiae (Sc.*Tup1*), Candida albicans* (*Ca.* Tup1), *Uromyces maydis* (*Um*. Tup1), *Zymoseptoria triciti* (Zt.Tup1) and *Verticillium dahlia* (*Vd*. Rco-1) and *Claviceps purpurea* (*Cp*. Rco-1) by ClustalW with the following domains: N-terminal (residues 1–72) is in bold; middle region includes: two repression domains (R1and R2: residues 73–172 and residues 267–365 respectively) in gray; two glutamine-rich regions (Q1 and Q2: residues 96–116 and 173–194 respectively) are in bold and italics; and the ST domain (serine-threonine-rich region: residues 392–419) is placed in gray box. C-terminal (residues 317–682) with seven WD40 repeats are in open boxes and the *S. cerevisie* WD residues are in black boxes.

Sup. 2- Percent identity matrix between Tup1 protein from various fungi (created by Clustal2.1)

	Verticillium dahlia	*Candida purpurea*	*Zymoseptoria tritici*	*Ustilago maydis*	*Claviceps albicans*	*Saccharomyces cerevisiae*	*Saccharomyces pombe*
Verticillium dahlia	100.00	78.35	59.72	44.98	48.73	40.30	43.42
Claviceps purpurea	78.35	100.00	60.76	46.45	50.00	42.52	43.98
Zymoseptoria tritici	59.72	60.76	100.00	45.73	48.86	39.66	42.12
Ustilago maydis	44.98	46.45	45.73	100.00	41.78	34.08	38.85
Candida albicans	48.73	50.00	48.86	41.78	100.00	66.41	44.26
Saccharomyces cereviciae	40.30	42.52	39.66	34.08	66.41	100.00	37.95
Saccharomyces pombe	43.42	43.98	42.12	38.85	44.26	37.95	100.00

Sup. 3- Predicted secondary structure of the N-terminus in *Saccharomyces cerevisiae*, *Zymoseptoria tritici*, V*erticillum dahlia*, *Claviceps purpurea* and *Ustilago maydis* Tup1 proteins sequences (residues 1–72 and 73–128). The line HELIX shows residues likely to adopt a-helical structure.