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In silico structural and functional characterization of the Yap1 transcription factor in fungi of the *Tuber* genus: a study approach on the mechanisms of response to oxidative stress

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ABSTRACT

Tuber genus includes hypogeous ectomycorrhizal (ECM) fungi, widely recognized as truffles. Truffles spend much of their life in a vegetative form in the soil, a crucial phase to ensure the growth and the accomplishment of their life cycle. Adverse events to which they may be subjected, *i.e.,* rise in temperature, drought, and biotic stress factors, can influence the levels of oxidative stress, increasing the production of free radicals.

It is known that both prokaryotes and eukaryotes have evolved various primary antioxidant defenses to protect themselves from oxidative damage. In some eukaryotes, transcription factors are activated to regulate the genes responsible for producing enzymes and proteins necessary to reduce ROS levels. In yeasts the Yap1 protein is a key modulator of oxidative stress response.

In this study, we used an *in silico* approach to characterize the Yap1 transcription factor in fungi of the *Tuber* genus, using *Saccharomyces cerevisiae* as the main reference model. The phylogenetic analysis based on the Yap1 protein sequences from 24 fungal species showed clustering consistent with their taxonomic relationships, including those observed within *Tuber* genus.

The amino acid alignment revealed the conservation within the *Tuber* species of cysteine-rich domains (nCRD and cCRD) and motifs potentially involved in redox regulation. Finally, by *silico* investigation of Yap1 possible protein interactions and three-dimensional structure in *Tuber melanosporum*, it was possible to provide further elements to understand the molecular mechanisms underlying the functionality of Yap1 and the response mechanism to oxidative stress modulated by it in fungi of the *Tuber* genus.

The evidence obtained represents an essential starting point for understanding a complex process that can strongly influence the biological cycle of the truffle and compromise its fruiting.

1. Introduction

The *Tuber* genus (*Tuberaceae*, *Pezizales*) comprises hypogeous ectomycorrhizal (ECM) fungi of the phylum *Ascomycota*, commonly known as truffles (Leonardi et al., 2017). *Tuber* includes more than 180 species distributed primarily in Europe, North America and Southeast Asia, with some species endemic and others occasionally found across continents (Wang et al., 2024). Several truffles are appreciated worldwide due to the unique aroma and flavour of their fruiting bodies. Considering the economic point of view, the main species with a commercial value are *Tuber melanosporum* Vittad. (Périgord black truffle) in the Melanosporum, *Tuber borchii* Vittad. (Bianchetto truffle) in the Puberulum, *Tuber magnatum Picco* (Italian white truffle) and *Tuber aestivum* Vittad. (Burgundy truffle) which are both inserted in the Aestivum (Graziosi, Hall and Zambonelli, 2022; Bonito et al., 2013). In particular, *T. magnatum*, is the most expensive edible fungi on the international gastronomic sector (Wang et al., 2024) Despite their economic and culinary value, truffles have a crucial role in the ecosystem, providing support for the development of their host plants as well as nutrients for mycophagous mammals (Bonito and Smith, 2016).

Tuber species exhibit considerable morphological and genetic diversity, which has complicated their taxonomic classification. However, the whole-genome sequencing of *T. melanosporum* has provided valuable insights into the evolution of the genus (Martin et al., 2010). Phylogenetic analysis and the use of molecular markers have proven to be

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Abbreviations

ROS – reactive oxygen species
SOD – superoxide dismutase
CAT – catalase
GPx – glutathione peroxidase
GR – glutathione reductase
nCRD – n-terminal cysteine-rich domain
cCRD – C-terminal cysteine-rich domain
NLS – nuclear localization sequence
NES – nuclear export sequence
bZIP – basic leucine zipper
VLCFAs very-long-chain fatty acids

fundamental tools for clarifying evolutionary relationships within the genus, showing that *Tuber* can be divided into eleven major clades (Bonito et al., 2013). Subsequently, the comparative genomic analyses of other *Pezizomycetes* support the previously works (Murat et al., 2018).

Tuber spp. during their complex life cycle, tackle with several biotic and abiotic factors which can influence their growth, symbiosis and productivity (Graziosi et al., 2022; Mello et al., 2006; Lee et al., 2020).

Previous genomic works on several *Tuber* species uncovered a repertoire of genes related to stress responses, including those involved in oxidative stress, metabolite detoxification, cell wall organization, mechanical stress, metal ion homeostasis and drought stress (Martin et al., 2010; Murat et al., 2018; Vita et al., 2020). Transcriptomic analysis performed in *Tuber indicum* and *T. borchii* revealed that environmental conditions such as cold storage and heat stress can induce significant changes in the expression of genes related with oxidative metabolism and redox balance (Zhang et al., 2024; Leonardi et al., 2017).

Furthermore, biochemical and pharmacological studies on *T. melanosporum* and *T. magnatum* have confirmed the antioxidant and anti-inflammatory activities of metabolic extracts, supporting the idea that these fungi have evolved efficient defence mechanisms against various stress stimuli. Phenolic profile, antioxidant, anti-inflammatory, and cytotoxic activities found in *Tuber* species suggest their potential as nutraceuticals (Beara et al., 2014).

As mycorrhizal fungi, *Tuber* spp. spend a large part of their life cycle in the soil, where they are exposed to environmental factors and microbial threats, leading to cellular oxidative stress (Mello et al., 2006).

Oxidative stress is a condition where reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot-}$), and hydroxyl radicals (HO•) accumulate and cause oxidation of lipids, proteins, and DNA, leading to cellular dysfunction and cell death (Fang et al., 2002; Fridovich, 1999).

To counteract the adverse effects of reactive oxygen species (ROS), organisms, including fungi, have developed advanced antioxidant defense mechanisms. These defenses consist of enzymatic and non-enzymatic components that work together to neutralize ROS, repair oxidative damage, and maintain cellular balance (Yaakoub et al., 2022). Primary antioxidants are specific enzymes that directly neutralize reactive oxygen species (ROS). These include superoxide dismutase (SOD), which converts superoxide radicals ($O_2^{\cdot-}$) into hydrogen peroxide (H_2O_2); catalase (CAT) which breaks down hydrogen peroxide into water and oxygen; glutathione peroxidase (GPx) and glutathione reductase (GR), which protect cells from oxidative damage by reducing peroxides with the help of glutathione (Halliwell and Gutteridge, 2007). Secondary, or preventive, antioxidant mechanisms deactivate metal ions, prevent the breakdown of lipid hydroperoxides, and regenerate primary antioxidants (Kozarski et al., 2015). Many of these protective mechanisms are conserved across species. In *Saccharomyces cerevisiae*, the Yap1 transcription factor regulates the oxidative stress response, activating the gene transcription involved in ROS detoxification and repair pathways (half of the 70 genes involved) (Mendoza-Martínez et al., 2020; Rodrigues-Pousada et al., 2010). Yap1 acts as a redox sensor through conserved cysteine residues in its N-terminal (nCRD) and C-terminal (cCRD) cysteine-rich domains (Mendoza-Martínez et al., 2020; Rodrigues-Pousada et al., 2010). The oxidation of these cysteine residues in response to ROS modulates Yap1's subcellular localization by affecting the recognition of its nuclear localization (NLS) and nuclear export (NES) signals (Mendoza-Martínez et al., 2020; Rodrigues-Pousada et al., 2010; Yaakoub et al., 2022).

Studies have shown that Yap1 plays a role in the regulation of penicillin production by *Penicillium chrysogenum* and may be involved in the regulation of aflatoxins, ochratoxins, and trichothecenes in *Aspergillus ochraceus* and *Fusarium graminearum* (Pérez-Sánchez et al., 2023). Despite the importance of this regulatory mechanism, the structure, function, and role of Yap1 in *Tuber* species remain completely unexplored. In *S. cerevisiae*, Yap1 oxidation is regulated by forming intramolecular disulfide bonds involving conserved cysteine residues, stabilizing the protein in an oxidized conformation. This conformational change promotes its retention in the nucleus, activating antioxidant gene expression. Based on sequence conservation and structural predictions, a similar redox-dependent regulatory mechanism is hypothesized in *Tuber* spp. Within the *Tuber* genus, *T. melanosporum* Vittad. was the primary focus for detailed *Tuber*'s Yap1 characterization due to the availability of a fully sequenced and functionally annotated genome. This comprehensive genomic information enabled accurate domain identification and structure–function predictions. Insights gained from the analysis of *T. melanosporum* were subsequently used to guide the investigation of Yap1 homologs in other *Tuber* species with less complete genomic data.

Understanding Yap1's function in *Tuber* spp. is crucial to clarify how truffles adapt to and survive under environmental stress conditions. This study aimed to investigate the structure and function of Yap1 in *Tuber* spp., through *in silico* analysis. Using bioinformatics tools, Yap1 homologs in *Tuber* spp. were compared with those of *S. cerevisiae* and 23 other fungi. The research provides the first step to explain the complex molecular processes underlying the oxidative stress response in *Tuber* spp.

2. Materials and methods

2.1. Database searches and selection of Yap1 sequences

In this study, Yap1 homologs from 23 fungal species and *Saccharomyces cerevisiae* were utilized (Table 1). The sequences data were obtained from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/), MycoCosm of Joint Genome Institute (JGI) fungal portal (http://jgi.doe.gov/fungi), and from the known yeast yap protein (Accession number: KZV08838.1). The deduced homologous protein of *Tuber melanosporum* was searched using BLAST and the corresponding sequence (NW_003298869.1) was found in *T. melanosporum* Whole Genome Shotgun Sequence Assembly, scaffold_204, strain Mel28, using the tool tBLASTn (Altschul, 1997). First, the mRNA (XM_002837868.2) was identified, and the corresponding predicted protein sequence was determined (Accession number: XP_002837914.1). The query sequence XP_002837914.1 from *T. melanosporum* was the starting point for identifying homologous sequences in other *Tuber* spp. (Martin et al., 2010). Using the BLASTP alignment with composition-based matrix adjustment, we identified sequence identity, similarity, E-value, and alignment coverage through the NCBI platform (Table 2) (Altschul, 1997).

2.2. Multiple alignment and phylogenetic relationships

Evolutionary analyses were conducted in MEGA 11 (Tamura et al., 2021). The amino acid sequence alignments were produced via multiple sequence alignments using ClustalW algorithm in the MEGA version 11

Table 1

List of the 24 fungal species selected for Yap1 analysis.

Species	Accession number	Sequence Length	Definition
Saccharomyces cerevisiae	KZV08838.1	650	Yap1
Schizosaccharomyces pombe	NP_593662.1	552	DNA-binding transcription factor, oxidative stress-responsive Pap1/Caf3
Candida albicans	KAF6072508.1	485	bZIP transcription factor AP-1/Yap1
Aspergillus fumigatus Af293	XP_750882.1	615	bZIP transcription factor AP-1/Yap1
Aspergillus nidulans FGSC A4	CBF79538.1	577	bZIP transcription factor AP-1/Yap1
Aspergillus parasiticus SU-1	KJK63566.1	583	transcription factor PAP1
Asperigillus flavus	EED48711.1	584	AP-1-like transcription factor yap1
Aspergillus niger	CAK45991.1	595	conserved hypothetical protein
Aspergillus oryzae RIB40	XP_001819128.1	563	AP-1-like transcription factor yap1
Pyricularia oryzae 70-15	EHA50719.1	576	unnamed protein product
Talaromyces marneffei ATCC 18224	XP_002145732.1	592	bZIP transcription factor AP-1/Yap1
Ustilago maydis (strain 521/FGSC 9021)	CAL48250.1	758	bZIP transcription factor
Colletotrichum gloeosporioides Cg-14	EQ B46862.1	566	transcription factor PAP1
Epichloe festucae	AGC94641.1	580	Ap1-like transcription factor
Botrytis cinerea	CAX15423.1	601	Ap1-like transcription factor
Morchella sextelata	XP_044700045.1	601	uncharacterized protein
Choiromyces venosus 120613-1	RPA96627.1	639	PAP1-domain-containing protein
Terfezia boudieri ATCC MYA-4762	RPB24434.1	594	PAP1-domain-containing protein
Tuber indicum	KAG0130860.1	621	transcription factor PAP1-domain-containing protein
Tuber aestivum	CUS13007.1	644	unnamed protein product
Tuber magnatum	PWW80753.1	616	PAP1-domain-containing protein
Tuber brumale	KAG0640787.1	616	transcription factor PAP1-domain-containing protein
Tuber borchii	PUU75379.1	615	transcription factor PAP1-domain-containing protein
Tuber melanosporum Mel28	XP_002837914.1	615	uncharacterized protein

software (Thompson et al., 1994). The evolutionary history was inferred using the Neighbor-Joining method (“The neighbor-joining method,” 1987). The analysis was conducted according to the following parameters: gaps were removed for each sequence pair, resulting in 888 residues in the final dataset; the statistical significance of individual nodes was assessed by bootstrap test (1000 replicates with only >70 % displayed on the final tree) and the evolutionary distances were computed using the p-distance method, leaving related parameters as default (Nei and Kumar, 2000). The final tree figure was obtained using the iTOL (https://itol.embl.de/) tree editor with the newick extension of MEGA NJ output as query (Letunic and Bork, 2024).

Table 2

BLASTP alignment performed using the query sequence of *T. melanosporum Mel28* XP_002837914.1 against the protein sequences of other *Tuber* species. The table includes the sequence identity, similarity, E-value, and alignment coverage.

Species	Sequence ID	Identities	Positives	Gaps
T. indicum	KAG0130860.1	607/621 (98 %)	611/621 (98 %)	6/621 (0 %)
T. aestivum	CUS13007.1	558/645 (87 %)	579/645 (89 %)	33/645 (5 %)
T. magnatum	PWW80753.1	565/616 (92 %)	585/616 (94 %)	1/616 (0 %)
T. brumale	KAG0640787.1	595/618 (96 %)	601/618 (97 %)	5/618 (0 %)
T. borchii	PUU75379.1	562/616 (91 %)	580/616 (94 %)	2/616 (0 %)

2.3. *Conserved amino acid residues identification within Yap1 motifs in Tuber species*

The distribution of conserved amino acid residues within Yap1 motifs in *Tuber* spp. was obtained using the WebLogo software (http://weblogo.berkeley.edu/logo.cgi) with default parameters and submitting the specific portion of each motif, previously analyzed via multiple sequence alignments, as queries in Clustal format (Crooks et al., 2004).

2.4. *Deduction of T. melanosporum Yap1 structure and protein interactions*

The three-dimensional structure of *T. melanosporum Mel* 28 Yap1 protein was analyzed using the UniProt database, which integrates data from AlphaFold (alphafold.ebi.ac.uk) (Jumper et al., 2021). AlphaFold is an artificial intelligence system by DeepMind that employs deep learning techniques to predict protein structures with high accuracy (Jumper et al., 2021; Varadi et al., 2022). The system utilizes evolutionary, structural, and biophysical information to generate structural models, significantly advancing the understanding of protein folding and function (Jumper et al., 2021; Varadi et al., 2022). In addition, the interactive protein network for the Yap1 of *T. melanosporum* (UniProt ID: D5CG62_TUBMM) was predicted using the STRING database (Search Tool for the Retrieval of Interacting Genes/Proteins) (string-db.org). The STRING analysis provides information about potential interaction partners, biological pathways, and the protein's role in the cellular context (Szklarczyk et al., 2023).

3. Results and discussion

Organisms have evolved antioxidant response mechanisms to sense and eliminate ROS (Sachdev et al., 2021). One of the developed mechanisms involves a series of cascade reactions that lead to the activation of Yap1, the first transcription factor identified as a member of the JUN family (Moye-Rowley et al., 1989). This protein is involved in a mechanism used by the cell to sense and translate ROS signals. The yap1 gene has been identified as an essential gene for survival against oxidative stress caused by hydrogen peroxide (H_2O_2), diamide compounds, cadmium, and other heavy metals (Schnell and Entian, 1991). Furthermore, the increase in Yap1 influences drug resistance (Hertle et al., 1991; Schnell and Entian, 1991). Yap1-mediated regulation of gene expression in response to oxidative stress is highly conserved in yeast fungi. Yeast activator proteins (YAPs) are an important subfamily involved in yeast adaptation to stress responses (Mendoza-Martínez et al., 2020; Rodrigues-Pousada et al., 2010). However, no information is available on the role of this factor in fungi of the *Tuber* genus. An alignment of the orthologous protein sequences of various fungal genera was carried out to examine the possible amino acid variations of the protein that could influence the antioxidant response. From the identification of the domains and the predictions of the three-dimensional conformation and interprotein interactions in *Tuber melanosporum,* it was possible to hypothesize that Yap1 in *Tuber* is involved in the oxidative stress mechanisms and can influence cellular differentiation. From these initial investigations, a preliminary vision of gene regulation and responses to oxidative stress in fungi of the *Tuber* genus was obtained, laying the foundations for understanding the mechanisms of adaptation and survival of these organisms in the vegetative phase.

3.1. Gene and predicted protein

The Yap1 protein of *Saccharomyces cerevisiae* (KZV08838.1) was used as a query in the NCBI genomic database, to identify the orthologous *Yap1* gene *of T.melanosporum*. The *T.melanosporum Yap1* gene (NCBI Gene ID: 9184797; locus tag GSTUM_00000600001) is located on scaffold_204 of the Mel28 genome assembly (RefSeq: NW_003298869.1, assembly accession GCF_000151645.1) (Martin et al., 2010). On the corresponding scaffold, the Y*ap* gene spans nucleotide 146 to 3646, with a total length of 3501 bp. Specifically, the gene codes for an mRNA (XM_002837868.2) composed of four exons and three introns, as shown in Table 3. The corresponding RefSeq protein (XP_002837914.1) contains the typical basic leucine zipper (bZIP) domain of Yap1 proteins, further found across the *Tuber* species analyzed in this work.

3.2. Phylogenetic analyses

The phylogenetic analysis of the Yap1 transcription factor is shown in Fig. 1. The resulting Neighbor-Joining tree was built using a dataset of 24 Yap1 amino acid sequences, clustered into five clades or evolutionary lineages distinguished by different colours. The overall clustering of Yap1 sequences reflects the taxonomic position of the analyzed species, rather than their biological habit as mutualistic, pathogenic, saprophytic, or other functional groups such as endophytes, mycoparasites, and lichenized fungi.

The *Tuber* species together with *Choiromyces venosus* (*C. venosus RPA96627.1*), *Morchella sextelata* (*M. sextelata XP_044700045.1*), and *Terfezia boudieri* (*T. boudieri RPB24434.1*), are grouped in the yellow clade with a strong bootstrap support. The phylogenetic tree (Fig. 1) confirmed the grouping of *T. indicum* with *T. melanosporum* in the *Melanosporum* subclade, and of *T. aestivum* with *T. magnatum* in the *Aestivum* subclade, consistent with previous evolutionary analyses of the genus (Bonito et al., 2013; Murat et al., 2018). As previously reported (Moreno et al., 2012), *C.venosus* is an independent species within the genus *Choiromyces* belonging to the family *Tuberaceae*. By contrast, *M. sextelata* and *T. boudieri*, even if are not members of the *Tuberaceae*, cluster in the yellow clade. It can explain considering their taxonomic affiliation within the order *Pezizales.* Notably, the presence of *M. sextelata* (Elata group), a saprophytic ascomycete, further confirms that clustering is determined by taxonomic affinity at the order level rather than by ecological strategy (Zhang et al., 2023).

The green clade includes *Botrytis cinerea* (*B. cinerea CAX15423.1*), *Epichloë festucae* (*Epichloe festucae AGC94641.1*), *Colletotrichum gloeosporioides* (*C. gloeosporioides EQB46862.1*), *and Pyricularia oryzae* (*P. oryzae EHA50719.1*). These species belong to four different families, *Sclerotiniaceae*, Clavicipitaceae, *Glomerellaceae*, and *Magnaporthaceae*, respectively. Despite that, their clustering is well supported by bootstrap values due to their broader placement within two classes. Specifically, *B. cinerea* (class *Leotiomycetes*) forms a separate branch, while *E. festucae*, *C. gloeosporioides*, *P. oryzae* (class *Sordariomycetes*) grouped in a subclade. The organization of the green clade indicates that Yap1 sequences are conserved even in lineages that have diversified into different families, reflecting the taxonomic separation between the sister classes *Leotiomycetes* and *Sordariomycetes* (Fitzpatrick et al., 2006). Consequently, the clustering reflects taxonomic affinity at the class level rather than biological habit.

The blue clade contains *Saccharomyces cerevisiae* (*S. cerevisiae KZV08838.1*), *Schizosaccharomyces pombe* (*S. pombe NP593662.1*), *Candida albicans* (*C. albicans KAF6072508.1*), and *Ustilago maydis* (*U. maydis CAL48250.1*). The first three species are ascomycetous yeasts belonging to the classes *Saccharomycetes* (*S. cerevisiae* and *C. albicans*) and *Schizosaccharomycetes* (*S. pombe*), while *U. maydis* is the only basidiomycete included in this study. Notably, they can all adopt a yeast-like growth form during part or all their life cycle (Fernandes et al., 1997; Jamieson et al., 1996). Thus, their clustering may be explained by convergent features in Yap1 sequences associated with yeast growth. The blue clade represents the only major exception to the overall taxonomic consistency observed in the phylogenetic tree (Fig. 1).

Aspergillus fumigatus (*A. fumigatus XP_750882.1*), *Aspergillus nidulans* (*A. nidulans CBF79538.1*), *Aspergillus oryzae* (*A. oryzae XP_001819128.1*), *Aspergillus parasiticus* (*A. parasiticus KJK63566.1*), *Aspergillus niger* (*A. niger CAK45991.1*), and *Aspergillus flavus* (*A. flavus EED48711.1*) are grouped within the purple clade. They form a monophyletic group supported by strong bootstrap values, in concert with their taxonomic classification within the genus *Aspergillus*. Moreover, they all belong to the family *Trichocomaceae*, order *Eurotiales*, class *Eurotiomycetes*, and phylum *Ascomycota*. In agreement with previous phylogenetic studies (Pérez-Sánchez et al., 2023), Yap1 is highly conserved within this genus.

Interestingly, *Talaromyces marneffei* (*T. marneffei XP_002145732.1*) is the unique species within the red clade and appears as an isolated lineage closely related to the genus *Aspergillus*. Indeed, *T. marneffei* clusters consistently with its taxonomic classification in the family *Trichocomaceae* (order *Eurotiales*, class *Eurotiomycetes*), the same family to which species of genus *Aspergillus* belong (Tsang et al., 2018). The division between the red and purple clades, originating from an ancestral node, indicates that Yap1 is conserved across the family *Trichocomaceae*, but the sequence divergence is sufficient to distinguish between the two lineages.

Table 3
Information about the exons and introns of the gene GSTUM_00000600001 (gene ID: 9184797).

No.	Exon/Intron	Start	End	Length nt
	5′ upstream sequence	1	358	358
1	Exon 1	359	860	502
	Intron 1	861	1173	313
2	Exon 2	1174	1662	489
	Intron 2	1663	1745	83
3	Exon 3	1746	2427	682
	Intron 3	2428	2511	84
4	Exon 4	2512	2686	175
	3′ downstream sequence	2687	3501	815

3.3. Yap1 domains

The Yap1 protein, from ectomycorrhizal fungi of the genus *Tuber*, was studied starting from the multiple alignment of the fungal sequences reported in Table 1. The Yap1 protein from *S. cerevisiae* contains five characteristic domains (Fernandes et al., 1997; Rodrigues-Pousada et al., 2010), also identified in its homologs (Fig. 2). Moreover, the frequencies of the amino acids at each position were evaluated for each domain with WebLogo 3 software, submitting separately as query the length of the specific sequences deducted from the multiple alignment (Fig. 2).

3.3.1. bZIP and NLS domain

Like numerous transcription factors, Yap of the fungi analyzed also contained the bZIP domain (Fernandes et al., 1997). The length of 64

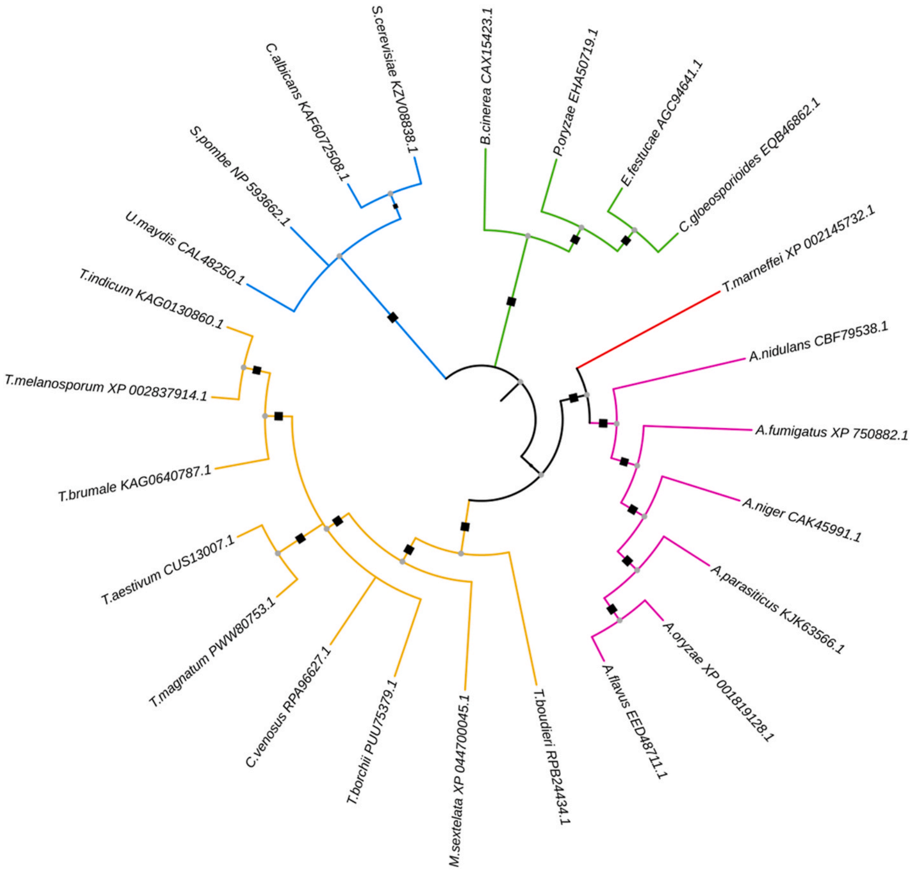

Fig. 1. Neighbor-Joining tree constructed based on the 24 protein sequences of Yap1. In the phylogenetic tree (sum of branch lengths = 4.65815398), colored branches represent different clades. GenBank accession numbers are shown next to each species name. The size of each black square on the nodes is proportional to the bootstrap value: 0.8, 0.85, 0.9, 0.95, up to 1.

amino acids is also unchanged, indicating the conservation of domain structure and functionality.

In the N-terminal region, we found the NLS sequence: N-terminal nuclear localization sequence. It is an 8-amino acid region, rich in lysine (K), arginine (R) and alanine (A) (Fig. 3). This domain is important for proper nuclear transport of Yap1 also in *Tuber* fungi, which is crucial for regulating gene expression in response to stress. NLS interacts with importins, allows Yap1 to bind the DNA, and activate the transcription (Lu et al., 2021). The NLS sequence is embedded with the basic domain that binds DNA, typically rich in R and K residues (Fig. 3).

In *Tuber* spp., it shows the high conservation of the “leucine zipper” structure, formed by two structures with α-helical residues, containing a dimerization domain and a basic domain. Leucine residues are placed every seven amino acids, like the bZIP in *S. cerevisiae* (Fernandes et al., 1997) (Fig. 3). Interestingly, in all *Tuber* spp. analyzed, the third leucine residue typically found in the bZIP leucine zipper motif is substituted by either an alanine or a threonine. This conserved variation may suggest a modulation of the dimerization strength of Yap1 in truffles, potentially representing an evolutionary adaptation to specific environmental or redox conditions.

3.3.2. nCRD

The nCRD is in the N-terminal region of the Yap1 protein and represents one of the two cysteine-rich domains (CRD). In all *Tuber* species analyzed in this study, three conserved cysteine residues were identified (Fig. 4), distributed along the nCRD (Rodrigues-Pousada et al., 2010). These cysteines are critical for sensing oxidative stress through the formation of intramolecular disulfide bonds. Their conservation across species suggests the preservation of the redox-sensing mechanism typical of Yap1 in *S. cerevisiae*.

3.3.3. cCRD and NES domain

The cCRD represents the second of the two cysteine-rich regions (CRD), and it is located in the C-terminal region of the Yap1 amino acid sequence. This domain varies in length among *Tuber* spp., ranging from 51 amino acids in *Tuber brumale* to 79 amino acids in *T. aestivum*. In all species analyzed, three conserved cysteine residues were identified, except for *T. indicum* and *T. borchii*, which possess an additional cysteine residue (Fig. 5). Moreover, within the cCRD, a C-terminal NES was identified. This NES region, rich in aspartic acid (D) and serine (S) residues, consistently spans 21 amino acids across all species (Fig. 5) and acts as the key signal regulating nuclear export of protein. Loss or masking of this region results in the nuclear accumulation of Yap1 (Mendoza-Martínez et al., 2020).

3.4. Yap1 interactome map prediction

We identified a list of proteins potentially involved in specific biological pathways using the Core Dat Resource STRING database (Szklarczyk et al., 2023). The STRING tool predicts protein–protein interactions (PPIs). In *T. melanosporum*, ten proteins could interact with Yap1 (Table 4), obtaining the interactome map shown in Fig. 6. Four of these proteins would seem to be involved in the response to oxidative stress (Martin et al., 2010): thioredoxin peroxidase (TPx), glutathione peroxidase (GPx) and reductase (GR), and thioredoxin-like proteins (Trx).

Two proteins that have been identified are involved in the thioredoxin pathway (Martin et al., 2010). The first of these, which we will define as Trx, has a thioredoxin domain that could probably form transient redox-active disulfide bonds that are alternately reduced and oxidized during an enzymatic reaction (Carmel-Harel et al., 2001). This

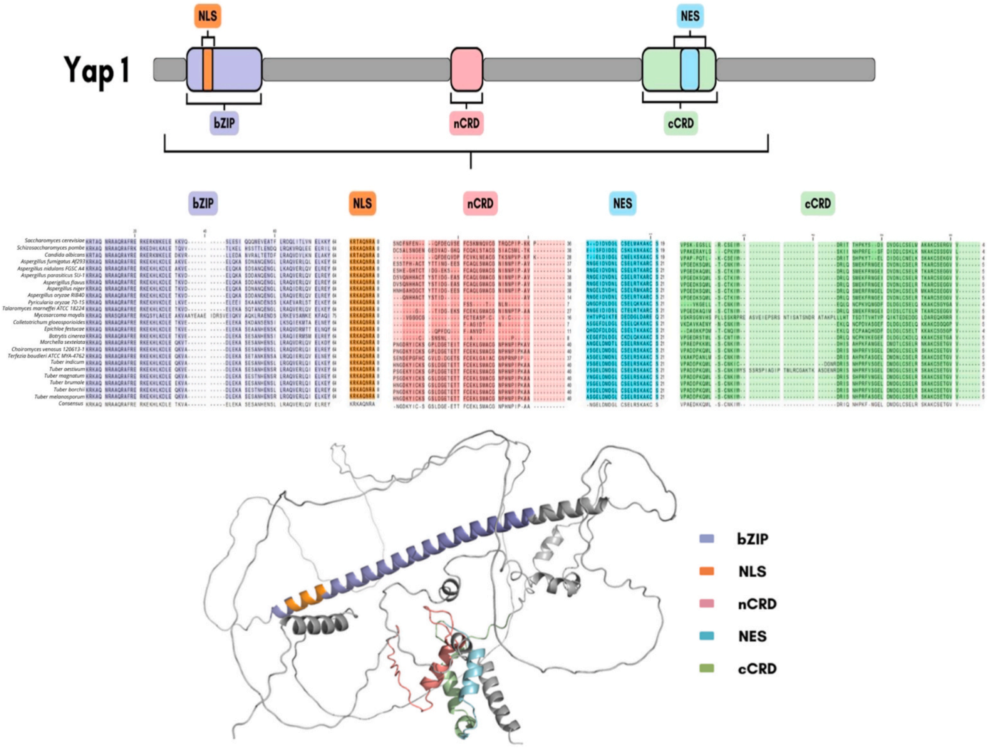

Fig. 2. Graphical representation of the conserved domains in yeast, filamentous fungi, and *Tuber* spp.: the basic leucine zipper (bZIP, violet), nuclear localization signal (NLS, orange), N-terminal cysteine-rich domain (nCRD, red), nuclear export signal (NES, cyan), and C-terminal cysteine-rich domain (cCRD, green). The multiple sequence alignment of Yap1 homologs from various fungal species highlights the conservation of these domains. At the bottom, a predicted 3D model of *T. melanosporum* Yap1 (D5GC62) from AlphaFold2, using PyMOL visualization tool, illustrates the spatial arrangement of the functional domains. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

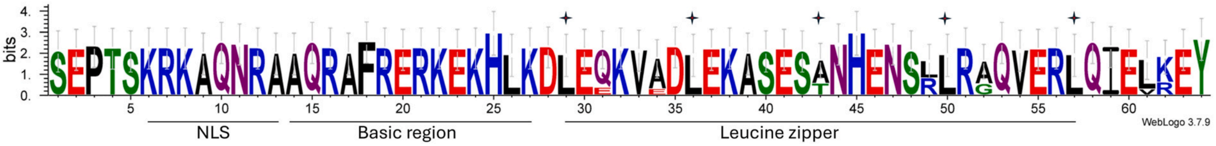

Fig. 3. Logo consensus sequences of bZIP and NLS domain. Black stars indicate conserved leucine residues, except for the third leucine as discussed in the text. Each stack's overall height represents the sequence conservation level at each position. Capital letters highlight amino acids that are conserved in more than 50 % of the sequences within the domains. Arabic numerals below the colored letters indicate the motif width, with each color corresponding to a different amino acid category. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

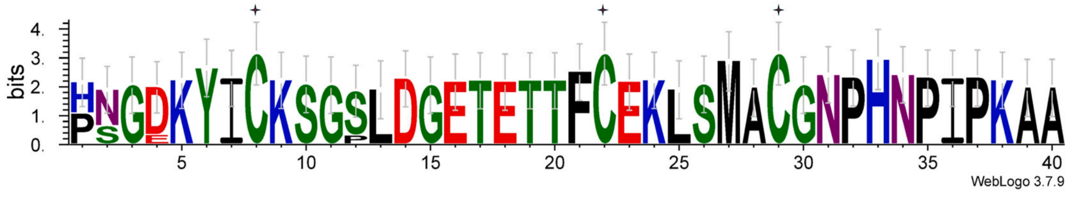

Fig. 4. Logo consensus sequences of nCRD domain.
Black stars indicate conserved cysteine residues. The overall height of each stack represents the level of sequence conservation at each position. Capital letters highlight amino acids that are conserved in more than 50 % of the sequences within the domains. Arabic numerals below the colored letters indicate the motif width, with each color corresponding to a different amino acid category. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

protein interacts with the second protein, a thioredoxin-dependent peroxiredoxin (TPx), that catalyzes the reduction of hydrogen peroxide and organic hydroperoxides into water and alcohols, respectively (Patenaude et al., 2005). It is predominantly present in the cytoplasm and has an active site of sulfenic acid (-SOH), forming disulfide bridges between cysteines, which is important for the oxidation of Yap1 (Patenaude et al., 2005).

A glutathione reductase (GR) is present, which is necessary to maintain low glutathione (GSH) levels in the cytosol, which is essential for the detoxification of ROS (Gostimskaya and Grant, 2016; Martin et al., 2010).

Another key protein, which could initiate a reaction cascade in case

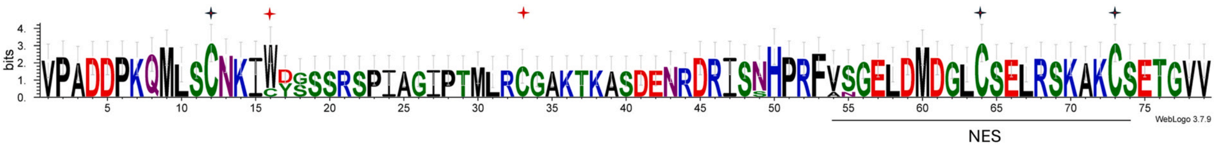

Fig. 5. Logo consensus sequences of cCRD and NES domains.
Black stars indicate conserved cysteine residues found in all Tuber species considered in this study. Red stars indicate the two more cysteines in *T. indicum* and *T. aestivum*. In particular, the cysteine residue at position 16 belongs to *T. indicum*; instead, the cysteine residue at position 33 corresponds to *T. aestivum*. The overall height of each stack represents the level of sequence conservation at each position. Capital letters highlight amino acids that are conserved in more than 50 % of the sequences within the domains. Arabic numerals below the colored letters indicate the motif width, with each color corresponding to a different amino acid category. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 4
List of proteins possibly interacting with Yap1 in *T. melanosporum* identified using the Core Data Resource STRING database.

Uniprot Code	Accession number	Gene model	Definition e/o best match	Name abbreviated
D5GMA9_TUBMM	XP_002841461.1	GSTUM_00010629001	tRNA nucleus export receptor	Exp-t
D5GFP7_TUBMM	XP_002839149.1	GSTUM_00001911001	Oxidoreductase activity, acting on the CH–CH group of donors	Oxidoreductase
D5GD35_TUBMM	XP_002838237.1	GSTUM_00000949001	DNA-binding transcription factor activity	TF
D5G450_TUBMM	XP_002835172.1	GSTUM_00003950001	Glutathione reductase	GR
D5GI33_TUBMM	XP_002839985.1	GSTUM_00008248001	Thioredoxin peroxidase activity	TPx
D5GEV7_TUBMM	XP_002838859.1	GSTUM_00001411001	Thioredoxin like protein	Trx
D5GCE5_TUBMM	XP_002837997.1	GSTUM_00005852001	Mitogen-activated protein kinase	Map-K
D5GB16_TUBMM	XP_002837518.1	GSTUM_00005402001	Glutathione peroxidase	GPx
D5GDS0_TUBMM	XP_002838472.1	GSTUM_00006244001	Very-long-chain 3-oxoacyl-CoA reductase	VLCF-oxoacyl-CoA R
D5GMP7_TUBMM	XP_002841599.1	GSTUM_00010871001	Oxidoreductase activity	oxidoreductase

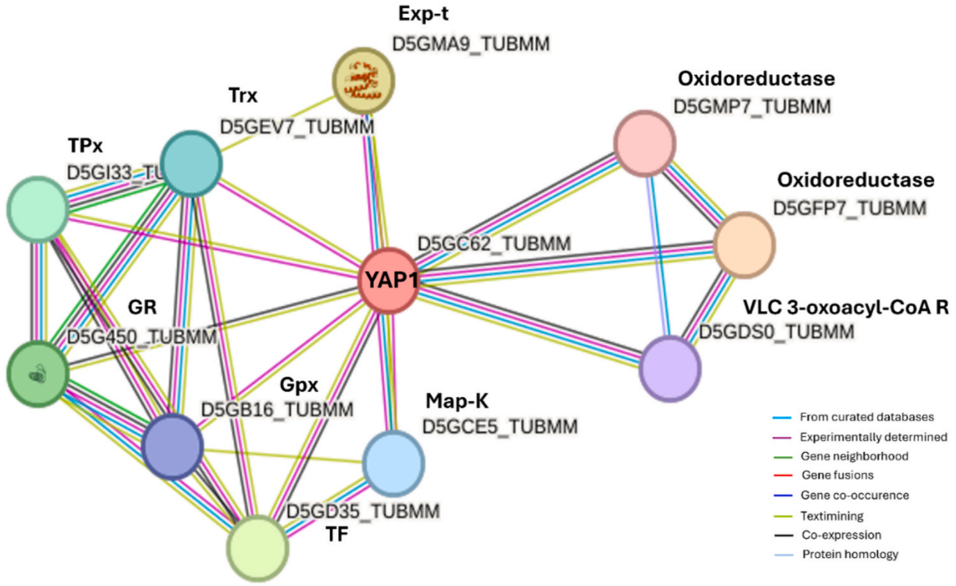

Fig. 6. Predicted Interactome map of the Yap1 protein in *T. melanosporum*. The known interactions are identified in light blue; the experimentally determined interactions are in fuchsia. The remaining interactions were presumed by gene proximity (in green), gene fusion (in red), gene co-occurrence (in blue) (string-db.org/). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

of oxidative stress caused by H_2O_2, is glutathione peroxidase GPx. Its function is important because it catalyzes two molecules of glutathione with H_2O_2, giving rise to one molecule of oxidized glutathione and two molecules of water (Vašková et al., 2023). The presence of glutathione peroxidase (GPx) and glutathione reductase (GR) suggests a functional glutathione system, which is part of the antioxidant response. Yap1 could regulate the expression of GR, creating a positive feedback loop: the more GSH is available, the more Yap1 is reduced and inactivated.

Another protein identified in the interaction network is TF, a transcription factor likely homologous to Skn7 in other fungi (Lamarre et al., 2007; Nikolaou et al., 2009; Tsuzi et al., 2004; Yaakoub et al., 2022). TF may act as a co-regulator with Yap1, enhancing the activation of stress-responsive genes, particularly under oxidative stress conditions. Additionally, the presence of Map-K (Mitogen-activated protein kinase) in the network suggests a potential role in regulating stress response pathways. In *S. pombe*, the Map-K Sty1 activates Pap1 (a Yap1 homolog) under stress, and a similar mechanism could occur in *Tuber* spp. (Chen et al., 2003). Here, Map-K might phosphorylate Yap1, modulating its transcriptional activity or subcellular localization, thereby integrating redox signaling with other stress-responsive pathways, a regulatory layer not yet described in yeast. Furthermore, the interaction network includes VLCF-oxoacyl-CoA reductase, an enzyme involved in synthesizing very-long-chain fatty acids (VLCFAs), which are critical for membrane integrity (Sassa and Kihara, 2014). Since oxidative stress often damages cellular membranes, it is hypothesized that Yap1 could recruit or upregulate enzymes like VLCF-oxoacyl-CoA reductase to facilitate membrane repair and maintain cellular homeostasis under stress conditions.

3.5. *Yap1 oxidation mechanisms*

The mechanisms characterized in *S. cerevisiae* provide a robust framework for hypothesizing analogous oxidative stress responses in *Tuber* spp. The evolutionary conservation of stress response pathways in fungi supports the likelihood of shared regulatory mechanisms. Under physiological conditions, Yap1 in *Tuber* spp. is predicted to shuttle dynamically between the nucleus and cytoplasm, mediated by the coordinated activity of importins (facilitating nuclear entry) and exportins (promoting cytoplasmic export). This shuttling is governed by the presence of conserved nuclear localization signals (NLS) and nuclear export signals (NES) within Yap1's structure (Mendoza-Martínez et al., 2020; Rodrigues-Pousada et al., 2010). Among the interacting partners identified, Exp-t (tRNA nucleus export receptor) is hypothesized to regulate this nucleocytoplasmic transport, akin to the role of Crm1/exportin-1 in *S. cerevisiae* (Maeta et al., 2004).

Reactive oxygen species (ROS) and hydrogen peroxide (H_2O_2), generated during aerobic respiration or under altered cellular conditions, trigger oxidative stress. In *T. melanosporum*, thioredoxin peroxidase (TPx), a functional homolog of *S. cerevisiae* peroxiredoxin (Prx), acts as the primary redox sensor (Carmel-Harel et al., 2001). Upon ROS accumulation, TPx undergoes oxidation, forming a sulfenic acid intermediate at its catalytic cysteine residue. This oxidized TPx transiently interacts with Yap1, initiating a disulfide relay. In *S. cerevisiae*, peroxiredoxin (Prx) transfers oxidative stress signals to Yap1 via intermolecular disulfide bonds, involving conserved cysteine residues in Yap1's cysteine-rich domains (CRDs) (Carmel-Harel et al., 2001). Specifically, three critical disulfide bonds form in yeast Yap1: Cys303-Cys598, Cys310-Cys629, and Cys315-Cys620, stabilizing its oxidized conformation and masking NES motifs to retain Yap1 in the nucleus. In *T. melanosporum*, sequence alignments and AlphaFold-predicted structural models suggest a similar mechanism (Fig. 2). The presence of conserved cysteine clusters in its N-terminal (nCRD) and C-terminal (cCRD) domains implies redox-sensitive conformational rearrangements. For instance, Cys554 (cCRD) may form an intramolecular disulfide bond with Cys414 (nCRD) (Fig. 7. B), analogous to the Cys303-Cys592 bond in yeast. A second bond between Cys587 (cCRD) and Cys421 (nCRD) (Fig. 7. A) could further stabilize the oxidized state, mirroring the yeast Cys310-Cys629 interaction.

Notably, Cys578 and Cys400 do not participate in disulfide bonding, as inferred from the AlphaFold-predicted CRD architecture, which lacks spatial proximity between these residues.

This redox-dependent conformational shift likely masks Yap1's NES motifs, preventing Exp-t-mediated nuclear export and ensuring sustained nuclear retention. Once in the nucleus, Yap1 activates transcription of antioxidant genes (e.g., glutathione peroxidases, catalases), restoring redox homeostasis.

The thioredoxin-like protein (Trx) is essential for reducing oxidized Yap1 by interruption its disulfide bonds (Carmel-Harel et al., 2001; Delaunay et al., 2002; Maeta et al., 2004). This reductive process restores the accessibility of the NES, enabling Yap1 to interact with exportins and return to the cytoplasm (Delaunay et al., 2000). The redistribution of Yap1 ensures the dynamic balance of the redox-dependent regulatory system, allowing fungal cells to reset their stress response machinery and maintain homeostasis under fluctuating oxidative conditions (Wood et al., 2004).

4. Conclusions

This study used an *in silico* approach to characterize Yap1 transcription factor in fungi of the *Tuber* genus and hypothesize its functional roles using homologous proteins from other filamentous fungi and *Saccharomyces cerevisiae*. Based on bioinformatics analysis, it is possible to assume its involvement in the mechanisms of action aimed at counteracting oxidative stress, especially during *Tuber* growth and development.

The proposed mechanism of Yap1 activation in *Tuber* spp. mirrors that of *S. cerevisiae:* under oxidative stress caused by H_2O_2, the thioredoxin-dependent peroxiredoxin (TPx) acts as the primary sensor of high concentrations of hydrogen peroxide. This protein oxidizes the cysteines of the Yap1 transcription factor (Carmel-Harel et al., 2001; Delaunay et al., 2000; Yaakoub et al., 2022). This oxidation triggers Yap1's nuclear accumulation, activating the genes for the synthesis of proteins involved in the antioxidant response (Belozerskaya and Gessler, 2007; Mendoza-Martínez et al., 2020). Subsequently, the glutathione system is engaged to reduce H_2O_2: a glutathione synthetase produces glutathione necessary as a substrate for the glutathione peroxidase (GPx) to convert H_2O_2 into water and oxidized glutathione (Tsuzi et al., 2004). This process is tightly regulated, with glutathione reductase (GR) potentially inhibited to maintain the balance between glutathione and H_2O_2 levels.

Beyond oxidative stress responses, Yap1, together with TF and the Map-k, is hypothesized to promote the transcription of genes involved in cell differentiation, suggesting a dual role for Yap1 in both stress adaptation and developmental processes.

In conclusion, the *in silico* predictions presented here suggest that Yap1 and its regulatory network are crucial for the growth and

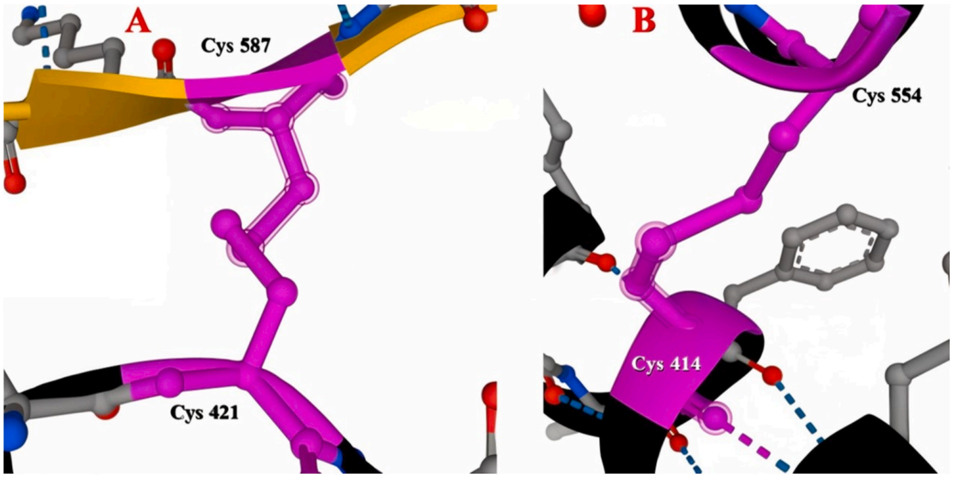

Fig. 7. A) Transient disulfide bonds form between Cys421 (nCRD) and Cys587 (cCRD), and between Cys414 (nCRD) and Cys554 (cCRD) **B)**, stabilizing a closed conformation that masks NES motifs. Structural predictions were generated using AlphaFold2.

development of *Tuber* spp., contributing not only to oxidative stress defense but also to pathways driving cellular differentiation. These findings provide a foundation for further exploration of the molecular mechanisms underlying *Tuber* spp. life cycle, particularly in response to environmental stressors.

Such insights could prove invaluable for developing strategies to protect *Tuber* spp. from environmental challenges, preserving their ecological roles and economic value as prized truffle-producing fungi. Future experimental studies, including functional characterization of Yap1 and its interactors, will be essential to validate these hypotheses and deepen our understanding of *Tuber* genus biology.

CRediT authorship contribution statement

B. Ranocchi: Writing – review & editing, Writing – original draft, Software, Resources, Methodology, Investigation, Data curation. **A. Pennesi:** Writing – original draft, Software, Methodology, Investigation. **E. Baglieri:** Writing – original draft, Software, Methodology, Investigation. **A. Amicucci:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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