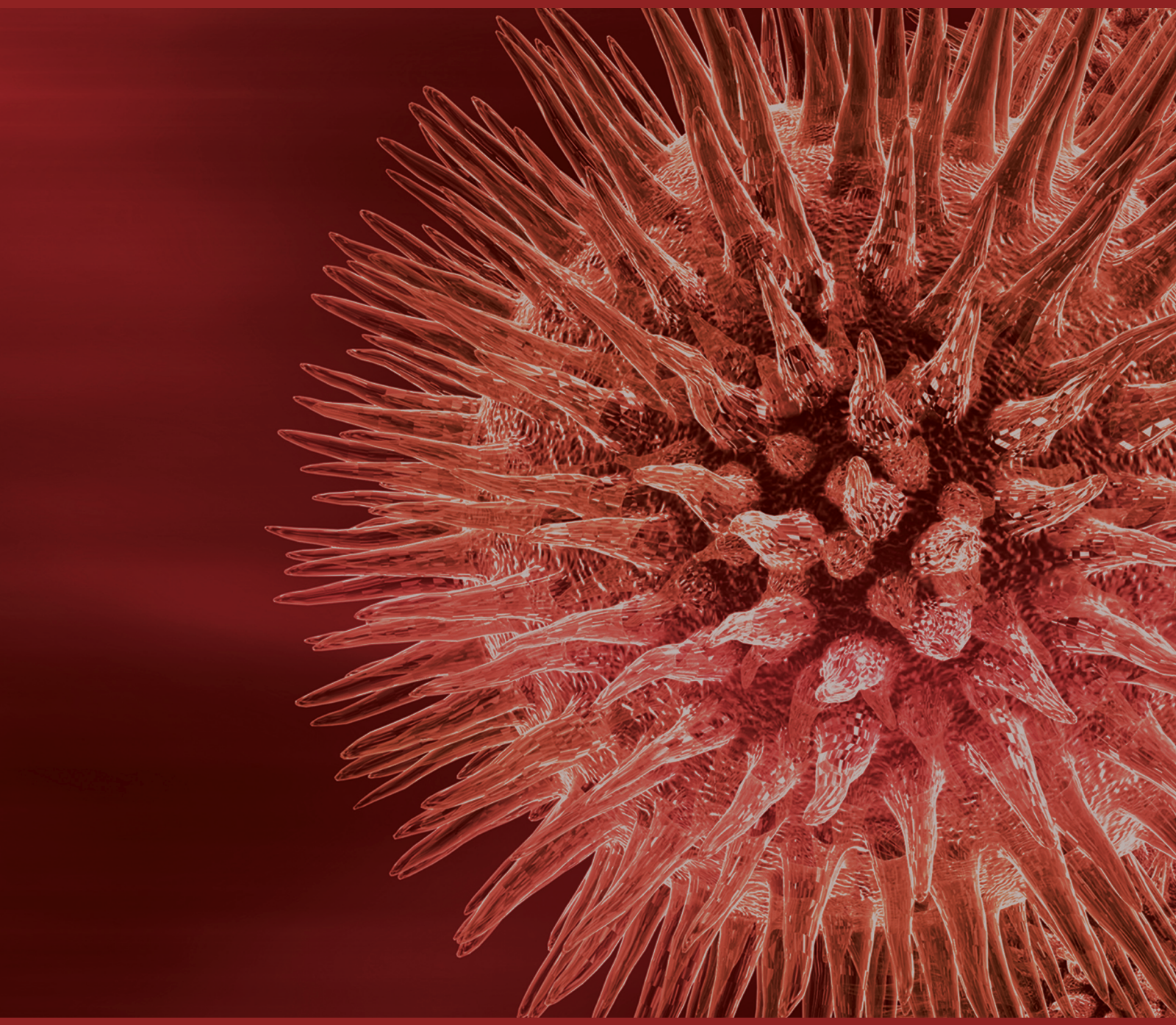
Journal of Biomedicine and Biotechnology

Contribution of Yeast and Plant Research for Improving Human Health

Guest Editors: Diego F. Gomez-Casati, Marina Clemente, Alberto Inga, Lucilia Saraiva, and Claudia P. Spampinato



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Editorial

Contribution of Yeast and Plant Research for Improving Human Health

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In the last years there was an increase in the use of several organisms such as plants and yeast for medical and biotechnological purposes. Both organisms are very useful when research in humans is nonpossible or unfeasible due to ethical issues. There are several advantages of using model organisms such as the availability of genomic information, the possibility to easily make genetic manipulation and screenings, and availability of mutants in desired genes. In addition, these organisms are generally easy to grow.

The conservation in many cases of various metabolic and signaling pathways and basic cellular processes, often accompanied by proteomic and metabolomic information, is also an advantage in medical research, while the presence of specific metabolic pathways may be an advantage in the production of compounds of industrial interest. The paper of P. Jeandet et al. presents a detailed review on the biosynthesis, metabolism, and production of resveratrol, a stilbenic derivative. This compound is produced in the phenylalanine/polymalonate pathway, with the stilbene synthase (STS) being the key enzyme in this process. The increased use of resveratrol as "nutraceutical" (for nutrition and pharmaceutical uses) or in plant disease control makes its production an important goal in biotechnology. Different approaches to increase the production of this compound include the manipulation of the biosynthetic pathway and the overexpression of STS and other specific genes.

Besides the production of compounds of industrial interest, the use of plants for bioremediation and for the production of vaccine antigens is other important achievement in plant biotechnology related to improving human health. Some toxic compounds that can accumulate in plants are heavy metals. This is one of the biggest problems of environmental pollution and they also have severe consequences for their toxic effects on human health. The paper of Z. Zhang et al. presents a study of the accumulation of Pb and Cd in elite maize inbred lines. This study shows that there are significant differences in Pb and Cd accumulation between maize hybrid lines. So, it is possible to select low-metal accumulation lines for nutritional purposes. In addition, the study also showed the existence of maize lines with high capacity for accumulation of heavy metals that might be useful for bioremediation purposes.

On the other hand, plants become an emerging alternative for the production of vaccine antigens. The use of plants has several advantages such as easy gene engineering and low cost in the production of antigens. M. Clemente et al. reviewed the strategies for the production of antigens against malaria in plants. Malaria is a major health problem worldwide due to its high mortality rate, estimated at around one million people per year. Such strategies include the production of codon-optimized surface antigens of *Plasmodium*, the agent responsible for the disease, and the production

of dual cholera-malaria antigens in tobacco plants and bacterial-made antigens.

There is also an extensive work using yeast, such as *Saccharomyces cerevisiae* and plants, mainly *Arabidopsis thaliana*, for the study of human pathologies. One example of this is the use of *Saccharomyces* for the study of the function of proteins of the human immunodeficiency virus (HIV) and the identification of its cellular partners common to yeast and humans. It is known that this retrovirus is the cause of AIDS, which affects more than 30 million people around the world. The paper of M. L. Andréola and S. Litvak provides a detailed review of the work done on the identification of cellular factors associated with different viral proteins, as well as the function and regulation of known enzymes encoded by the viral genome. The knowledge of the role of the viral proteins and cellular targets might allow the development of new therapeutic targets and strategies. In addition, this research could allow the discovery of new genes and proteins involved in virus proliferation and virus infection mechanisms, shortening the time necessary to develop different therapies against new viruses.

Besides the use of yeast, the study of plants, such as *Arabidopsis thaliana*, is a valuable tool in biomedicine, in the study of several mechanisms that might act similarly in producing human diseases, since human genes and proteins have been structurally and functionally conserved in plant organisms. The paper of C. P. Spampinato and D. F. Gomez-Casati presents a review of the current knowledge of human diseases of nuclear origin such as those related to defects in DNA repairing pathways and also mitochondrial pathologies, such as Friedreich's ataxia and respiratory complexes deficiencies, leading mainly to the production of neurological diseases. In addition, several studies in yeast could also contribute to uncover the etiology and pathogenesis of several neurodegenerative diseases as reviewed in the work of Pereira et al., including Alzheimer's, Parkinson's and Huntington's disease.

These papers represent an interesting compilation on the use of yeast and plants for biotechnological and biomedical purposes and the future prospects.

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Diego F. Gomez-Casati
Marina Clemente
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Review Article

Contribution of Yeast Models to Neurodegeneration Research

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As a model organism *Saccharomyces cerevisiae* has greatly contributed to our understanding of many fundamental aspects of cellular biology in higher eukaryotes. More recently, engineered yeast models developed to study endogenous or heterologous proteins that lay at the root of a given disease have become powerful tools for unraveling the molecular basis of complex human diseases like neurodegeneration. Additionally, with the possibility of performing target-directed large-scale screenings, yeast models have emerged as promising first-line approaches in the discovery process of novel therapeutic opportunities against these pathologies. In this paper, several yeast models that have contributed to the uncovering of the etiology and pathogenesis of several neurodegenerative diseases are described, including the most common forms of neurodegeneration worldwide, Alzheimer's, Parkinson's, and Huntington's diseases. Moreover, the potential input of these cell systems in the development of more effective therapies in neurodegeneration, through the identification of genetic and chemical suppressors, is also addressed.

1. Introduction

The budding yeast *Saccharomyces cerevisiae* has long been used as an eukaryotic model organism mostly due to its ease of manipulation and amenability to genetic modifications. This can also explain the forefront position of yeast in the development of large scale screening approaches, like DNA and protein microarrays [1–3], two-hybrid analysis [4, 5], and whole-genome deletion and overexpression libraries [6, 7]. Additionally, this contributed to the large amount of easily accessible online dataset for yeast which include genetic interactions, transcriptional changes, protein interactions, and localization (reviewed in [8]).

The use of yeast as a model organism was recently expanded to the dissection of the molecular mechanisms of human diseases, either by directly studying an endogenous protein orthologue of a human counterpart involved in the disease or through the heterologous expression of human disease-associated proteins. Though several aspects of the disease are beyond the reach of a unicellular organism like yeast, many processes and pathways are highly conserved in this organism, namely, mitochondria biogenesis, protein quality control, vesicular trafficking, and autophagic pathways. Actually, the high simplicity of yeast, when compared to human cells, has been widely explored to accelerate the discovery of new drugs and therapeutic targets in human diseases. In fact, when complemented with more physiologically relevant models where the hits discovered will be validated, yeast can be seen as a powerful first-line screening system for large genetic and chemical libraries.

With the increase in life expectancy, neurodegenerative disorders such as Alzheimer's, Parkinson's, and Huntington's diseases became major health problems in our society for which treatments are critically required [9].

The yeast models herein described, based on both classical and high-throughput methodologies, have widely contributed to the uncovering of the molecular mechanism underlying neurodegenerative disorders. Since many of them were adapted to target-directed screening approaches, a promising contribution of yeast to the identification of novel therapeutic opportunities against these pathologies can be envisaged.

2. Yeast Models of Neurodegeneration-Associated Proteins

2.1. Yeast Models Based on Orthologues. Neurodegenerative diseases are hereditary and sporadic conditions, which are

characterized by a progressive nervous system dysfunction. Sporadic diseases like Parkinson's disease (PD) also have rare familial forms that have been linked to mutations in several genes, providing research opportunities for pathogenic mechanisms [10]. Because yeast encodes orthologues of many disease-causing proteins, several disease models based on the study of protein functions in yeast were established (Table 1). For example, Yfh1p is the yeast orthologue of human frataxin whose decreased expression and/or function is associated with Friedreich's ataxia (FRDA), a neuro- and cardiodegenerative disorder [11]. Studies with Yfh1p were decisive in determining the function of frataxin. Absence of Yfh1p, likewise of its human orthologue, results in mitochondrial iron accumulation, mitochondrial dysfunction, and oxidative stress [12, 13]. Recently, the yeast model was applied to the high-throughput screening (HTS) of compounds able to rescue mitochondrial function [14]. This was possible because yeast is one of the rare eukaryotes with a good fermenting capacity allowing the analysis of commonly disease-associated mitochondrial defects that would be lethal in other systems [15]. Other proteins that were directly studied in yeast are associated with Batten's [16] and Niemann-Pick's [17, 18] diseases, Ataxia telangiectasia [19, 20], and Hereditary Spastic Paraplegia [21]. Though yeast has no true orthologues of the human prion protein (PrP), responsible in its prion form for the Creutzfeldt-Jakob disease, it has prions, with at least three forms [URE3], [PSI+], and [PIN+], that show similarities concerning transmission of phenotype in a protein-only mode [22]. The yeast prion system has been useful, for example, for the screening of inhibitors with promising activity against mammalian prions [23].

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by the selective degeneration of motor neurons. With the recognition of the involvement of copper-zinc superoxide dismutase (CuZnSOD1) gene in familial ALS [24], the respective yeast orthologue was studied in an attempt to clarify the role of disease-associated mutations [25]. More recently, TDP-43 (TAR DNA binding protein) and FUS/TLS (fused in sarcoma/translocated in liposarcoma) were recognized as major pathological proteins in sporadic cases of ALS. TDP-43 and FUS/TLS are both RNA-binding proteins that are found in ubiquitinated protein aggregates in the cytoplasm of ALS and in frontotemporal lobar degeneration patients [26].

2.2. Yeast Models Based on Heterologous Expression. In many cases, the human gene encoding the neurodegeneration-causing protein has no orthologue in yeast. As such, its heterologous expression in this organism (the so-called "humanized yeast") has been widely used (see Table 1). For example, yeast cells provided a useful system for investigating ALS through TDP-43 and FUS/TLS expression. Similarly to that observed in ALS patients, yeast expressing human TDP-43 exhibit cytoplasmic TDP-43 aggregates that correlate with toxicity [27, 28]. Likewise, expression of FUS/TLS in yeast was recently described to form protein aggregates and to induce cytotoxicity, with two ALS-associated mutants showing increased cytotoxicity [29, 30]. Several other proteins involved in neurodegeneration, namely, α-synuclein and Lrrk2 in PD, tau and amyloid-β (Aβ) peptide in Alzheimer's disease (AD), and Huntingtin with expanded polyglutamine (polyQ) tracts in Huntington's disease (HD), have been studied in yeast through heterologous expression [31–35]. Expression of these human proteins in yeast recapitulated important aspects related to protein pathology, such as the aggregation phenotype and toxicity.

Recombinant human proteins most thoroughly addressed in yeast include Aβ peptide, α-synuclein, and Huntingtin with expanded polyQ tracts, causally associated with the three most common neurodegenerative disorders worldwide, AD, PD, and HD, respectively. As such, these proteins will be discussed in more detail in the next sections.

3. Amyloid-β Peptide

Amyloid-β disorders include several progressive neurodegenerative disorders characterized by the oligomerization and deposition into plaques of an amyloid peptide, known as Aβ. These disorders include not only AD, that is the most recognized, but also Down syndrome, inclusion body myositis, and cerebral amyloid angiopathy [36].

Aβ is a cleavage product derived from the amyloid precursor protein (APP), by the action of specific proteases known as secretases. APP is usually delivered to the cell surface membrane where it is subjected to proteolytic processing by α-secretase. APP molecules that fail to be cleaved by α-secretase can be internalized into endocytic compartments and subsequently cleaved by β-secretase (identified as BACE-1) and γ-secretase, a multimeric complex containing the presenilins. γ-Secretase produces the C-terminal at the end of the Aβ peptide and it can generate the Aβ40 peptide or more rarely the longer Aβ42. The Aβ42, which is more aggregating and cytotoxic, is considered the pathologically important form, responsible for the formation of amyloid plaques [37]. Despite its toxicity, recent evidence suggests that increased amounts of Aβ peptide may have a normal function as an antimicrobial peptide [38]. Mutations in either APP or presenilin genes associated with early-onset AD have been noted to increase the relative production of Aβ42. Therefore, the modulation of the activity of secretases responsible for APP processing can be a potential therapeutic strategy for AD.

One of the initial contributions of *S. cerevisiae* to the understanding of Aβ disorders has been in the study of APP processing. In an early study, it was found that when APP is expressed in yeast, it gets cleaved at the C-terminus of the α-proteolysis site. This indicated that yeast possessed α-secretase activity [33, 39]. Additionally, the responsible proteases for the α-secretase-like activity in yeast were identified as being two aspartyl proteases, Yap3 and Mkc7 [39], though Mkc7 alone is apparently sufficient to cleave APP [40]. Since the secretases responsible for APP processing in humans are still not completely identified, the discovery of secretases in yeast may help in the identification of new human secretases by homology.

Table 1: Proteins associated with human neurodegenerative disorders studied in yeast.

Disease	Protein(s) involved	Yeast orthologue	References
Batten's disease	CLN3	YHC3/BTN1	[16]
Friedreich's Ataxia	Frataxin	YFH1	[12, 13]
Ataxia-telangiectasia	ATM	TEL1, MEC1	[19, 20]
Niemann-Pick disease	NPC1	NCR1	[17, 18]
Hereditary Spastic Paraplegia	mAAA-proteases (Afg312 and paraplegin)	mAAA proteases (Yta10 and Yta12)	[21]
Creutzfeldt-Jakob disease	PrP	—	[23]
Amyotrophic lateral sclerosis	SOD1	SOD1	[25]
	TDP-43	—	[27]
	FUS/TLS	—	[29, 30]
Parkinson's disease	α-synuclein	—	[31]
	Lrrk2	—	[32]
Alzheimer's disease	Amyloid β	—	[33, 34]
	tau	—	[35]
Huntington's disease	Huntingtin	—	[41]

—: No orthologue.

Though a possible β-secretase-like enzymatic activity was also reported in yeast [42], this finding was not supported by other works. Overcoming the lack of β-secretase-like activity, a fragment derived from the APP cleaved by β-secretase (C99) was expressed in yeast [43]. In an attempt to clarify the role of the proteasome in the C99 proteolytic processing, authors used proteomic analysis to compare the proteolysis fragments obtained in the presence and in the absence (using proteasome mutants) of the proteasome. In agreement with the concept that APP proteasomal degradation may be beneficial in mammalian cells [44], in yeast it was observed that the proteasome is involved in C99 processing and that most of the C99 cleavage products are mainly nonamyloidogenic [43]. Interestingly, it was reported the existence in yeast of a compensatory mechanism to overcome the absence of proteasomal activity [43]. This may be relevant for AD since proteasomal function is known to decline with age [45]. With the study of APP processing in yeast, it was found that secondary modifications, like O-glycosylation, are also important for protein solubility and processing in this organism [46].

One of the advantages of using yeast models to study human APP processing is the possibility to individually express human secretase complex subunits. This allowed dissecting the contribution of each subunit, a difficult goal to achieve in mammals due to the high redundancy of these proteases. As such, the γ-secretase activity was reconstituted in yeast by expressing four components of the γ-secretase multimeric complex, which were therefore established as the minimal components required for γ-secretase activity [47]. More recently, reconstitution of γ-secretase activity in yeast was used to produce microsomes. This made possible the study of the enzymatic properties of this complex *in vitro* upon the addition of its substrates, C99 and C55 [48].

Due to the amenability of yeast to HTS, several systems were developed to search for new proteases. One of those systems took advantage of the yeast *GAL* reporter system to screen for proteases that cleaved at the C-terminal of APP. When APP was processed, the Gal4 transcription factor was released activating the *GAL*-reporter genes. Among other hits obtained, the screening of a library of human cDNAs led to the discovery of caspase-3 and -8 [49]. This finding validated this approach for the screening of APP processing proteases since these caspases cleave APP generating a fragment also implicated in the pathogenesis of AD [50]. Another novel approach to identify APP processing secretases used an engineered APP (fused to invertase) that only allows the growth of invertase-null yeast upon processing of APP [51]. The efficacy of this approach was demonstrated by the absence of yeast growth upon the deletion of proteases Yap3 and Mkc7 that, as referred above, cleaved APP in yeast at the α-proteolysis site. Also, expression of human BACE-1, responsible for the β-secretase activity in humans, restored the growth in the absence of yeast proteases, indicating processing of APP. This system, initially developed for the identification of novel secretases, was also adapted for the screening of compounds that inhibit BACE-1 activity [52].

Additionally to the different approaches and tools developed in yeast to study APP processing, yeast has been used as a cell system to study the toxicity of the amyloidogenic APP fragment Aβ42. In humans, Aβ peptide was first identified as a component of extracellular amyloid plaques, but the existence of intracellular Aβ is now recognized [53]. The intracellular Aβ was found in several cellular locations such as cytosol, secretory pathway, autophagosomes, and mitochondria [54], thus potentially affecting diverse processes. In yeast, the effects of both extra- and intracellular Aβ were assessed. Since most of the Aβ is secreted, the addition of APP end products to the culture medium is the most straightforward way of investigating their cellular effects. As in mammals, the physical form in which these products were provided to *S. cerevisiae* (oligomeric or fibrillar) was important for the obtained effects [34, 55]. While an early study found no toxicity in adding soluble Aβ to yeast cells [34],

another group performed the studies in water to better control the physical form of Aβ. Using this approach, authors reported a dose-dependent cytotoxicity for the oligomeric Aβ, while the fibrillar form had a lower toxicity [55]. This observation was consistent with studies in mammalian cells, suggesting the soluble oligomeric Aβ42 as the major determinant of neuronal dysfunction in AD [56]. Unlike the exogenously provided Aβ42, its intracellular expression caused only a mild toxicity, as evidenced by the slight reduction on yeast cell growth [57]. Besides the growth stress, it was observed that Aβ42 activated the yeast heat shock response [57]. This may explain the unexpected report that Aβ42 protected yeast from oxidative stress [58]. However, it was recently reported that directing Aβ42 to the secretory pathway in order to mimic its trafficking in human cells leads to a higher toxicity [59] (Figure 1(a)). This phenotype allowed authors to perform a genome-wide overexpression screen for toxicity modifiers, which resulted in the discovery of several toxicity suppressors. Interestingly, in this study, authors uncovered a previously unknown connection between this peptide and the phosphatidylinositol binding clathrin assembly protein (PICALM), also involved in AD [59].

Along with the fact that Aβ42 is found in yeast predominantly in the insoluble protein fraction, Aβ42 formed lipidic patches, indicating aggregated protein [57]. Taking advantage of the fact that, like in humans, Aβ aggregates in yeast, this model was used to screen for compounds that reduce the aggregation/oligomerization of Aβ peptide. One of such studies used a system consisting of Aβ fused to the green fluorescence protein (GFP), in which Aβ misfolding contributes to GFP misfolding and consequent loss of fluorescence. Using this system, folate was validated as an inhibitor of Aβ misfolding [60]. Bagriantsev and Liebman [61] used an interesting approach to monitor the formation of Aβ42 low-n oligomers formed at the early stage of Aβ42 oligomerization and considered the most toxic Aβ species. Authors used a fusion of Aβ42 with a Sup35p (a translational termination factor) reporter that becomes inactive when low-n oligomers are formed, resulting in a robust phenotype (growth and colony color) that can be easily scored. In a following work, authors adapted this system to (HTS) of small molecule inhibitors of the early oligomerization, since they are potentially more effective than inhibitors of later steps of oligomerization (e.g., fibrillization) [62]. After validation of the approach, by screening several known antiaggregation compounds, authors expanded the screening to a large library of small molecules, resulting in the identification of two new promising antioligomeric compounds: AO-11 (2-(4-methoxyphenyl)-1,3-benzoxazol-5-amine) and AO-15 (1-(2,3-dimethylphenyl)-4-(2-furoyl) piperazine) [62]. Another strategy developed to study aggregation of Aβ peptides took advantage of the same Sup35 protein as a reporter (that is also a prion protein) and consisted in replacing the prion domain in the Sup35 protein by the Aβ42 peptide [60]. Authors found that Aβ42 peptide can restore the Sup35p ability to aggregate and that the Aβ42 aggregates have different properties compared to the original prion aggregates. This model also offers the possibility for genetic and chemical screenings of suppressors of aggregation.

The yeast studies described above allowed better understanding the processing of APP and, more importantly, resulted in a series of high-throughput models for the genetic screening of secretases and genetic/chemical screenings of modulators of Aβ42 aggregation and toxicity with potential therapeutic applicability.

4. α-Synuclein

α-Synuclein plays a crucial role in PD, the second most common neurodegenerative disorder after AD [63]. Though the majority of PD cases arise sporadically, there are some rare (around 10%) familial cases. α-Synuclein was the first gene to be associated with the familial cases, with several missense mutations (A53T, A30P, E46K) as well as locus duplication and triplication causing PD [64, 65].

α-Synuclein is a presynaptic protein with a role in vesicle dynamics. α-Synuclein exhibits a nonclassical chaperone activity that in conjunction with the cysteine-string protein-α (CSPα) helps to promote SNARE complex assembly, crucial for dopamine neurotransmission, vesicle recycling, and synaptic integrity [66, 67]. In both sporadic and familial cases of PD, α-synuclein in the form of amyloid fibrils is the main constituent of Lewy bodies, proteinaceous aggregates that are the major feature of this disease [68]. The first attempt to study α-synuclein in yeast was performed by Outeiro and Lindquist [31]. Three levels of α-synuclein expression were tested in yeast, low (one integrated copy), intermediate (two integrated copies), and high (several copies using a multicopy vector). In addition to the wild-type (wt) α-synuclein, two of the PD-associated mutations, A53T and A30P, were also studied. At low levels, α-synuclein associated with the plasma membrane with no effect on the cell viability [31]. At intermediate expression levels, α-synuclein redistributed from the cell surface into cytoplasmic foci and became cytotoxic (Figure 1(b)). At high expression levels, α-synuclein cytotoxicity was increased, with inhibition of phospholipase D, retardation of endocytosis, and accumulation of cytosolic lipid droplets [31]. This dependence of α-synuclein toxicity on its expression levels in yeast resembled the gene dosage-dependent premature onset of PD found in humans [31]. Despite being PD-associated mutations, and as such we would expect a higher toxicity, A53T and A30P had similar and lower toxicities, respectively, than wt [31]. Following this first study, several other groups used yeast to study α-synuclein pathobiology. The α-synuclein yeast cytotoxicity was shown to be mediated by increased levels of reactive oxygen species (ROS), activation of the heat shock response, endoplasmic reticulum (ER) stress, decreased proteasome function, and altered lipid metabolism [69–76]. In addition, it was shown that α-synuclein triggers an apoptotic cell death, involving ROS accumulation and cytochrome c release from mitochondria [71], which was decreased in the absence of functional mitochondria (lacking mitochondrial DNA) [77]. Supporting an important role of mitochondria on α-synuclein toxicity, microarray data also revealed that

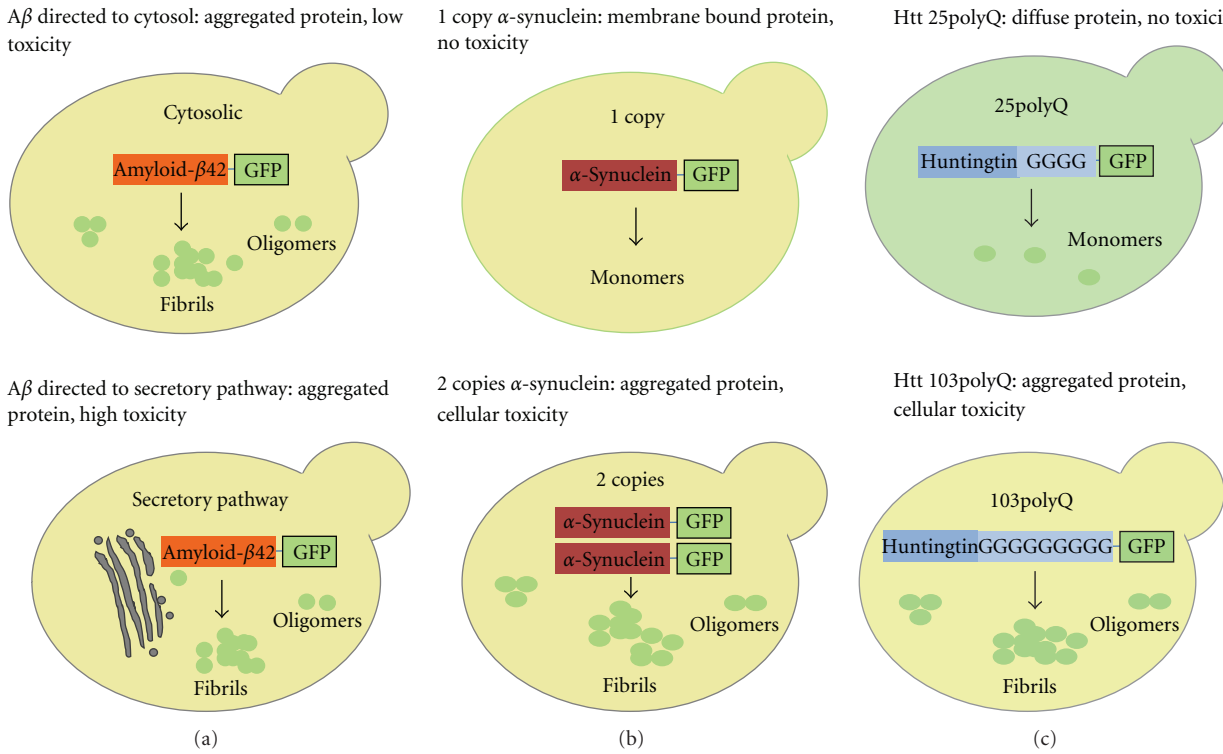

Figure 1: Yeast models of (a) AD, based on amyloid-β42 expression; (b) PD, based on α-synuclein expression; (c) HD, based on Huntingtin's polyQ tract expression. (a) The toxic Aβ42 peptide expressed in yeast causes aggregation and moderate stress, but when expression is directed to the secretory pathway, the toxicity is increased. (b) At low expression levels, α-synuclein associates with the plasma membrane with no effect on cell viability. At doubling expression levels, α-synuclein redistributes from the cell surface into aggregates and induces toxicity. (c) Expression of a Huntingtin polyQ tract with 25 glutamines (normal length) does not lead to aggregation or toxicity. Expression of a polyQ tract with 103 glutamines (HD-associated expansion) leads to the formation of aggregates and toxicity (dependent on the presence of active prions).

mitochondrial stress is an early feature of α-synuclein expression in yeast [78]. The association of α-synuclein with mitochondria is particularly interesting since other PD-associated proteins, for example, Parkin and Pink, have been associated with mitochondrial function/dysfunction [79]. An important feature of α-synuclein expression in yeast is a defect in ER-to-Golgi complex trafficking [73, 80–82]. In addition, other steps of the vesicular trafficking (e.g., vacuolar fusion) were also disrupted by direct interference of α-synuclein with the yeast transport machinery [81–83]. Also, as a result of this vesicular defect, cytoplasmic vesicles that are unable to dock or fuse due to the presence of α-synuclein formed aggregates [80, 81, 83]. Human orthologues of vesicular trafficking yeast proteins involved in α-synuclein toxicity, namely, Rab1, Rab3A, and Rab8A, were found to also suppress α-synuclein toxicity in neuronal models of PD [74, 81].

Aggregation of α-synuclein is the major disease-associated feature of this protein [68], and as such it has been often addressed in the yeast-based α-synuclein model. Using this cell system, sequence determinants for fibrillogenesis were uncovered [84] as well as risk factors for increased α-synuclein fibrillation [85, 86]. In spite of this, the contribution of α-synuclein fibrillation to its toxicity is still dubious since in most studies a correlation between fibrillation rate and α-synuclein toxicity was not found [87]. Rather, through the screening of several random point mutants, the N-terminal amino acids were shown to contribute to α-synuclein cytotoxicity as well as membrane affinity [87, 88]. Amino acid residues in the hydrophobic central core of α-synuclein also contributed to the cytotoxicity, but not interfered with the protein solubility. This further indicated that harmful α-synuclein species were not the insoluble aggregates [89]. In agreement, the defibrillation of α-synuclein fibers with dequalinium, an amphipattic molecule described as a modulator of α-synuclein fibrillation [90], increased its toxicity [41]. This suggests that early misfolded forms of α-synuclein are the toxic species, as has been reported for the Aβ peptide (discussed in the previous section). Since they are not aggregated, this can be explained by the availability of these forms to interact with cell membranes. This membrane interaction-dependent toxicity of α-synuclein is supported by the observation that A30P, with poor membrane binding affinity, is not toxic to yeast cells [31, 73]. In addition, while some yeast strains exhibited α-synuclein inclusions without showing a significant growth defect [41], in other strains α-synuclein proved to be extremely toxic in the absence of visible aggregates [31, 72]. Together, these data indicated that the aggregation or fibrillation of α-synuclein *per se* is not toxic. Instead, it may be the interference of α-synuclein with membranes and the obstruction of the vesicular trafficking the culprit for its pathogenicity.

Yeast was also used to clarify the association of α-synuclein with other PD-related genes. Coexpression of α-synuclein with Dj-1, Parkin, Pink1, ubiquitin C-terminal hydrolase-L1 (UCH-L1), or Synphilin-1 did not significantly alter the α-synuclein toxicity in exponentially growing yeast cells [87]. Yet, in aged cultures, likewise in mammalian cells, Synphilin-1 increased α-synuclein aggregation and cytotoxicity [91]. Similarly, coexpression of α-synuclein with tau, a protein involved in AD and occasionally associated with α-synuclein inclusions [92], caused synergistic toxicity [93]. Moreover, overexpression of the yeast orthologue of the human *ATP13A2*, a risk factor for PD, was shown to suppress α-synuclein toxicity in yeast, an effect confirmed in animal and neuronal PD models [94].

The α-synuclein model was also adapted to genetic large-scale screenings, which allowed the identification of several factors that influence the toxicity/aggregation of α-synuclein. A genetic screening of a collection of deleted nonessential genes led to the identification of several mutants with enhanced α-synuclein toxicity [69]. This approach was also used to identify genes that alter the α-synuclein localization or aggregate formation [82]. Not surprisingly, in both screens many of the hits found were mutants affected in lipid metabolism, vesicular transport, and vacuolar degradation. This reinforced the idea of the importance of these processes to α-synuclein cytotoxicity. With these data, a targeted screen using strains deleted on genes involved in lipid metabolism was recently performed. With this approach, ER lipid elongases were identified as suppressors of α-synuclein toxicity [95]. Using a gene overexpression screening approach, proteins involved in vesicular trafficking were also identified as suppressors of α-synuclein toxicity [74]. Remarkably, with this approach, it was identified the protein Ypt1, whose mammalian homologue Rab1 rescued the loss of dopaminergic neurons in animal models of PD [72]. Other two gene overexpression screens were performed for suppressors of the lethality of wt α-synuclein and A30P in response to hydrogen peroxide [96, 97]. In this study, little overlap was found between the suppressors identified for the wt α-synuclein and the two familial mutations, pointing for distinct mechanisms of toxicity.

A new mathematic algorithm (ResponseNet), allowing the combination of data obtained from microarray expression profiles with genetic screens, was applied to the α-synuclein yeast model [98]. This algorithm revealed new potential cellular responses involved in the toxicity of α-synuclein, including ergosterol biosynthesis and the target of rapamycin (TOR) pathway [98].

Finally, the use of the yeast-based α-synuclein cell model as a drug screening tool also allowed the identification of several toxicity suppressors from large libraries, such as two cyclic peptides and four 1,2,3,4-tetrahydroquinolinones, with promising therapeutic potential [78, 89, 99].

Altogether, the yeast model has provided several insights into the α-synuclein cytotoxicity. The ease to perform HTS allowed the identification of genetic and chemical suppressors of α-synuclein, many of them already validated in higher eukaryotes, with promising applicability in PD therapy.

5. Polyglutamine-Containing Proteins

Inclusions of proteins containing polyglutamine (polyQ) expanded tracts are causatively associated with a group of about 9 fatal hereditary neurodegenerative disorders globally referred as polyglutamine disease, from which HD is the most recognized one [100]. PolyQ expansion in different proteins induces different pathologies; however, these pathologies share the fact that the longer the polyQ expansion, the more severe the disease and the earlier the onset. Huntingtin is a multi-domain protein essential for cell survival and it is thought to play a role in several processes, including transcriptional regulation, vesicular trafficking, metabolism, and synaptic function. It is believed that underlying HD is not only the loss-of-function of the Huntingtin protein due to the expansion of the polyQ tract, but also a new toxic gain-of-function associated with the expansion [101]. Despite the intensive research in this area, the mechanisms underlying the neurodegeneration induced by polyQ expansions remain unclear. Based on this, several research groups have developed yeast models to study the mechanisms of aggregation and toxicity of proteins with expanded polyQ tracts as well as to identify molecular regulators that could serve as therapeutic targets.

Most of the studies performed in yeast used the N-terminal of Huntingtin (which includes the polyQ tract) as a surrogate for proteins with expanded polyQ tracts. When first expressed in yeast, polyQ constructs with different lengths, namely, Q25 (corresponding to a normal polyQ length) and Q47, Q72 and Q103 (representing expanded HD-related polyQ lengths) were analyzed [102]. Authors showed that the Q25 protein did not aggregate. However, the formation of aggregates increased with the polyQ length (Figure 1(c)). With this, it was possible to recapitulate in yeast the dependency of the Huntingtin aggregation on the expansion of the polyQ tract. Despite this, the expression of polyQ in yeast exhibited minimal toxicity, as further confirmed by other authors [103]. However, the presence of the protein Rnq1 in its prion conformation [Pin+] was sufficient to convert the polyQ tract into a toxic protein to yeast, establishing an unexpected link between prions and HD [104, 105]. Toxicity was proposed to be partially due to sequestration of essential proteins, like Sup35 and Sup45, in the polyQ aggregates [106]. Yeast prions are responsible for the transmission of a phenotype but, unlike mammalian prions, they are not harmful to the cell (reviewed in [107]). The connection between polyQ and yeast prions was strengthened by the fact that the polyQ domain facilitated the appearance of prion states [108] and that both proteins can coaggregate [105, 109].

As for yeast prions, the aggregating ability of the expanded polyQ tracts was strongly dependent on chaperones, such as Hsp70 and its cochaperone Hsp40, Hsp26, Hsp104, and the chaperonin TRiC [69, 102, 103, 110–112]. Chaperones bind aggregates in an orderly fashion, with Hsp70 and Hsp90 binding in an early phase and Hsp26 and Hsp104 in a late phase of polyQ aggregate expansion [112]. Chaperones can decrease polyQ toxicity either by decreasing prion propagation or by directly affecting the size of the

polyQ aggregates [111]. Besides chaperones, other cellular factors affected the aggregation of Huntingtin polyQ in yeast, including the microtubule cytoskeleton [113, 114], proteins like Cdc48 and cofactors [114], and components of the late endocytic complex [115]. Making clear that unfolded protein aggregation is not a spontaneous process, as initially assumed, the uncovering of aggregation pathways reveals novel opportunities for intervention [116]. Although most studies point to the formation of aggregates as beneficial, whether aggregation should be hindered or promoted to relieve polyQ toxicity is still controversial [113, 114, 117].

In addition to the polyQ length, the sequences flanking the polyQ domain also modulate toxicity. In particular, the polyQ flanking proline-rich region of Huntingtin has an important role in the toxicity [114, 117, 118]. The presence of this domain strongly reduced the polyQ toxicity, and along with the N-terminal N17 domain was proposed to be a sequence determinant for the addressing of the polyQ to the large protective perinuclear aggregates [114]. The proline-rich region can also operate in *trans*, that is, the coexpression of the polyQ containing the proline-rich domain converted the toxic polyQ without the proline-rich region into benign variants [105]. Also operating in *trans* and affecting polyQ toxicity were other glutamine rich proteins, such as yeast prions [105].

While many studies addressed the properties of polyQ aggregates, other authors tried to understand the cellular dysfunction underlying toxicity. Most of the cellular effects of polyQ observed in yeast were consistent with reports for HD animal models and HD patients, which validated the yeast model for the study of polyQ toxicity. For instance, it was reported that polyQ tracts led to transcriptional dysregulation [119], caused impairment in ER-protein homeostasis [120] and in endocytosis [115, 121]. Huntingtin polyQ also altered mitochondrial morphology and compromised mitochondrial function, with early alterations in complexes II and III of the respiratory chain associated with an increase in ROS production [122, 123]. Stimulation of mitochondrial biogenesis was able to rescue mitochondrial dysfunction and cellular toxicity, indicating that mitochondria contribute significantly to polyQ toxicity [124]. In addition to mitochondrial dysfunction, the occurrence of DNA fragmentation and caspase activation indicated the activation of an apoptotic pathway by Huntingtin polyQ tracts [123]. The same authors reported a disturbance in the cell cycle that was also involved in polyQ cytotoxicity. This effect on cell cycle was attributed to the accumulation of cell cycle substrates likely due to a hindrance in protein degradation caused by the accumulation of polyQ [123, 125]. Another consequence of the polyQ expression in yeast is the alteration of the cellular concentration of several metabolites, namely, alanine, glycerol, glutamine, and valine. Alterations in these metabolites were proposed as promising biomarkers for HD [126]. Genome-wide screens in yeast identified several modifiers of mutant Huntingtin aggregation and toxicity, which could potentially serve as therapeutic targets. The proteins identified as loss-of-function suppressors were majorly associated with stress, protein folding, vesicular transport, vacuolar degradation, and transcription [69, 127]. An interesting suppressor was the mitochondrial protein kynurenine 3-monooxygenase involved in the kynurenine pathway of tryptophan degradation [127]. The kynurenine pathway was found to be activated in HD patients and in animal models of this disorder and is regulated by histone deacetylase enzymes [128]. Notably, a previous work had shown that the transcriptional profile of yeast expressing a polyQ tract was similar to those of yeast strains deleted for components of the histone acetyltransferase complex [119]. Transcriptional deregulation was rescued by treating the cells with a histone deacetylase inhibitor [119]. Inhibition of histone deacetylase also suppressed the kynurenine pathway in yeast, microglia, and mice expressing the Huntingtin polyQ [129]. This pointed out the kynurenine pathway as a promising therapeutic target for HD. Consistent with the previously established connection between polyQ and yeast prions, the prion form of Rnq1 and proteins involved in prion-like aggregation were also amongst the hits obtained. In one of the screenings, the major functional categories obtained for Huntingtin polyQ was compared to the ones obtained for the PD-associated protein α-synuclein. The overlap obtained for both proteins was minimal. This indicated that despite the coalescence of both proteins into aggregates, they had distinct pathogenic mechanisms [69].

In addition to genetic screenings, yeast polyQ models have also been widely used as a cell-based HTS system of small molecules with potential therapeutic application. The compounds found have already shown promising results to ameliorate polyQ toxicity in higher eukaryotes [130–133].

Overall, yeast models of polyQ-containing proteins provided new insights into the molecular determinants for polyQ aggregation. Importantly, studies in yeast uncovered promising therapeutic compounds and new molecular targets in HD, namely, chaperones and kynurenine 3-monooxygenase.

6. Concluding Remarks

Though this study has been focused on *S. cerevisiae,* other yeast species like the fission *Schizosaccharomyces pombe* and the methylotrophic *Pichia pastoris* have also been used as model organisms of neurodegeneration. Likewise, only proteins strictly involved in the most common neurodegenerative disorders have been addressed. However, other human proteins recognized as central players in apoptotic diseases as neurodegeneration, including several members of the caspase, Bcl-2, p53, and protein kinase C family, have also been widely studied in yeast. Supported by the valuable contribution of yeast as a model organism towards the understanding of the molecular basis underlying neurodegenerative disorders, it is expectable that many other disease-associated proteins will be studied in this unicellular eukaryote. Even though yeast has no orthologues of the majority of these proteins, most of them exhibit some assessable phenotype that can be explored in the development of target-specific approaches easily adapted to HTS analysis. In fact, an enormous input in the identification of new therapeutic opportunities against these pathologies

has been given through the development of fast and reliable yeast target-based HTS approaches. Though the obvious limitations of using a microorganism to address human diseases, when used in the early phase and with complementary mammalian systems, yeast may greatly contribute to the identification of novel targets and to the discovery of novel agents against neurodegeneration. Therapeutic options for many patients with neurodegenerative disorders have changed little. A typical delay between the early steps in the discovery of a lead compound, in which yeast participate, and its clinical use make us believe that many great contributions of the yeast model to the discovery of new therapeutic agents will be realized in the near future.

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Review Article

Overview of Plant-Made Vaccine Antigens against Malaria

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This paper is an overview of vaccine antigens against malaria produced in plants. Plant-based expression systems represent an interesting production platform due to their reduced manufacturing costs and high scalability. At present, different *Plasmodium* antigens and expression strategies have been optimized in plants. Furthermore, malaria antigens are one of the few examples of eukaryotic proteins with vaccine value expressed in plants, making plant-derived malaria antigens an interesting model to analyze. Up to now, malaria antigen expression in plants has allowed the complete synthesis of these vaccine antigens, which have been able to induce an active immune response in mice. Therefore, plant production platforms offer wonderful prospects for improving the access to malaria vaccines.

1. Introduction

Malaria is a major global health problem caused by parasites of the genus *Plasmodium*. This disease is responsible for the death of over one million people annually, and approximately three billon people are at risk of infection by both *Plasmodium falciparum* and *Plasmodium vivax* parasites [1, 2]. About 90% of the disease burden occurs in the most poverty-stricken sub-Saharan African countries. However, countries of Asia, Latin America, Middle East, and Europe are also affected [3]. Among the species of *Plasmodium* known to cause disease in humans: *P. falciparum* and *P. vivax* have received special attention for vaccine development. *P. falciparum* is responsible for most malaria-induced deaths and has therefore been the focus of the greater part of research [4]. On the other hand, *P. vivax* is often the most prevalent malaria infection in tropical and subtropical areas [5, 6].

Vaccination against malaria is considered the most efficient method to prevent this infectious disease [7]. However, although several vaccines are under development, a completely effective vaccine against malaria is not yet available. In particular, RTS,S, a potential vaccine targeting the preerythrocytic stage of the parasite, has shown the most promising results so far [8]. However, this vaccine is not fully effective, and thus more efficacious, second-generation vaccines are needed. In this sense, good progress is being made in the development of potential vaccines directed to the asexual blood and sexual stages of the parasite [9, 10]. In the first case, vaccines are designed either to stop invasion of erythrocytes or to kill intraerythrocytic parasites [8]. In the second case, vaccines aim to prevent mosquitoes from becoming infected by preventing parasite fertilization and/or by inhibiting early development in the insect vector. This is referred to as transmission-blocking (TB) immunity and is mediated by specific antibodies and other factors ingested during the blood meal, which inhibit parasite development in the mosquito [11, 12]. These antibodies recognize proteins expressed on either gametocytes or parasite stages that develop in the mosquito midgut and are considered to be potential malaria vaccine candidates [13]. This interruption of parasite transmission from patients to mosquitoes provides the basis for the feasibility of TB vaccines (TBV) of stopping transmission from infected to noninfected people [10]. These findings have stimulated research into the human immune mechanisms involved in

protection against infection and disease [14, 15] and into the blockage of parasite transmission from the host to the mosquito [13, 16].

Most of the vaccines available today belong to one of three categories: attenuated microbes, a killed version of the original pathogen, or protein subunits [17]. Each of them has its own associated advantages and disadvantages in terms of safety, immunogenicity, and general feasibility in terms of the ability to develop in to a commercial product. The disadvantages of live, attenuated vaccines in primates and humans include their side effects and risks for breakthrough infection. In contrast, recombinant subunit vaccine approaches offer one of the safest alternatives [18, 19].

Since a malaria vaccine based on attenuated parasites is not feasible in humans, several antigens from malaria parasites are being expressed in different heterologous expression systems like bacteria, yeast, insects, and mammalian cells [2, 8, 20–23]. However, the high costs estimated for a successful immunization strategy and scalable methods for vaccine production, distribution and delivery, together with the lack of infrastructure in malaria-endemic areas, are the main challenges in the development of a subunit malaria vaccine [20]. In this context, the capacity of transgenic plants to produce properly folded proteins at low costs makes them attractive expression systems [24, 25].

Plant-based expression systems represent an interesting production platform due to their reduced manufacturing costs and high scalability. In addition, plants have the ability to generate complex recombinant proteins with desired structures, maintaining biological functions and offering greater safety because plants do not harbor mammalian pathogens or microbial toxins [22, 26–29]. However, plant-expression systems have been poorly explored for development of vaccines against human parasite pathogens. In fact, only few antigens from *Plasmodium* ssp. and *Toxoplasma gondii* have been expressed in plants [25, 30–39]. At present, whereas *T. gondii* antigen expression in plants is just beginning, different *Plasmodium* antigens and expression strategies have been optimized in plants. Furthermore, malaria antigens are one of the few examples of eukaryotic proteins with vaccine value expressed in plants, making plant-derived malaria antigens an interesting model to analyze. For this reason, this paper is an overview of vaccine antigens against malaria produced in plants.

2. Plant-Made Vaccines

In the last twenty years, plants have been used as bioreactors for the biosynthesis of products with biotechnological interest, such as subunit vaccines [40, 41]. An increasing number of antigens derived from either viruses or bacteria have been expressed using transgenic and transient expression in whole plants or plant cell cultures [42]. Among them, mucosal vaccines for diarrheal diseases, hepatitis B and rabies, as well as injectable vaccines for non-Hodgkin's lymphoma, H1N1 and H5N1 strains of influenza A virus, and Newcastle disease have been assayed [43]. Many of these antigens have demonstrated efficacy and safety in animal models and preclinical studies [42]. In fact, Dow AgroSciences [44] obtained the first commercial plant-made vaccine approved for Newcastle illness in chicken. This vaccine was developed and purified from tobacco cell cultures [44].

In addition to their use as bioreactors, plants can be used as potential delivery systems for oral vaccines [43]. In particular, plant tissues provide protection and prevent degradation of the antigen when it passes through the gut [45, 46]. It has been demonstrated that plant-made vaccines applied to mucosal surfaces in the absence of adjuvant are able to induce a protective immune response, suggesting that some phytochemicals could synergistically affect the immunogenicity of plant-expressed antigens acting as endogenous adjuvants [47–53]. In addition, plants are known for their natural immune-stimulating or antimicrobial activity due to secondary metabolites as lectins, saponins, alkaloids, phenolic compounds, and flavonoids [54]. Moreover, some commonly occurring plant components, such as unmethylated CpGs motifs of DNA, carotenoids and immunogenic proteins, have adjuvant properties [55, 56]. Hence, it might be assumed that plants can be used in the short term as oral or injectable vaccine producers and as a source of endogenous adjuvants as well.

3. Plant-Made Antigens against the Asexual Blood Stage of *Plasmodium*

A small number of merozoite surface proteins have been analyzed as vaccines against the asexual bloodstage of *Plasmodium* by inducing an immune response that either blocks invasion of erythrocytes or inhibits its subsequent multiplication [57–59]. Apical membrane antigen-1 (AMA1), merozoite surface protein-1 (MSP1), and erythrocyte surface antigens are under clinical trials [11, 60–64]. Some of them have been expressed in plants, and their immunogenicity assayed (Table 1) [25, 33, 35, 36, 39, 65].

The 19-kDa C-terminal fragment of *P. falciparum* merozoite surface protein 1 ($PfMSP1_{19}$) was the first malaria antigen expressed in plants by stable transformation, with a very low expression level (~0.0035% of the total soluble protein (TSP)) [30]. Afterwards, a plant-codon-optimized version of the 42 kDa C-terminal fragment of PfMSP1 ($PfMSP1_{42}$) was designed and synthesized, and expressed in transgenic *Arabidopsis thaliana* seeds [35]. Also, for protein-stable accumulation, the plant-optimized $PfMSP1_{42}$ gene was fused to the phaseolin peptide signal [66] or to the lysine-rich protein [35]. These strategies resulted in a substantial improvement of $PfMSP1_{42}$ expression (approximately 5% of TSP) [35].

Plant codon optimization has also been implemented to improve the expression level of *Plasmodium yoelii* merozoite surface protein 4/5 (PyMSP4/5) [25], another important vaccine candidate with high effectiveness in protecting mice against a lethal challenge [67, 68]. In this case, this optimized PyMSP4/5 version was expressed in transgenic tobacco plants and its expression level was six-fold higher than that obtained with the native protein [25]. In general,

Table 1: Malaria vaccine antigens expressed in plants.

Malaria antigens	Plant/expression system	Expression level	Functional evaluation	Refs.
Asexual blood stage antigens				
P. falciparum $MSP1_{19}$ ($PfMSP1_{19}$)	Tobacco/nuclear transformation	0.0035% TSP	Not reported	[30]
P. falciparum $MSP1_{42}$ ($PfMSP1_{42}$)	*A. thaliana* seeds/nuclear transformation	5% TSP	Immunoreactivity to sera from malaria-infected patients	[35]
P. yoelii MSP4/5 (PyMSP4/5)	Tobacco/nuclear transformation Tobacco/magnICON	0.02–0.04% TSP 10% TSP (1-2 mg/g FW)	Immunogenic by i.p. and oral delivery to mice Induction of specific antibodies by oral delivery or primed by a DNA vaccine to mice	[25] [33]
P. yoelii $MSP1_{19}$ ($PyMSP1_{19}$)	Tobacco/magnICON	23% TSP (3-4 mg/g FW)	Immunogenic by i.p. delivery with Freund's adjuvant or oral administration without any adjuvant	[65]
P. falciparum AMA1 (PfAMA1)	Tobacco/chloroplast transformation Lettuce/chloroplast transformation	13.1% TPS 7.3% TSP	Induction of specific antibodies in s.c. and orally immunized mice Immunoreactivity to native parasite proteins	[36]
P. falciparum MSP1 (PfMSP1)	Tobacco/chloroplast transformation Lettuce/chloroplast transformation	10.1% TSP 6.1% TSP	Induction of specific antibodies in s.c. and orally immunized mice Immunoreactivity to native parasite proteins	[36]
P. vivax MSP1 (PvMSP1)	*B. napus*/nuclear transformation	Not reported	Induction of antigen-specific IgG1 and increase in Th1-related cytokines IL-12 (p40), TNF, and IFN-γ in orally immunized mice	[39]
P. vivax CSP (PvCSP)	*B. napus*/nuclear transformation	Not reported	Induction of antigen-specific IgG1 and increase in Th1-related cytokines IL-12 (p40), TNF, and IFN-γ in orally immunized mice	[39]
Sexual stage antigens				
P. falciparum P230 (Pfs230)	Tobacco/agroinfiltration	800 mg/kg FW	Induction of transmission-blocking antibodies in s.c. and i.m. immunized mice Immunoreactivity to native parasite proteins	[38]
P. falciparum P25 (Pfs25)	Tobacco/agroinfiltration	0.25 and 0.4 mg/g FW	Induction of transmission-blocking antibodies in i.m. immunized mice Immunoreactivity to native parasite proteins	[37]

TSP: total soluble protein. FW: fresh weight. i.p.: intraperitoneal. i.s.: intrasubcutaneous. i.m.: intramuscular.

rare codons, AU-rich destabilizing sequences and putative polyadenylation and splicing signals may contribute to rapid mRNA decay, thus limiting the expression of foreign genes in plants. In the plant codon-optimized PyMSP4/5 antigen, the A+T content is reduced from 67% to 53%, and the AT-rich regions are disrupted, allowing the reduced A+T gene version to be more efficient in antigen production than the native version [25]. Codon optimization has also been implemented in protozoan parasite *T. gondii* antigens expressed in plants [31]. In this last case, the A+T content (54%) in the optimized *T. gondii* SAG1 version is increased, negatively affecting SAG1 expression [31]. These results, together with the results obtained in *Plasmodium*, support the idea that a high A+T content is correlated with a low protein expression.

More recently, Webster et al. [33] have expressed the plant codon-optimized PyMSP4/5 in *Nicotiana benthamiana* leaves, using the deconstructed tobacco mosaic virus-based transient expression system, magnICON, from ICON Genetics (Germany) [69] and found expression levels of up to 10% TSP (~1-2 mg/g of fresh weight) [33]. The use of magnICON has also been found to increase the expression level of the plant codon-optimized *P. yoelii* $MSP1_{19}$ ($PyMSP1_{19}$) to at least 23% TSP (~3-4 mg/g of fresh weight) [65]. All together, these results show that not only codon optimization but also the viral provector system improves the expression level of plant-made malaria antigens [33, 65]. Indeed, plant codon optimization combined with the magnICON expression system has been shown to allow an increase of up to 100 fold with respect to the transgenic tobacco system [25, 33].

Studies on the immunogenicity of these plant-made malaria antigens have shown that PyMSP4 and $PyMSP1_{19}$ expressed in plants are able to elicit an immune response by intraperitoneal (i.p.) or oral vaccination in a murine model [25, 33, 36, 39, 65]. These findings provide a rationale for the development of a plant-made oral vaccine against malaria. The report by Wang et al. [25] was the first to demonstrate the immunogenic properties of PyMSP4/5. First they found that oral immunization with *E. coli*-produced PyMSP4/5 in the presence of the cholera toxin B subunit of *Vibrio cholera* (CTB) induced a significant increase in the systemic-specific antibodies and a partial protection against a lethal challenge with *P. yoelii* [24]. Then, they found that oral immunization of mice using plant-made malaria antigens elicited an effective immunity against this parasite [25].

They demonstrated that oral immunization using plant-made malaria antigens elicited an effective immunity against this parasite [25]. However, immunization with plant-made antigens elicited a lower immune response than *E. coli*-made antigen immunization. Thus, Webster et al. [33] proposed increasing the antigen dose using the magnICON system [33]. This strategy allowed them to increase the antigen dose orally administered. However, a higher antigen dose (leaf powder containing 100 μg of PyMSP4/5) was not enough to induce a protective response against *P. yoelli* [33].

Thus, they hypothesized that the presence of dimeric antigen forms produced in *E. coli* enhances its immunogenicity, while the presence of monomeric forms in the plant-made antigen would explain its inability to induce protection. Similar results were obtained by Ma et al. [65]: although the plant-made $PyMSP1_{19}$ was immunogenic when delivered i.p. with Freund's adjuvant or orally without any adjuvant, the specific antibody titers were lower than those induced with the same dose as that of *E. coli*-made antigens [65]. Like that observed in PyMSP4/5, the higher proportion of dimers and multimers contained in *E. coli*-made $PyMSP1_{19}$ suggests that the multimers are more stable and immunogenic [65]. In case that this hypothesis is demonstrated, further innovations will be required to induce a protective immunity.

Finally, *P. falciparum* apical membrane antigen-1 (PfAMA1) and PfMSP1, both fused to CTB, have been expressed in transplastomic plants [36]. This strategy allowed accumulating CTB-PfAMA1 and CTB-PfMSP1 fusion proteins up to 13.1% and 10.1% of TSP in tobacco plants. In addition, since the expression of antigenic proteins in tobacco plants is not suitable for oral delivery in humans, Davoodi-Semiromi et al. [36] expressed these same chimeric proteins in lettuce chloroplasts and found that the expression levels of these malaria antigens fused to CTB in lettuce were up to 7.3% and 6.1% of TSP, sufficient for oral delivery evaluation [36, 39]. These authors combined two attractive strategies to improve the level of expression: plastid expression and chimeric protein production. While many chloroplasts in each cell and multiple copies of the plastid genome per cell make the high level of protein expression possible [20, 23], the *carrier* proteins contribute to a higher stability of the target protein in plants [43]. The levels of dual cholera-malaria vaccine antigens were ~50 fold higher in tobacco and ~30 fold higher in lettuce than other malaria antigens expressed in tobacco transgenic lines [36]. On the other hand, the lettuce chloroplast transformation opens the possibility of producing a green vaccine that could be orally administered without cold chain requirements. Indeed, Davoodi-Semiromi et al. [36] also found that subcutaneous and oral vaccination with candidate PfAMA1 and PfMSP1 genes fused to CTB are highly immunogenic in mice. Moreover, they also showed a significant parasitic inhibition, providing evidence that the antibodies generated from immunized mice are effective in preventing parasite invasion of red blood cells [36].

Several reports have assessed plant production of chimeric proteins as potential vaccines [36, 43]. The expression of chimeric proteins carrying epitopes from different pathogens, linkers, or adjuvant sequences would allow increasing the immunogenicity of the recombinant antigen [43]. In addition, a great number of models have demonstrated the feasibility to elicit broad cellular and humoral immune responses using such a type of molecules [43]. In particular, Davoodi-Semiromi et al. [36] demonstrated that CTB-PfAMA1 and CTB-PfMSP1 are able to elicit a humoral response by mucosal routes. However, they did not evaluate the cellular immune response. In addition, it is necessary to identify new, safe, and nontoxic adjuvants, particularly for those capable of strongly boosting cellular immune responses. In this context, leaves have a large number of heat shock proteins (HSPs), which have been demonstrated to have adjuvant properties in other organisms [70, 71]. Indeed, plants expressing HSP90 are able to induce *in vitro* B-cell proliferation from naïve mice, suggesting that these proteins could be excellent *carriers* of interesting vaccine antigens and peptides expressed in plants [72].

Other edible plant species has been explored to produce malaria antigens [39]. *Brassica napus* has been used to express *P. vivax* MSP1 (PvMSP1) and the *P. vivax* circumsporozoite protein (PvCSP), and to synthesize a chimeric recombinant gene containing PvMSP-1, a Pro-Gly linker motif, and PvCSP [39]. Oral immunization of mice with plant-made MLC chimeric recombinant protein successfully induced antigen-specific IgG1 production [39]. Additionally, the Th1-related cytokines IL-12, TNF, and IFN-γ were significantly increased in the spleens of BALB/c mice, supporting the idea that oral vaccines could be developed based on the use of food plant platforms for the production and delivery of vaccine antigens, contributing to a substantial cost reduction [22].

4. Plant-Made Antigens against Sexual Blood Stage of *Plasmodium*

As mentioned above, an alternative strategy to develop an efficient malaria vaccine is to obtain vaccines directed against proteins found in the sexual stages of *P. falciparum* parasites present in the *Anopheles* mosquito midgut, known as transmission-blocking vaccines (TBVs). A positive aspect of this type of TBV is that they attack the parasite at

a vulnerable stage of its life cycle, when it has to transfer from one host to another and when its numbers are very small [73]. Clinical trials have been conducted with vaccines based on the ookinete antigens of *P. falciparum* and *P. vivax* with induction of TB antibodies [10, 74].

Farrance et al. [38] used the transient *N. benthamiana* expression system to express the *P. falciparum* gamete surface antigen P230 (Pfs230) (Table 1). They demonstrated that antibodies against this protein block the progression of the parasite's life cycle in the mosquito and thus block the transmission to the human host [8, 75]. Pfs230 is present on the surface of *P. falciparum* gametocytes and gametes and is expressed during human infection [8, 75]. The N-terminal region of Pfs230 spanning aminoacids 444 to 730 was fused to the ER retention sequence (KDEL) and produced using plant-based transient-expression system by agroinfiltration [38]. The recombinant protein was expressed in approximately 800 mg/kg in whole fresh leaf tissue, being 100% soluble [38]. This plant-made Pfs230 elicited high titers of anti-Pfs230 antibodies when administered to rabbits in the presence of complete/incomplete Freund's adjuvant. Furthermore, the antibodies generated combined with this adjuvant were shown to bind specifically to the native protein in *P. falciparum* on the surface of gametes/zygotes, resulting in significant TB activity (TBA) [38].

Farrance et al. [37] also expressed an ookinete antigen present in the sexual form of the parasite—Pfs25—using the transient *N. benthamiana* expression system (Table 1). Pf25 is able to induce TB antibodies and is present in the zygote and ookinete stages but is not expressed during human infection [8]. In order to evaluate the role of the Lichenase molecule (LicKM) as a *carrier* [54] and the effects of N-glycosylation on the generation of a functional immune response, Farrance et al. [37] obtained four constructs carrying the recombinant Pfs25 protein for transient expression in plants. Independently of the protein versions evaluated, Pfs25 expression levels were between 0.25 and 0.4 mg/g of fresh leaf tissue [37]. Also, these authors observed that this recombinant protein with N-glycans had the lowest titers, while the Pfs25 lacking glycans (Pfs25-LG) showed the highest IgG titers in mice immunized intramuscularly (i.m.). However, the fusion to LicKM elicited a higher titer than that of the Pfs25 lacking glycans alone. The authors concluded that nonglycosylated antigens generated high antibody titers and enhanced TBA. In addition, they were able to eliminate the negative effect of the presence of glycans on the Pfs25 protein's ability to induce a functional immune response when Pfs25 was fused to LicKM [38]. Glycosylation of some antigens can affect their immunogenicity [37]. According to this, the plant-produced nonglycosylated and glycosylated versions determined that the nonglycosylated antigens generated higher antibodies titers and enhanced TBA [37]. Since glycosylation does not occur in chloroplasts and the natural pathogen organism (*Plasmodium*) does not glycosylate the target proteins, these results support the idea that the chloroplast would be the best compartment to express this group of proteins, where the glycosylation may affect their antigenicity.

Conclusion

Development of an effective malaria vaccine has been a great challenge for medical science. Up to now, malaria antigen expression in plants has allowed the complete synthesis of these vaccine antigens, which have been able to induce an active immune response in mice. Therefore, plant production platforms offer wonderful prospects for improving the access to malaria vaccines. Indeed, plant-derived multiantigen malaria vaccines could be formulated through mixing transgenic plant materials or polycistronic operons in chloroplasts expressing several malaria antigens. In addition, efficient malaria antigen expression in the lettuce chloroplast for oral immunization is a significant advancement that allows the oral delivery of subunit vaccines combined with a potent mucosal adjuvant. However, the local immune response induced by plant-made malaria antigens has not been analyzed yet. Indeed, most of these published reports are centered in the systemic humoral response. In this sense, further research is needed to understand the mucosal and systemic cellular immune response elicited by plant-made malaria antigens orally delivered.

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Review Article

Yeast and the AIDS Virus: The Odd Couple

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Despite being simple eukaryotic organisms, the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have been widely used as a model to study human pathologies and the replication of human, animal, and plant viruses, as well as the function of individual viral proteins. The complete genome of *S. cerevisiae* was the first of eukaryotic origin to be sequenced and contains about 6,000 genes. More than 75% of the genes have an assigned function, while more than 40% share conserved sequences with known or predicted human genes. This strong homology has allowed the function of human orthologs to be unveiled starting from the data obtained in yeast. RNA plant viruses were the first to be studied in yeast. In this paper, we focus on the use of the yeast model to study the function of the proteins of human immunodeficiency virus type 1 (HIV-1) and the search for its cellular partners. This human retrovirus is the cause of AIDS. The WHO estimates that there are 33.4 million people worldwide living with HIV/AIDS, with 2.7 million new HIV infections per year and 2.0 million annual deaths due to AIDS. Current therapy is able to control the disease but there is no permanent cure or a vaccine. By using yeast, it is possible to dissect the function of some HIV-1 proteins and discover new cellular factors common to this simple cell and humans that may become potential therapeutic targets, leading to a long-lasting treatment for AIDS.

1. Introduction

Since the genetics and cell biology of higher eukaryotes are extremely complex, scientists looking for a good model have turned to the use of yeast as a simpler system for the study of various pathologies including virus proliferation and assay new drugs against these pathogenic agents.

The yeast *Saccharomyces cerevisiae* is a simple eukaryotic organism with approximately 6,000 genes. It is inexpensive to cultivate, can exist in either haploid or diploid states, and is extremely simple for genetic manipulations. The complete sequence of this yeast genome, the fruit of a worldwide collaboration of more than 100 laboratories from 1989 to 1996, was the first determined for a eukaryote cell. More than 75% of the genes have an assigned function, while more than 40% share conserved sequences with at least one known or predicted human gene ([1]; http://www.yeastgenome.org/).

Owing to the high conservation of fundamental biochemical pathways, yeast has been used as a model to unravel biological processes in many higher eukaryotes (for a short review see [2]). A very important tool facilitating the use of these cells is the availability of yeast libraries in which each nonessential gene has been deleted. It has been used for multiple studies including genome wide screenings for human disease genes and host factors that support virus replication [3–5]. The above collection is commercially available and covers more than 90% of all yeast genes [6].

An exciting example of the beneficial using this model system is found in two very recent reports, although not related to virus research, on the use of the yeast model to study a severe human pathology. Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's Disease, is a neurodegenerative disease in which the motor neurons of the central nervous system that control muscles die off leading to paralysis and death within 3–5 years of onset. The cause of the disease is unknown, and there is no treatment to stop or slow it. A gene related to ALS, called FUS, has been used to explore its biology in yeast and highlight the potential for modeling elements of complex diseases in this simple eukaryotic cell. Results from both studies suggest

that defects in RNA processing and transport may be a key element of ALS pathophysiology. In the cytoplasm of motor neurons of ALS patients, proteins aggregate to form insoluble aggregations, called inclusions, that may involve FUS and another ALS causing protein, called TDP-43 [7]. When the two research groups overexpressed human FUS in yeast, they observed cytoplasmic inclusions. In humans, the majority of FUS is found in the nucleus, and some ALS-associated mutations reduce the nuclear/cytoplasmic ratio of the protein, suggesting that transfer to the cytoplasm, rather than mutation, may be important in this pathology. This hypothesis was supported by the observation that decreasing the overexpression of wild-type FUS in the yeast nucleus diminished its toxic effect. Both FUS and TDP-43 are RNA binding proteins. Genome-wide screens to identify yeast genes that specifically decreased cell toxicity were performed and the identified genes included some coding for other DNA/RNA binding proteins. One of these yeast genes, called ECM32, has a human homolog, hUPF1, which was found to eliminate toxicity. Among other functions hUPF1 plays an important role in mRNA quality control, supporting the idea that pathways involving RNA are defective in FUS-induced ALS. Interestingly, expression of hUPF1 was able to rescue FUS toxicity in yeast without driving FUS out of the inclusions or sending it back to the nucleus, suggesting that it may be possible to overcome therapeutically the effects of abnormally localized FUS without addressing the difficult problem of restoring it to its original compartment. Two important consequences may result from this approach. By identifying new genes that can diminish ALS-linked toxicity, they point to RNA processing as a therapeutic target in this disease. It has also been shown that yeast has the potential to be a much simpler and exciting system for modeling aspects of ALS. Since testing ideas about pathogenesis and treatment is much faster and cheaper in yeast, these results may open the way for more rapid progress in understanding the disease, its treatment, and the role of this new gene in ALS development. Important details of other neurodegenerative diseases have been obtained using yeast. For example, protein misfolding associated with many human diseases, including Alzheimer's, Parkinson's, and Huntington's disease, has been studied in yeast. As in the case of ALS mentioned above, protein misfolding often results in the formation of intracellular or extracellular inclusions whose role in protein misfolding diseases is unclear. Studies on the implication of protein aggregation and toxicity in yeast provide an excellent experimental and conceptual paradigm that contributes to understanding the differences between the toxic and protective roles of protein conformation changes. Results from these studies using yeast have the potential to transform basic concepts of protein folding in human diseases and may help in identifying new therapeutic strategies for their treatment [8].

In virus research, *S. cerevisiae* is a very useful organism. In terms of public health, the use of yeast to produce vaccines was a remarkable breakthrough. For example, the first recombinant vaccine, the hepatitis B surface antigen expressed in yeast, has become a safe and efficient prophylactic vaccine worldwide [9]. In addition, yeast has proved useful for drug discovery as illustrated by the power of applying genomic approaches using the yeast model to characterize the biological activity of small molecules and to identify their cellular targets, an important step towards understanding the mode of action of human therapeutic agents [10]. This study and other recent reports show that the path from sequence and functional analysis to direct therapeutic applications is not necessarily long, at least using the yeast model, a simple organism, which could well be considered among "man's best friends."

In basic research in Virology, yeast has assisted the elucidation of the function of individual proteins from important pathogenic viruses such as *Hepatitis C virus*, and *Epstein-Barr virus* ([11, 12]; for a review and further references see [13]). Furthermore, studies of viruses that infect yeast have provided many important contributions to the dissection of the life cycle of many other higher eukaryotes RNA viruses and the host factors involved [14].

The study of the replication of RNA plant viruses was the first to be attempted using yeast cells. The great majority of RNA+ viruses carry their own RNA polymerase and are replicated in the cytoplasm while entry to the nuclear compartment during the viral cycle is confined to retroviruses (see below). The first higher eukaryotic virus reported to replicate in yeast was *Brome mosaic virus*, a positive-strand RNA ((+)RNA) virus that infects plants ([15], for an excellent review see [16]).

Since the pioneering work of the Ahlquist laboratory, a growing list of viruses has been reported to undergo replication in yeast. These include RNA and DNA viruses that infect plants, insects, mammals, and humans (Table 1) [17–22].

This wide range of viruses emphasizes the general applicability of the yeast system. In this paper, we will focus on the contribution of the yeast model to the study of retroviruses with special emphasis on the human immunodeficiency virus type 1 (HIV-1).

2. The Search of Cellular and Viral Partners Using the Yeast Two-Hybrid System

The yeast two-hybrid system, also called double-hybrid, has been extensively used to search and characterize the potential partner(s) of a given protein ([23], for a review see [24]).

The basis of this method allows searching and characterizing proteins capable of interacting with a known protein leading to obtaining the cloned genes of these interacting proteins. Plasmids are constructed to encode two hybrid proteins (Figure 1). One hybrid consists of the DNA-binding domain of the yeast transcriptional activator protein GAL4 fused to the known protein; the other hybrid consists of the GAL4 activation domain fused to protein sequences encoded by a library of yeast (or other sources) genomic DNA fragments. Interaction between the known protein and a protein encoded by one of the library plasmids leads to transcriptional activation of a reporter gene easy to detect containing a binding site for GAL4. In addition of thousand of examples described in the literature using this system many cellular or viral partners of HIV-1 proteins have been

Table 1: Viruses that replicate in yeast.

(+)RNA viruses	Virus	Natural host
Bromoviridae	*Brome mosaic virus*	Plants
Tombusviridae	*Carnation Italian ringspot virus*	Plants
	Tomato bushy stunt virus	Plants
Nodaviridae	*Flock house virus*	Animals
	Nodamura virus	Animals
DNA circular viruses	**Virus**	**Natural host**
Papillomaviridae (dsDNA)	*Human papillomavirus*	Animals
	Bovine papillomavirus	Animals
Geminiviridae (ssDNA)	*Mung bean yellow mosaic India virus*	Plants

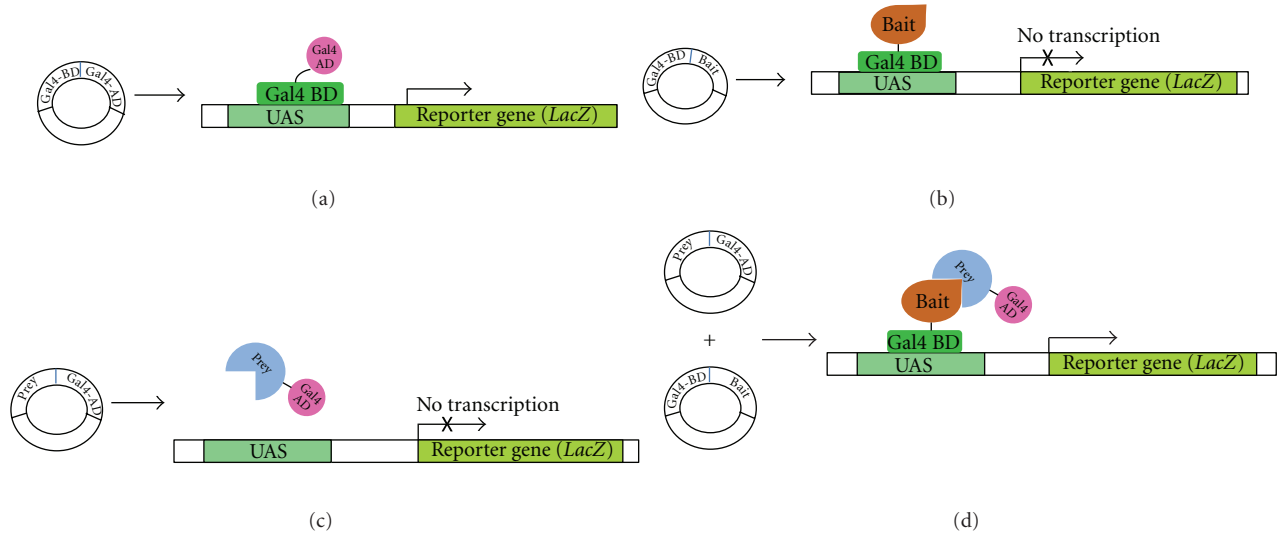


Figure 1: The two-hybrid assay, checking for interactions between two proteins, here called Bait and Prey. (a) Gal4 transcription factor gene produces two domain proteins (BD and AD), which are essential for transcription of the reporter gene (LacZ). (b) and (c), Two fusion proteins are prepared: Gal4BD + Bait and Gal4AD + Prey. None of them is usually sufficient to initiate the transcription (of the reporter gene) alone. (d) When both fusion proteins are produced and the Bait part of the first interacts with Prey part of the second, transcription of the reporter gene occurs (adapted from http://www.wikimediafoundation.org/).

detected using this strategy. In the case of the HIV-1 enzymes in addition of studies with RT and protease several cellular or retroviral partners of integrase have been described. Some examples are the interactions between the human and its ortholog yeast transcription factor SFN5/InI1 described below and that of the lens epithelium-derived growth factor (LEDGF/p75), the latter playing an important role by stimulating the integration step and facilitating the interaction with the host cell chromatin [25, 26]. The interaction of HIV-1 IN with microtubules proteins [27] using the two-hybrid approach was confirmed later by using other methods [28]. More recently were developed the three-hybrid and one-hybrid systems derived from the two-hybrid system.

3. Human Immunodeficiency Virus Type 1 (HIV-1)

Even though retroviruses carry an RNA+ genome, their replicative cycle involves the cell nucleus. These viruses are characterized by the fact that they carry an RNA-dependent DNA polymerase (reverse transcriptase (RT)) that synthesizes a double-strand DNA called proviral DNA in the cytoplasm of the infected cell by using the viral RNA genome as template. Proviral DNA and other viral and cellular factors forming the preintegration complex (PIC) enter the nuclear compartment and integrate the proviral double-stranded DNA in the host genome via an encoded retroviral enzyme, integrase (IN). In this paper we will focus on the use of the yeast model system to study the following proteins encoded in the HIV-1: the enzymes reverse transcriptase, integrase and protease, Vpr, Rev and the translation machinery of HIV-1.

3.1. Enzymes Encoded in the HIV-1 Genome

HIV-1 Encoded Enzymes. All retroviruses carry three enzymes, an RNA- and DNA-dependent DNA polymerase or reverse transcriptase (RT), integrase (IN), and protease

(PR) (see [29]). The organization of these proteins in several retroviruses is shown in Figure 2 while their 3D structure is shown in Figure 3. RT also contains an additional enzymatic activity, RNase H, which has been mapped to a separate, contiguous portion of the polypeptide. First identified in the avian viruses, the retroviral enzymes are organized in domains on the Gag-Pro or Gag-Pro-Pol precursor polypeptide. These domains are not always cleaved into separate mature proteins. In most genera, all enzymes are translated together as a Gag-Pro-Pol precursor, which are processed to yield the mature forms of the enzymes. Whether expression of *pro* and *pol* is due to frameshifting or termination suppression, they account for approximately 5% of RT and IN on a molecular basis but are synthesized and packaged in the virion at the same level as the Gag protein. For most retroviruses, the same holds for PR. A scheme describing the retroviral frameshifting process is shown in Figure 7. Since RT, IN, and PR are essential for viral replication and have characteristics that distinguish them from related cellular enzymes, they have all become privileged targets for drugs against the acquired immunodeficiency syndrome (AIDS).

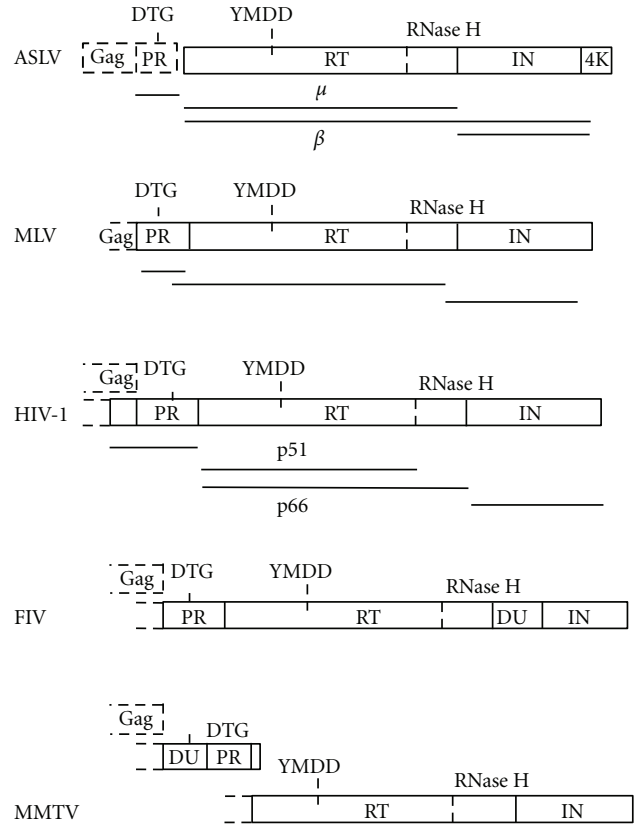


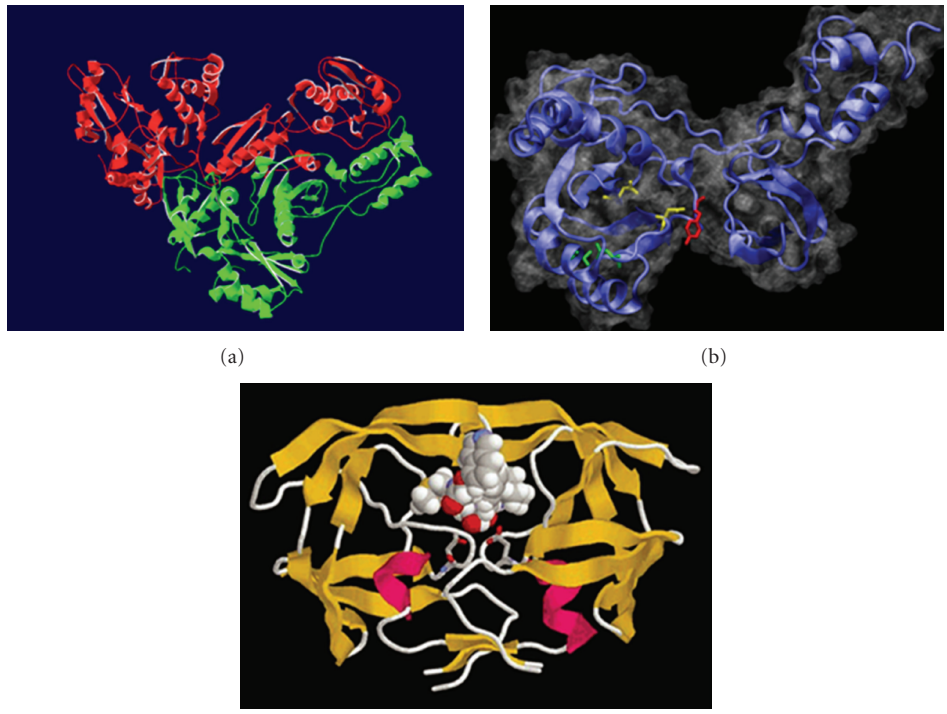
Figure 2: Organization of Pro and Pol proteins. Schematic representations of the mature Pro and Pol proteins and their precursors are drawn for examples from several retroviruses. The sequences representing the mature proteins PR, RT, and IN are indicated. Rectangles, precursor proteins, with solid vertical lines marking major cleavage sites and thick horizontal bars indicating mature proteins. DTG or DSG and YMDD indicate the conserved active site residues in PR and RT. The RNase H domain of RT is also indicated (ASLV: avian sarcoma leucosis virus, MMTV: murine mammary tumor virus, MLV: murine leukemia virus, and FIV: feline immunodeficiency virus).

3.2. Reverse Transcriptase (RT). At a time when the only assay to measure RT activity was *in vitro* assay, it seemed attractive to use the yeast *S. cerevisiae* as a model to test the in vivo activity of this enzyme. For this purpose constructions of hybrid retrotransposons composed of the endogenous yeast Ty1 element and the reverse transcriptase (RT) of HIV-1 were shown to be active in the yeast *Saccharomyces cerevisiae*. The RT activity of these hybrid Ty1/HIV-1 elements can be monitored by using a simple genetic assay. The reverse transcription of yeast carrying this hybrid RT depends on both the DNA polymerase and RNase H domains of HIV-1 RT.

Most HIV-1 RT inhibitors can be divided into two classes, nucleoside analog RT inhibitors (NRTIs) and nonnucleoside RT inhibitors (NNRTIs). NRTIs such as AZT (3-azido-3-deoxythymidine) and ddI (dideoxyinosine) inhibit reverse transcription by a chain-termination mechanism; that is, when they are added to a growing DNA chain they block further synthesis while the NNRTIs act by blocking the synthesis by binding specifically an hydrophobic pocket of the HIV-1 RT. The reverse transcriptase activity measured in yeast cells was shown to be sensitive to inhibitors of HIV-1 RT like 8Cl-TIBO, a well-characterized nonnucleoside RT inhibitor of HIV-1 RT while the hybrid constructions that express NNRTI-resistant RT variants of HIV-1 are insensitive to8Cl-TIBO demonstrating in yeast the specificity of inhibition in this assay. These hybrid Ty*1*/HIV-1 (*his3AI*/AIDS RT), called HART, elements carrying NNRTI-resistant variants of HIV-1, RT were used to identify compounds that are active against drug-resistant viruses [30].

The reverse transcriptase enzyme purified from the virus is a heterodimer with subunits of 66 and 51 kDa called p66 and p51 (Figure 3(a)). p51 subunit is a shortened version of p66 after cleavage by the HIV-1 encoded protease. As with many other recombinant proteins the yeast *S. cerevisiae* has been used to express HIV-1 RT. Only the HIV-1 gene for the p66 subunit was carried by the expression vector used and surprisingly the recombinant enzyme purified after expression in yeast was the heterodimeric p66/p51 form. Protein sequencing showed that the cleavage to produce the p51 subunit gave a product identical to the native viral enzyme showing that yeast possesses a protease with the same exquisite specificity as the HIV-1 protease [31].

3.3. Integrase. HIV-1 IN catalyzes the insertion of proviral DNA into the host-cell genome (for general reviews on HIV-1 integrase and further references see [32–36]). In the first step of the integration reaction, termed 3′-end processing, two nucleotides are removed from each 3′-end of the double strand viral DNA to produce new "hydroxyl ends" (CA-3′OH). This reaction occurs in the cytoplasm within a large viral nucleoprotein complex (PIC). After entering the nucleus via the PIC multimeric complex, the 5′-ends of processed viral DNA are joined covalently to the host



(a) (b)

(c)

Figure 3: Tertiary structure of RT, IN, and PR: (a) RT: p66 in red and p51 in green; (b) catalytic core of IN in blue. Aminoacid 143 in red, 97 and 120 in green, and 64 and 116 in yellow. (c) Dimeric PR in complex with a protease inhibitor.

DNA. The joining reaction includes a coupled 4–6-base-pair staggered cleavage of the target host DNA and the ligation of the CA-3′OH viral DNA ends to the 5′-phosphate ends of the target DNA. Although the mechanism is not fully understood, host cell DNA repair enzymes probably accomplish the repair of the remaining gaps. HIV-1 IN is composed of three functional domains. The N-terminal region (residues 1–50) is characterized by an HHCC zinc finger-like sequence able to bind zinc. This binding induces proper folding of the N terminus and promotes tetramerization of IN. The central region (residues 50–160, Figure 3(b)) is characterized by three highly conserved amino acid residues, D64, D116, and E152, forming the catalytic triad DD(35)E. Each one of these residues is essential for enzyme activity. The C-terminal region is the least conserved domain and is involved in nonspecific DNA binding. The 3D structure of the three domains of HIV-1 IN has been determined by RMN or X-ray crystallography. Despite considerable efforts, the low solubility of the native HIV-1 integrase has hampered the determination of the crystallographic structure of the entire protein. The three HIV-1 IN domains are required for in vitro 3′-end processing and DNA strand transfer. Mutation analyses of the viral integrase gene showed that this enzyme is required for retroviral replication and that it is a legitimate target for the design of antiretroviral drugs. Recently, the 3D structure of the human foamy retrovirus integrase was determined, taking profit of the increased solubility and yield of this recombinant retroviral enzyme, and confirming a tetramer quaternary structure [37].

3.4. Expression of HIV-1 IN in the Yeast Saccharomyces cerevisiae. Based on results dating from 1985 where the expression of the bacterial EcoR1 restriction endonuclease expressed in yeast cells led to the appearance of a lethal phenotype [38] the idea emerged that the expression of retroviral IN also carrying an endonuclease activity may produce a similar phenotype. The idea behind this approach was that the feasibility of such a system may allow the setting up of an easy and rapid procedure for screening antiretroviral drugs. However, the weak entrance of molecules through the yeast wall and cell membrane hampered this project. The yeast model has been extremely useful to study many aspects of the integration step in retroviral replication. An expression plasmid containing the retroviral integrase gene under the control of the inducible promoter ADH2/GAPDH regulated by the glucose concentration of the medium was constructed. Haploid yeast strain W303-1A did not appear to be clearly sensitive to HIV-1 integrase expression (Figure 4). However, disruption of the *RAD 52* gene, which is involved in the repair of double-strand DNA breaks, strongly increased the deleterious effects of the retroviral enzyme in this yeast strain. The diploid strain constructed with W303-1A and an isogenic strain of the opposite mating type also showed strong sensitivity to the HIV-1 IN. The lethal phenotype was suppressed by missense integrase mutations in the catalytic domain that are known to abolish HIV-1 IN activities *in vitro* (Figure 4) [39]. Subsequent studies were performed in order to determine the critical amino acid(s) and/or motif(s) required for the induction of the lethal phenotype in the

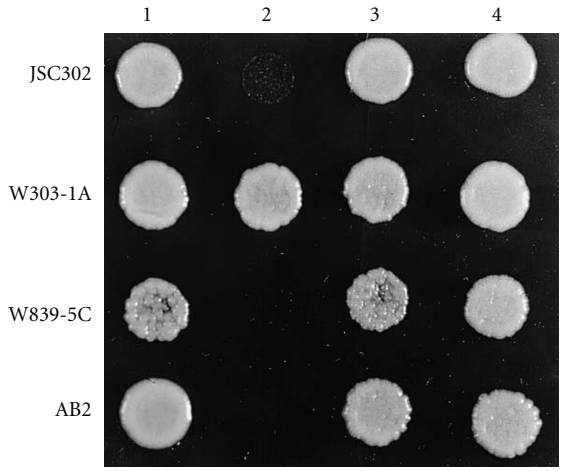


Figure 4: Drop test experiment: 3 μL droplets of plasmid-containing standard yeast suspensions containing about 20 000 ura+ colony-forming units were deposited on the appropriate selective media to allow expression of the HIV-1 integrase in yeast. When the drops had dried, plates were incubated for 3 days, and the effect of integrase expression on yeast was determined by visual observation of the Petri dishes. Lethality is observed when IN is expressed in yeast (2) but not in the presence of empty plasmid (1) or when expressing inactive D116A or E152A IN (3 and 4). JSC302 and W303-1A: RAD52+, W839-5C: identical to W303-1A except for *RAD52* gene disruption. AB2: diploid obtained from W303-1A (adapted from [39]).

yeast and to elucidate the molecular mechanism that causes this phenotype. The HIV-1 IN mutants, V165A, A179P, and KR186, 7AA, located in the C-terminal region of the catalytic core domain of IN were shown to be unable to induce the lethal phenotype in yeast. Further mutagenic and virologic experiments led to the conclusion that these mutations inhibit the IN-induced lethal phenotype in yeast by inhibiting the binding of IN to the host chromatin. These results demonstrate that the C-terminal region of the catalytic core domain of HIV-1 IN is important for binding to host chromatin and is crucial for both viral replication and the promotion of the IN-induced lethal phenotype in yeast [40]. The question remained whether the lethal effect was related to the nonspecific endonuclease activity of the viral IN or whether the mechanism involved was due to a pleiotropic effect of this protein. Lethality in yeast seems to be related to the mutagenic effect of the recombinant HIV-1 IN, most probably via the non-sequence-specific endonuclease activity carriedout by this enzyme. This non-sequence-specific endonuclease activity was further characterized. Although the enzyme was active on DNA substrates devoid of viral long terminal repeat (LTR) sequences characteristic of the retroviral proviral genome, the presence of LTR regions significantly stimulated this activity. Genetic experiments showed that both the mutagenic effect and the level of recombination events were affected in cells expressing the active retroviral enzyme, while expression of the mutated inactive IN D116A had no significant effect [41].

As mentioned above a nucleoprotein complex (PIC) comprising the proviral DNA, the IN, and other viral and cellular proteins is formed in the cytoplasm and enters the nucleus by an unknown mechanism where the retroviral DNA is inserted into the host nuclear genome. The exact composition of the PIC is a controversial matter since different laboratories have described various viral and cellular proteins. The yeast two-hybrid system was used to identify a human gene product that binds tightly to the human immunodeficiency virus type 1 (HIV-1) integrase *in vitro* and stimulates its DNA-joining activity. This protein has been suggested as being part of the PIC. The sequence of the gene suggests that the protein is a human homolog of yeast SNF5, a transcriptional activator required for high-level expression of many genes. The gene, termed INI1 (for integrase interactor 1), may encode a nuclear factor that promotes integration and targets incoming viral DNA to active genes [25]. The analogy of the yeast and human orthologs prompted study of whether yeast SNF5 is involved in the lethal effect of HIV-1 IN expression in yeast. The effect of the inactivation of the yeast gene encoding for SNF5 on the lethality induced by the yeast expression of HIV-1 IN has been described. Results showed that the retroviral IN is unable to perform its lethal activity in cells where the SNF5 gene has been disrupted, suggesting that SNF5 may play a role in the lethal effect induced by IN in yeast [42]. SNF5 inactivationdoes not affect neither yeast viability nor expression of HIV-1 IN. Given the homology between SNF5 and its human counterpart INI1, these results suggest that this factor may be important for IN activity in infected cells. Moreover, given the important role proposed for this transcription factor in the integration step and the fact that it is not essential for cell viability, the interaction between INI1/ySNF5 and HIV-1 IN should become a potential target in the search for new antiretroviral agents. Given the proximity of many human and yeast proteins the expression of the human INI1 rescued the lethal phenotype in yeast cells where SNF5 was inactivated, again revealing the functional analogy of many human and yeast genes [42].

A question that has not yet been fully answered concerning the HIV-1 cycle is the mechanism of entry of the PIC into the nucleus previous to integration in the host DNA. This question about intracellular HIV-1 trafficking has been tackled using the yeast system.Based on previous results using the two-hybrid strategy and showing that HIV-1 IN interacts with two yeast microtubule-associated proteins, Dyn2p (dynein light chain protein) and Stu2p, a centrosomal protein [43], the role of these proteins in intracellular trafficking and nuclear import in yeast was studied since it is thought that these biological mechanisms are evolutionarily conserved in eukaryotic cells. IN was found to accumulate at the perinuclear microtubule organizing center (MTOC) called the yeast spindle pole body (SPB) via Stu2p colocalization (Figure 5). Disruption of the microtubule network by nocodazole or IN expression in a dynein2-deficient yeast strain prevented IN accumulation in the nuclear periphery and additionally inhibited IN transport into the nucleus. By mutagenesis, the results indicated that trafficking of IN towards the SPB requires the C-terminus of the molecule.

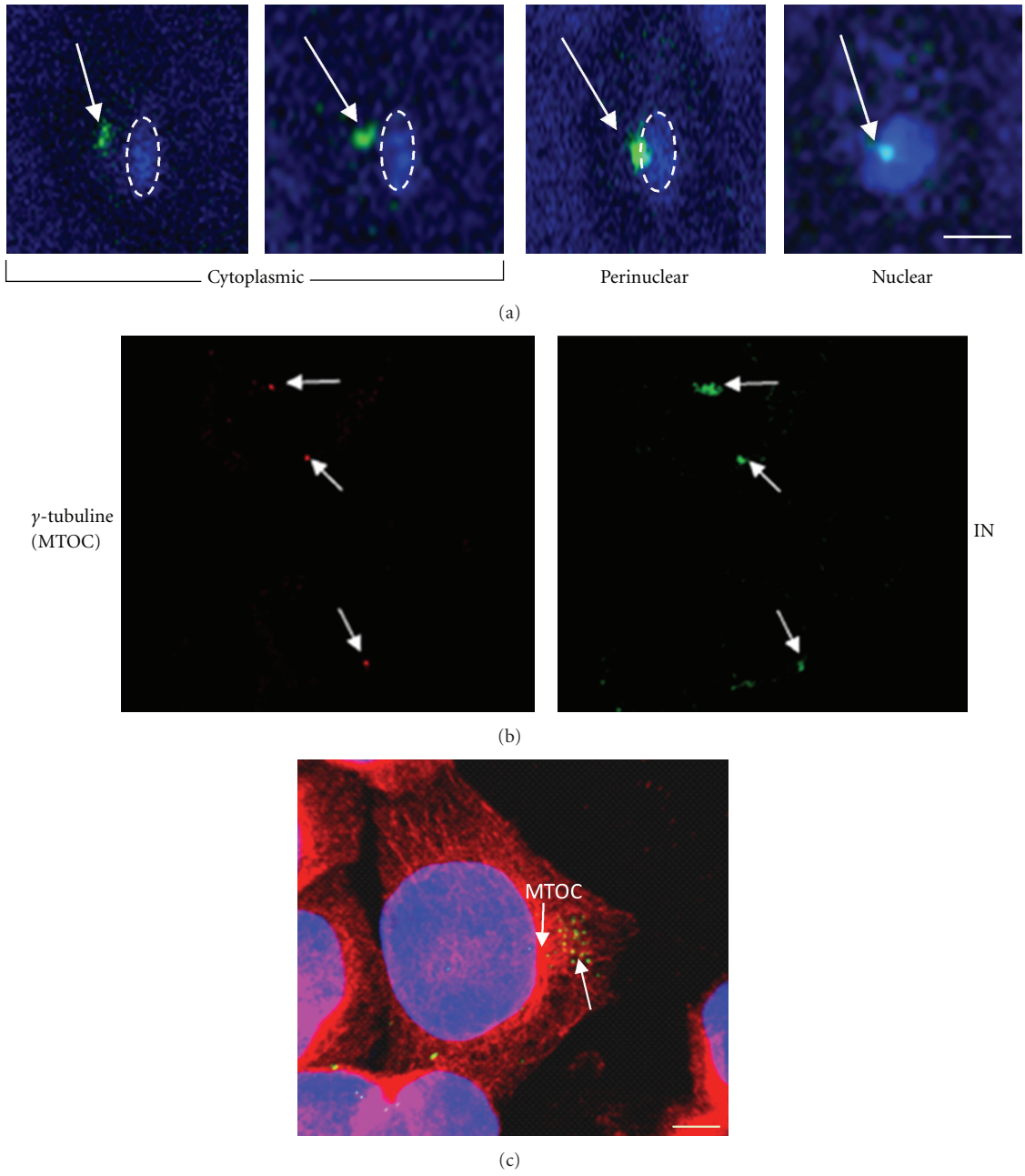


Figure 5: IN expressed in yeast and in human cell. (a) Expression of IN in yeast several times after induction. Cytoplasmic localisation: 6-7 h; perinuclear localisation: 8–10 h; nuclear localisation: 30 h. IN-GFP in green, nucleus in blue surrounded by dashed lanes. Barr = 1 μm. (b) Expression of IN in H9 cells 20 h after transfection. Left, MTOC in red after immunodetection of γ-tubulin (arrows). Right, IN in green immunodetected with anti-IN antibody (arrows). (c) Expression of IN in H9 cells at higher magnitude. IN in green, α-tubulin in red. Barr = 8 μm (Desfarges, unpublished results).

These findings strongly suggest that IN nuclear import seems to depend on an essential intermediate step involving the SPB and that Dyn2p and Stu2p play an important role in driving IN toward MTOC and optimizing nuclear entry of the retroviral enzyme [44]. Intracellular transport of HIV-1 IN in yeast cells is in good agreement with later results using immunofluorescence and electron microscopy of the trafficking in human cells of IN tagged with GFP and other labeling methods. These authors also observed an accumulation of labeled HIV-1 IN in the nuclear periphery and the involvement of the MTOC in the IN cellular *voyage* to the nucleus [28, 45].

The strong similarities between human infected cells and budding yeast demonstrate the relevance of the yeast model to obtain a detailed picture of the molecular mechanisms involved in the HIV-1 cycle (Figure 5).

As described above HIV-1 IN expressed in yeast in the absence of other viral proteins is able to enter the nucleus and cleave the host genome. An exciting question remained whether it is able to integrate a proviral-like DNA in the nuclear yeast genome when expressed alone. This was solved by setting up a system expressing only the IN and a short DNA mimicking the retroviral DNA by carrying the two LTRs flanking an antibiotic (zeocin) resistance gene (Figure 6). Multiple resistant yeast clones were observed under these conditions, while very few clones were obtained when using constructions carrying no IN or the inactive IN mutant D116A. Zeocin-resistant clones were confirmed to represent real integration events by detecting the 800 bp proviral-like DNA in the yeast genomic DNA by PCR or Southern experiments. Moreover, the sequence of the integration junctions showed that each integration event was disrupting a different open reading frame. Since the sequence of these links between integrated LTRs and the target DNA constitutes a specific signature of the IN involved in this process, they were carefully analyzed. The 5 bp repeats characterizing HIV integration were recovered for most clones, confirming that HIV-1 IN was responsible for the process. Therefore, in this simple eukaryotic model, the retroviral integrase is the only viral protein necessary for the insertion of a DNA containing viral LTRs into the genome, thereby allowing the study of the isolated integration step independently of other viral mechanisms. The identification of the yeast factors involved in the HIV-1 IN trafficking remains an interesting perspective. Another issue is whether these cellular factors, which are analogs to those forming the PIC in human infected cells, may be used to reconstitute a PIC-like complex [46]. Moreover these results may indicate that at least a fraction of the pool of integrase alone may enter the nucleus independent of the PIC.

The last step of the integration process involves the repair of the DNA junctions. It has been widely reported that the cellular DNA repair system plays a crucial role in the retroviral integration step. DNA repair factors like RAD18, RAD51, and RAD52 modulate this process [47]. The effect of RAD51 and other DNA repair factors can be easily studied in yeast since, contrary to what has been observed in human cells, no deleterious effects are observed when RAD51 or RAD18 are deleted in yeast. When RAD51 was deleted, a striking increase in integration events was observed after expression of IN and the viral-like DNA in yeast [46]. Very recently, the close interaction between IN and RAD51 and the molecular mechanism played by this protein in HIV-1 integration was described in yeast and human cells. *In vitro* integration assays performed under various conditions promoting or inhibiting hRAD51 activity demonstrated that the formation of an active hRAD51 nucleofilament is required for optimal inhibition involving the dissociation of the IN-DNA complex. Furthermore, the availability of the RAD51 stimulatory agent, RAS-1, made it possible to show that this inhibition mechanism could be promoted in HIV-1-infected cells by chemical stimulation of the endogenous hRAD51 protein. Stimulation of RAD51 induced both an enhancement of the endogenous DNA repair process and the inhibition of the integration step. These results showing the restriction of viral proliferation by RAD51 pave the way for a new concept of antiretroviral therapy based on the enhancement of endogenous hRAD51 recombination activity [48].

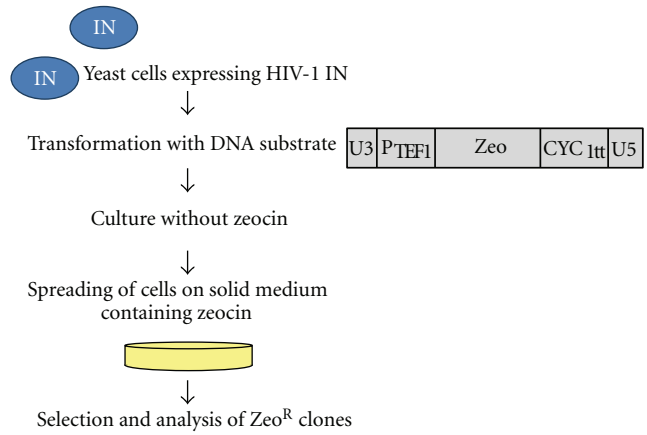


Figure 6: Integration assay in yeast. Adapted from [46].

3.5. Protease. A lethal effect similar to that obtained by the expression of HIV-1 IN in yeast was observed more recently when the retrovirus-encoded protease (HIV-1 PR, Figure 3(c)) was expressed both in *Saccharomyces cerevisiae* and in mammalian cells. These findings contribute to a deeper understanding of HIV-1-induced cytopathogenesis. Expression of HIV-1 PR stopped yeast growth followed by cell lysis. The lytic phenotype included loss of plasma membrane integrity and cell wall breakage leading to the release of cell content to the medium. Interestingly, this effect seems to be specific for HIV-1 PR since neither poliovirus 2A protease nor 2BC protein, which are both highly toxic for *S. cerevisiae*, was able to produce similar effects. Drastic alterations in membrane permeability preceded the lysis in yeast expressing the HIV-1 PR. The morphological changes after expression of HIV-1 PR in yeast and mammalian cells were similar in many aspects [49].

4. Expression of Retroviral Enzymes Is Controlled by Frameshifting

During the replication of retroviruses, large numbers of Gag molecules must be generated to serve as precursors to the structural proteins of the virions. However, the enzymes encoded by the *pro* and *pol* genes (PR, RT, and IN) are generally needed in lower amounts to carry out their catalytic functions. Retroviruses have developed a mechanism leading to the synthesis of the Gag protein at higher levels relative to the *pro* and *pol* gene products, while retaining coregulated expression (Figure 7). This is due to the use of the same initiation codon in the same mRNA to express the *gag*, *pro*, and *pol* genes. Translation of this RNA leads occasionally to synthesis of a fusion protein that is usually called the Gag-Pro-Pol precursor. Typically, 10–20 structural Gag molecules are made for every molecule of Gag-Pro-Pol. This device

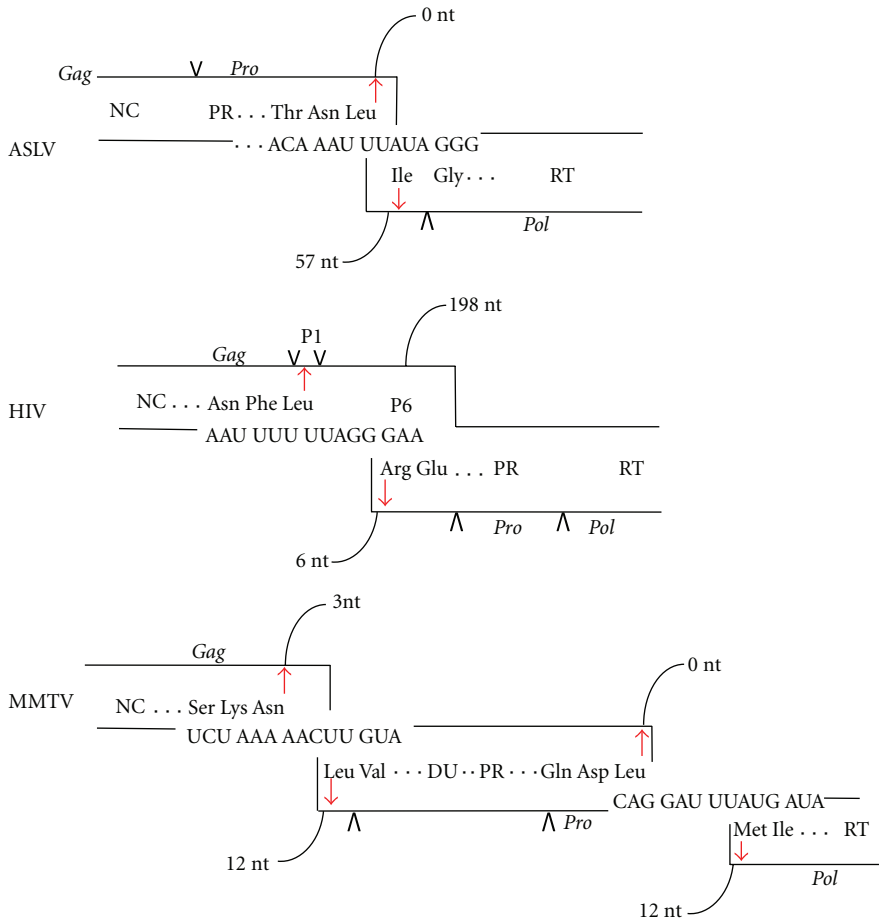


Figure 7: Frameshift suppression in the synthesis of Gag-Pro-Pol. Shown are the nucleotide sequences at the frameshift site and the amino acids encoded in the Gag-Pro-Pol precursors of the indicated viruses. The upper amino acid sequence is read from either the *gag* or *pro* reading frame, and the lower sequence is read from either the *pro* or *pol* reading frame, as shown. The boxes represent the indicated reading frames. The colored arrows indicate the position of the nucleotide (shown in color) that is read in both reading frames, the Vs represent the positions in the sequence that encode PR processing sites, and the numbers represent the number of nucleotides between the frameshift site and the end of the reading frame (irrespective of the nucleotide that is read in both frames). The nucleotides in the boxes include the beginning and ending codons in the reading frames shown (ASLV: avian sarcoma leucosis virus and MMTV: murine mammary tumor virus).

allows the same mechanism that targets the Gag precursor to the site of virion assembly also to direct the Gag-Pro-Pol precursor. In all retroviruses, the *gag* gene is positioned at the 5′ end of the viral genome, upstream of the *pro* and *pol* genes. The Gag-Pro-Pol precursor is generated using a strategy in which the termination codon that defines the 3′ terminus of the *gag* reading frame is bypassed, allowing translation to continue into the adjacent *pro* and *pol* reading frames. Bypass of the termination codon occurs by one of two mechanisms. The first mechanism (used by the mammalian type-C retroviruses) is read through (termination) suppression, in which the *gag* termination codon is occasionally misread as a sense codon. Translation then continues past the termination codon and into the *pro-pol* reading frame. The second mechanism, which is used by most retroviruses, is ribosomal frameshifting. Here, occasional ribosomes slip backward one nucleotide (–1 frameshift, i.e., in the 5′ direction) during translation of *gag*. Thus, the ribosome leaves the *gag* reading frame (with its downstream termination codon) and shifts into an overlapping portion of the *pro-pol* reading frame.

4.1. Vpr. The 14-Kd, 96-amino-acid HIV-1 encoded protein Vpr (viral protein R) [50, 51], plays several roles in the replication cycle of this retrovirus. Thus, it has been proposed that Vpr regulates the nuclear import of the preintegration complex (PIC) carrying the proviral DNA, the viral integrase, and other viral and cellular proteins. It is also required in the replication of HIV-1 in nondividing cells like the macrophages, and much evidence indicates that it is able to induce the arrest of cell cycle at the G2 step in proliferating cells, the latter effect likely playing an important role in the immunosuppression process in AIDS patients (for a recent review see [52]).

4.2. Arrest of G2/M by Vpr. Several lines of evidence in yeast and human cells show that Vpr has an important

effect on the G2 to M transition of the cell cycle [53]. It has been reported that the induction of Vpr expression in the fission yeast *Schizosaccharomyces pombe* leads to several defects in the assembly and function of the mitotic spindle. Some spindle pole body proteins were delocalized in Vpr-expressing yeast cells perturbing its integrity. In addition, nuclear envelope structure, contractile actin ring formation, and cytokinesis were also disrupted. As similar problems in mitosis and cytokinesis were observed in human cells, it has been suggested that these defects account for some of the pathological effects associated with HIV-1 infection.

4.3. Apoptosis. When VPR was expressed in the fission yeast *Schizosaccharomyces pombe* the induction of cell death observed was reminiscent of the effect of HIV-1 infection in mammalian cells, suggesting that VPR may affect a conserved cellular process. In recent years proteins have been identified as Vpr suppressors that are able to overcome Vpr-induced cell death in fission yeast as well as arrest Vpr-induced apoptosis in mammalian cells. Although not conclusive these results may suggest that Vpr-induced cell death in yeast resembles some of the apoptotic processes in mammalian cells. Like Vpr-induced apoptosis in mammalian cells where mitochondria play an essential role, Vpr in yeast promotes phosphatidylserine externalization and induces hyperpolarization of mitochondria, leading to changes in mitochondrial membrane potential. Moreover, Vpr triggers production of reactive oxygen species (ROS), indicating that cell death via an apoptotic-like mechanism might be mediated by these reactive species. As mitochondria play a crucial role in apoptosis, it is intriguing that, in fission yeast, Vpr induces unique morphologic changes in these organelles. The Vpr suppressor factors EF2, Hsp16, and Skp1 that suppress Vpr-induced apoptosis in mammalian abolished cell death mediated by Vpr and restored normal mitochondrial morphology in yeast cells [54]. The similarity of Vpr-induced cell death in fission yeast with the mammalian apoptotic process reinforces the idea that fission yeast may be used as a simple model organism to study the apoptotic-like process induced by Vpr and other pro-apoptotic agents.

4.4. Rev. The 116-amino-acid HIV-1 Rev, an 18 kDa phosphoprotein discovered in 1986, is capable of being imported into the nucleus and binding specially to the Rev responsive element (RRE) a viral sequence-specific RNA. Rev is able to form multimers and direct the nuclear export of large RRE-containing RNP complexes. Rev activity is crucial in the nuclear export of intron-containing HIV-1 RNAs [55–57]. Rev shuttles between the nucleus and the cytoplasm and has a nuclear localization signal (NLS) as well as a nuclear export signal (NES). These essential peptide motifs have now been shown to function by accessing cellular signal-mediated pathways for nuclear import and nuclear export. In addition to NLS and NES a nuclear import inhibitory signal (NIS) that inhibits the entry of low molecular weight proteins has been described in Rev [58]. HIV-1 Rev is therefore an excellent system to study aspects of transport across the nuclear envelope [59]. Human immunodeficiency virus type 1 (HIV-1) replication requires the expression of two classes of viral mRNA. The early class of HIV-1 transcripts is fully spliced and encodes viral regulatory gene products. The functional expression of Rev induces the cytoplasmic expression of the unspliced or incompletely spliced mRNAs that encode the viral structural proteins, including Gag and Env. Based on experiments that indicate a similar function of Rev in the yeast *S. cerevisiae,* a yeast protein interacting with the effector domain of Rev was found [60]. This protein called Rip1p is a novel small nucleoporin-like protein, some of which is associated with nuclear pores. Its closest known yeast relative is a nuclear pore component also involved in mRNA transport from nucleus to cytoplasm. Analysis of yeast strains that overexpress Rip1p or which are deleted for the RIP1 gene show that Rip1p is important for the effect of Rev on gene expression, indicating that the physical interaction is of functional significance in vivo. These results suggest that Rev directly promotes the cytoplasmic transcripts transport by targeting them to the nuclear pore.

The NES domain of the Rev protein is required for Rev-mediated RNA export in mammals as well as in the yeast *Saccharomyces cerevisiae* [61]. As mentioned above Rev NES has been shown to specifically interact with a human (hRIP/RAB1) and a yeast (yRip1p) protein in the two-hybrid assay. Both of these interacting proteins are related to FG nucleoporins on the basis of the presence of typical repeat motifs. Rev is able to interact with multiple FG repeat-containing nucleoporins from both *S. cerevisiae* and mammals. Moreover, the ability of Rev NES mutants to interact with these FG nucleoporins parallels the ability of the mutants to promote RNA export in yeast, Xenopus oocytes, and mammalian cells [62].

HIV-1 Rev-mediated nuclear export of viral RNAs involves the interaction of its leucine-rich NES with nuclear cofactors. Using an extensive panel of nuclear export positive and negative mutants of the functionally homologous NES of the HIV-1 Rev, physiologically significant interactions of human Nup98 (hNup98) with NESs from various viral sources were demonstrated. Recently, a cellular factor called CRM-1 was shown to be an essential nuclear export factor interacting directly with nuclear export signals including the Rev NES in a RanGTP-dependent manner. It was shown that NLP-1, like the previously described Rev-interacting protein hRIP/Rab and several nucleoporins, also interacts with CRM-1 in both yeast and mammalian cells. Missense mutations in the yeast nuclear export factor Crm1p abrogated Rev NES interaction with the XXFG repeat-containing nucleoporin Rab/hRIP. These mutations had minimal effects on the interaction with GLFG repeat-containing hNup98. Functional analysis of the Nup98 domains required for nuclear localization demonstrated that the entire ORF was required for efficient incorporation into the nuclear envelope. A putative nuclear localization signal was identified downstream of the GLFG repeat region. Whereas overexpression of both full-length Nup98 and the amino-terminal GLFG repeat region, but not the unique carboxy-terminal region, induced significant suppression of HIV unspliced RNA export, lower levels of exogenous Nup98 expression

resulted in a relatively modest increase in unspliced RNA export. These results suggest a physiological role for hNup98 in modulating Rev-dependent RNA export during HIV infection [63].

4.5. Antifungal-Effect of Rev NIS-Derived Peptide. The NIS signal encoded in Rev has been used as a therapeutic agent with anticandidal effects. In addition this property was used to study the mechanism of action of this factor by studying the intracellular localization of the peptide. The result showed that Rev rapidly accumulated on the fungal cell surface. The cell wall regeneration test also indicated that Rev exerts its anticandidal activity on the fungal plasma membrane rather than on the cell wall. By using fluorescent probes, the membrane-disruption mechanism of Rev was further confirmed, suggesting that it may be a potential therapeutic agent for treating fungal diseases caused by *Candida* species in humans. Further studies showed that the antifungal effects of the nuclear entry inhibitory signal peptide of HIV-1 Rev protein had no hemolytic effects [64, 65].

5. Yeast and HIV-1 Translation (for a Recent Review See [66])

Retroviral frameshifting (see above) is a change in reading frame during gene expression and is a mechanism that allows to keep at a low level the synthesis rate of the functional proteins relative to that of the structural proteins. Using *S. cerevisiae* to decipher viral frameshifting mechanisms Wilson et al. were the first to provide an in vivo demonstration of a frameshifting event, in *S. cerevisiae* [67]. They inserted the Gag-Pol fragment containing the potential frameshifting site of HIV-1 (without the stimulatory element) into a yeast expression plasmid, upstream from the interferon (IFN) cDNA. They monitored production of the frameshifted protein by Western blotting. It is now clear that a stimulatory secondary structure is required for maximal frameshifting efficiency although the precise nature of this structure remained unclear for a long time. Results using a dual reporter system in yeast showed that there is a direct correlation between HIV frameshifting efficiency and the stability of the stem loop [68]. The stem loop analyzed in these studies was the upper part of the complete stimulatory element observed by NMR. Under these conditions, the stability of this structure is clearly linked to frameshifting efficiency. A structure was recently identified on the basis of the complete yeast genome sequence, and it seems interesting to explore frameshifting efficiency with the complete sequence. The structure of the tetraloop is similar to the motif found in the RNase III recognition site from *S. cerevisiae* [69]. As the ACAA motif in the tetraloop is poorly recognized by RNase III, the possibility of engineering the *S. cerevisiae* RNase III for selective targeting of the HIV-1 tetraloop followed by the expression of this protein in HIV-1-infected cells has been suggested. This hypothetical approach remains to be confirmed but would provide an interesting therapeutic strategy, derived from experiments in yeast, to limit HIV-1 proliferation. The similar slippage efficiencies of the HIV frameshifting site in vivo in yeast and in vitro in a mammalian system demonstrate the high level of conservation of frameshifting mechanisms. Bidou et al. [66] suggested that the demonstration that frameshifting is conserved from yeast to humans paves the way for the use of yeast mutants to analyze retroviral frameshifting as already reported by several groups for other viruses. For instance, SARS coronavirus (sometimes shortened to SARS-CoV) is the virus that causes severe acute respiratory syndrome. SARS-CoV carries a frameshifting signal [70]. The minimal frameshifting signal in this virus is a U UUA AAC slippery sequence and a stimulatory structure folding into a pseudoknot [71]. This pseudoknot has several unusual features, including the third stem in loop 2 and the presence of two unpaired adenosine residues within the structure ([70, 72]. Plant and Dinman [70] demonstrated the ability of this new site to frameshift in *S. cerevisiae*. The frequency of frameshifting in yeast was much lower (3%) than for other coronavirus sites tested in yeast (12% for infectious bronchitis virus (IBV)). This may indicate the existence of subtle differences in terms of frameshifting mechanisms. Indeed, the importance of the unpaired adenosine residues remains unclear as this part of the pseudoknot is thought to lie outside the ribosome. It would be interesting to investigate the possible binding of a transacting factor, although the binding of such a factor has never been detected with the well-studied IBV coronavirus pseudoknot. The yeast mak8-1 mutant is known to have a specific defect in frameshifting [73]. It carries an altered form of ribosomal protein L3 in the ribosomal peptidyl-transferase center. This strain was reported to have a slightly higher SARS-CoV frameshifting efficiency than wild-type strains [70], in the first demonstration that this newly discovered frameshifting site is used.

6. Summary and Perspectives

In summary, although much of the potential promise of yeast is still to be revealed, it has proved extremely valuable in virus research. In addition to techniques like the double-hybrid system, this simple eukaryotic cell has been very useful for producing recombinant viral proteins whose purification from native virions is difficult or simply impossible. It also allows the mechanism of action of viral proteins to be studied thanks to the close analogy between human and yeast proteins, and this has led to the emergence of new therapeutic targets. By associating the toxic phenotype induced by some viral proteins in yeast cells with the genetic manipulation facilitating the entry of drugs with potential therapeutic properties, it may become possible to establish a simple cheap system allowing faster screening of the antiviral agents of the future. Moreover, future work should lead to the discovery of new cellular factors involved in virus proliferation, thus shortening the time necessary to develop new therapies against current and new viruses. The ultimate information on the behavior of viruses or virus proteins inside the cell should be attained with plant,

animal, and human cells or in vivo in whole organisms, although it may be difficult and costly for most laboratories to develop these approaches. Since experiments with yeast will always be technically easier, more rapid, and cheaper than those with human and other complex eukaryote cells, yeast will remain a method of choice for studying virus infections mechanisms and the search for new drug targets.

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Review Article

Research on Plants for the Understanding of Diseases of Nuclear and Mitochondrial Origin

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Different model organisms, such as *Escherichia coli*, *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster*, mouse, cultured human cell lines, among others, were used to study the mechanisms of several human diseases. Since human genes and proteins have been structurally and functionally conserved in plant organisms, the use of plants, especially *Arabidopsis thaliana*, as a model system to relate molecular defects to clinical disorders has recently increased. Here, we briefly review our current knowledge of human diseases of nuclear and mitochondrial origin and summarize the experimental findings of plant homologs implicated in each process.

1. Introduction

Sequencing of the human genome has been fundamental to progress in the study of genetic diseases. In recent years, the research on various human disorders and the influence of protein and gene interactions to disease state have increased. Several model systems have been used to investigate different human diseases such as cell lines (i.e., fibroblasts, human, and mammalian cell lines), yeast (i.e., *Saccharomyces cerevisiae*), as well as other organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster* [1]. Besides these organisms, plants, especially *Arabidopsis thaliana*, have proven to be a powerful additional model system to study eukaryotic mechanisms that might act similarly in the onset of human diseases [2]. In fact, Arabidopsis encodes several orthologs of human proteins [2]. In addition, Arabidopsis present some advantages for the study of human diseases: (i) short life cycle, (ii) fast and simple growth on MS medium, (iii) availability of mutants and homozygous lines for almost all genes, (iv) fast and simple plant transformation techniques, (v) fast and easy cell culture methods, and (vi) few ethical requirements [1].

The following sections review aspects of some human diseases of nuclear and mitochondrial origin and describe experimental advantages and recent studies of plant homologs implicated in each process.

2. DNA Repair Genetic Disorders

2.1. MMR Pathway

2.1.1. Hereditary Nonpolyposis Colon Cancer. Hereditary nonpolyposis colon cancer (HNPCC) or Lynch syndrome is an autosomal dominant disease characterized by the early occurrence of cancers of colon, endometrium, and other organs. Tumors are recognized by a high occurrence of microsatellite sequence instability (MSI) [3]. Microsatellites are tandem repeat nucleotides comprising 1–6 bp that occur ubiquitously throughout the genomes. These sequences undergo changes in the number of repeat units due to

slippage and inefficient proofreading activity of replicative DNA polymerases and to failure of mismatch repair (MMR) system.

The MMR system is best known for its role in the correction of mispaired and unpaired bases that arise during DNA replication and genetic recombination [4–10]. The system involves several nuclear proteins, which function in sequence. First, MutS proteins recognize DNA damage. Subsequently, MutS proteins recruit MutL proteins at the DNA lesion site in the presence of ATP. Then, proteins implicated in multiple DNA metabolic pathways (exonuclease I (Exo I), proliferating cell nuclear antigen (PCNA), replication factor C (RFC), single-strand binding protein RPA, high-mobility group box 1 (HMGB1), DNA polymerase δ and DNA ligase) excise the damaged DNA section and resynthesize the correct DNA sequence.

MutS and MutL proteins form heterodimers in eukaryotes. MutS heterodimers are composed of MutS homologs (MSH) subunits, which assemble as MSH2-MSH6 (MutSα) and MSH2-MSH3 (MutSβ). MutSα recognizes base-base mismatches and short insertion/deletion loops (IDLs) [11–16], while MutSβ binds larger IDLs [11, 14, 17–20]. MutL heterodimers are composed of MutL homologs (MLH) and postmeiotic segregation (PMS) subunits, which in humans assemble as MLH1-PMS2 (MutLα), MLH1-MLH3, and MLH1-PMS1 [7, 21, 22].

Inherited mutations in one of four different MMR genes (*MSH2*, *MSH6*, *MLH1,* and *PMS2*) are responsible for predisposition to HNPCC development [23]. Germline mutations in *MSH2* or *MLH1* lead to complete loss of DNA MMR activity, whereas inactivation of *MSH6* or *PMS2* shows a less severe form of cancer associated to the functional redundancy of *MSH3* and *MLH3* genes, respectively (Table 1). Once MMR activity is reduced, genes are prone to base and frameshift mutations and loss of function. The most critical mutated genes are either involved in the regulation of growth, the regulation of apoptosis, or in MMR system itself, which leads to a progressive inactivation of the entire system [23].

Consistent to the essential genome maintenance function performed by MMR system, evolutionary conservation of MMR genes in plants is not surprising. Besides MSH2, MSH3, MSH6, MLH1, MLH3, and PMS1 (ortholog to human PMS2), plants encode a unique mismatch-recognition protein named MSH7 [24–29]. MSH7 forms heterodimers with MSH2 leading to the formation of MutSγ. MutSγ preferentially recognizes some base-base mismatches and plays a specific role in meiotic recombination [25, 30–32]. Like human MMR proteins, plant counterparts are critical to efficiently promote genomic stability (Table 1). Inactivation of Arabidopsis *MSH2* by T-DNA insertion or RNA interference generated microsatellite instability at several dinucleotide repeat alleles [33]. These insertion/deletion mutations accumulated during propagation since the fifth generation of mutant plants showed up to 3-fold more allele shifts per plant than the first generation [34]. Cumulative mutations over generations produced abnormalities in morphology and development, fertility, germination efficiency, seed/silique development, and seed set [34]. Loss of a functional Arabidopsis *MLH1* gene also led to a significant reduction in fertility in both homozygotes and heterozygotes [35].

Some advantages of Arabidopsis as a model system to study DNA repair mechanisms are worth mentioning: (i) short generation time for propagating progeny, (ii) availability of both homozygous and heterozygous seeds for comparison, and (iii) different reporter systems based on histochemical staining, bioluminescence generation, or herbicide resistance for mutagenesis analysis. In this regard, several reporter constructs were developed to examine *Arabidopsis thaliana* MMR function *in vivo* (reviewed in [36]). Assays were designed to analyze microsatellite instability or monitor the frequency of somatic recombination by restoring β-glucuronidase activity or conferring tolerance to the herbicide phosphinothricin. Results confirmed that frameshift mutations were dependent on *MSH2* [37]. In addition, *MSH2*, *MLH1,* or *PMS1* were suggested to play essential roles in suppressing recombination between diverged sequences and indicated the involvement of *MLH1* and to a lesser extent *MSH2* in the stimulation of plant homologous mitotic recombination [35, 38–41].

Research in *Arabidopsis* MMR also included overexpression of functionally impaired mutated proteins. Transgenic plants harboring a truncated form of human PMS2, first identified in kindreds affected with HNPCC, exhibited a dominant negative effect [42]. A similar strategy was used to analyze the role of the highly conserved ATP binding domain of AtPMS1. Introduction of mutant alleles were shown to inhibit the MMR system in *Arabidopsis* [43].

A better understanding of MMR genes, and the mechanisms in which they contribute, requires the isolation and characterization of the proteins they encode. We successfully overexpressed AtMSH2 and AtPMS1 en *Escherichia coli* and raised polyclonal-antibodies against these subunits [44, 45]. In addition, in studies to be reported elsewhere, we found that expression of MutLα or MutSγ in *Saccharomyces cerevisiae* leads to a clear increase in yeast mutator rate, suggesting that the expression of the plant proteins somehow affects yeast MMR mechanism.

Taken together, the above-cited studies indicate that MMR system shows high conservation from humans to plants. Disruption of plant MMR genes, either by inactivation or dominant negative inhibition, confirmed the function of their orthologs in humans. Studies can be further extended to analyze DNA damage induction and repair. Considering that human MMR proteins also recognize modified bases generated in response to endogenous or exogenous DNA damaging agents and that Arabidopsis seedlings are relatively sensitive to chemical mutagens and reasonably transparent to UV light, Arabidopsis MMR research promises to yield insights into the processing of such lesions. Recently, the contribution of Arabidopsis and maize MutSα (MSH2–MSH6) to UV-induced DNA lesion repair was investigated [46]. *MSH2* and *MSH6* genes were reported to be upregulated by UV-B. Consistent with these results, *Atmsh2* and *Atmsh6* mutant plants accumulated more DNA lesions relative to wild-type plants. These data

Table 1: Overview of studies linking DNA repair pathways and disorders in humans and plants.

Genetic deficiency[a]	DNA repair pathway defect[b]	Human disease[c]	Reference	Plant disorder[d]	Reference
MSH2 MSH6 MLH1 PMS2(1)	MMR	HNPCC	[23]	MSI Homeologous recombination	[33, 34, 37–41]
XPA XPB XPC XPD XPE XPF XPG	NER	XP	[48]	Hypersensitivity to UV	[90, 93–95, 97, 98]
ATM	DSB	AT	[101]	Hypersensitivity to γ radiation, X-ray, radiomimetic agents	[115, 117, 118]

[a]MSH: MutS homolog; MLH: MutL homolog; PMS: postmeiotic segregation; XPA-XPG: xeroderma pigmentosum A-G; ATM: ataxia telangiectasia mutated.
[b]MMR: mismatch repair; NER: nucleotide excision repair; DSB: double-strand break.
[c]HNPCC: hereditary nonpolyposis colon cancer; XP: xeroderma pigmentosum; AT: ataxia telangiectasia.
[d]MSI: microsatellite instability.

provide evidence that plant MutSα is associated with the repair of UV-induced DNA lesions.

2.2. NER Pathway

2.2.1. Xeroderma Pigmentosum. Xeroderma pigmentosum (XP), meaning parchment pigmented skin, is a rare, autosomal inherited neurocutaneous disorder. XP patients are extremely sensitive to sun exposure (ultraviolet radiation, UV): 45% develop skin cancer, comprising mostly basal and squamous cell carcinomas, and to a lesser extent melanomas, angiomas, and sarcomas [47–49]. Besides skin cancers, 20% of the XP patients can develop progressive neurological disabilities. These patients are unable to repair UV-induced DNA damage because they are deficient in nucleotide-excision repair pathway (NER). Different genetic variants occur, thus patients are classified into eight complementation groups of XP named XP-A through XP-G for the respective mutated gene and XP-V for the variant form (Table 1). XP-C and XP-A are the most common complementation groups [50].

The NER pathway removes bulky DNA adducts, including cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidinone dimers (6-4 PPs) caused by UV radiation. The repair of DNA damage occurs through different steps: (i) damage recognition, (ii) assembly of a preincision complex, (iii) excision of the damaged strand, and (iv) gap-filling DNA synthesis. Two subpathways that differ in the initial damage recognition step operate in parallel. The global genome NER (GG-NER) removes lesions throughout the genome, while the transcription-coupled NER (TC-NER) functions on actively transcribed strands [51, 52].

The GG-NER subpathway involves recognition of the lesion by a heterotrimeric complex containing XPC, HR23B, and centrin together with damaged DNA-binding protein 1 and 2 (DDB1, DDB2, or XPE) [53]. The initial damage detection process involves cycles of XPC (together with DDB1 and DDB2) binding and dissociation from the DNA in search of structure distortions [54–57]. Once a lesion is encountered, XPC changes its conformation and binds DNA with single-stranded character opposite the lesion [56, 58–62].

The TC-NER involves recognition of the lesion through a stalled RNA-polymerase II, which triggers the recruitment of Cockayne syndrome type A (CSA), Cockayne syndrome type B (CSB), and XPA binding protein 2 (XAB2) to the damage [59, 63].

After DNA recognition, the ten-subunit complex transcription factor IIH (TFIIH, comprising XPD and XPB among others) is recruited and GG-NER and TC-NER converge into the same pathway. The DNA helicases XPB and XPD facilitate the partial unwinding of the DNA duplex leading to the recruitment of XPA, RPA, and XPG proteins and formation of a stable preincision complex around the damage site [64–69]. Then, ERCC1-XPF and XPG make DNA incisions, which result in the excision of a 24–32 nucleotide single-strand fragment containing the damaged site [70]. The gap formed is filled by DNA polymerases δ, ε, or κ and associated factors [71]. NER is completed by nick sealing by DNA ligase I or IIIα [72].

Plants have developed different strategies to counteract UV-induced DNA damage: the accumulation of UV absorbing flavonoids and related phenolic compounds in the upper epidermal layers of leaves [73–77], and the excision of UV-induced DNA adducts by photoreactivation or NER [2, 78–81]. Photoreactivation is mediated by photolyases. Arabidopsis contains two photolyase genes, the *UVR2* gene which encodes a CPD photolyase [82, 83], and the *UVR3* gene, which encodes a 6-4 PP photolyase [84]. In addition to this direct repair process, NER also contributes to maintain genome integrity in plants [85]. Orthologs of XPB and

XPD helicases have been isolated. Unlike other eukaryotic organisms, *A. thaliana* genome contains two *XPB* copies, named *XPB1* and *XPB2*, arranged as tandem repeats in head-to-tail fashion [86–89]. Expression of both genes varies with developmental stages and across tissues and is modulated by light [88]. *Atxpb1* mutant plants are viable but exhibit growth delay, lower seed viability, and loss of germination synchrony, indicating a partially functional redundancy of both XPB1 and XPB2 in DNA repair and transcription [87].

XPD was also shown to function in plant DNA repair. Liu et al. [90] characterized two Arabidopsis mutant lines. A T-DNA disruption of *XPD* was found to be homozygous lethal. However, plants harboring a point mutation in the *XPD* gene, which resulted in a substitution of a highly conserved glycine residue (G521E), are viable. These plants show growth defects, decreased UV resistance, and excision of UV photoproducts [90]. Results thus suggest that *XPD* gene is essential for plant development and is required for UV resistance.

DDB2 (XPE) is also critical for UV-B tolerance in plants. The transcript was reported to be expressed in rice and Arabidopsis proliferating tissues [91, 92] and anthers of Arabidopsis flowers [92]. These transcripts are rapidly induced after UV irradiation [91, 92]. Consistent with these results, *ddb2* mutant plants demonstrated a hypersensitivity to UV radiation [93, 94] and a dark repair deficiency of UV-induced DNA damage [94]. Combining the *ddb2* mutation with a CPD photolyase mutation (*uvr2*) further sensitized the plants to UV. These findings suggest the involvement of NER system besides photolyases for the repair of UV-induced DNA damage in plants.

Orthologs of human XPF (AtRAD1/UVH1) and XPG (AtRAD2/UVH3) endonucleases have also been characterized in Arabidopsis. *AtRAD1* transcript is expressed in all tissues but strongly accumulates in meristems and flowers [95, 96]. *AtRAD1* defective plants (*uvh1*) display a higher sensitivity to DNA damaging agents than wild-type [95, 97, 98]. More specifically, γ radiation of *uvh1* plants generated cell expansion but inhibited cell division [98]. This response was reported to be due to a G2-phase cycle arrest [97].

Finally, *AtRAD2* transcript appears to be ubiquitously expressed at moderate levels [99]. Plants deficient in *AtRAD2* (*uvh3* mutant) are substantially more UV-sensitive than the wild-type parent, exhibiting severe leaf yellowing and tissue damage after UV irradiation [99].

Overall, these findings indicate essential roles of XP genes in suppresing toxic effects of UV and another DNA-damaging compounds (Table 1). There are, however, differences among human and plant NER pathways. Arabidopsis has 2 copies of *XPB* and seems to lack *XPA*. In addition, *AtXPF* confers sensitivity to γ radiation while the corresponding human gene does not. Further studies are needed to understand NER pathway in higher plants.

2.3. Double-Strand Break Repair

2.3.1. Ataxia Telangiectasia. Ataxia telangiectasia (AT) is a rare human autosomal recessive neurodegenerative disorder that is characterized by ataxic movements due to cortical cerebellar degeneration and ocular and cutaneous telangiectasia (dilation of small blood vessels) [100, 101]. Other features of the disease include increased risk of cancer, with ~70% of malignancies being lymphomas and T cell leukemias [100], immunodeficiency [102], sterility, and extreme cellular and chromosomal sensitivity to ionizing radiation [103].

AT cells are defective in the ataxia telangiectasia-mutated (*ATM*) gene [101, 103, 104] (Table 1). ATM is a member of the family of phosphatidylinositol-3-OH-kinase-like kinases (PIKK) of serine/threonine protein kinases [105]. Activation in response to double-strand break (DSB) damage involves autophosphorylation and dimer dissociation [106, 107]. Activated ATM phosphorylates different downstream proteins involved in cell cycle arrest and/or apoptosis [100, 108]. Failure to activate ATM in response to DNA damage might attenuate repair and prevent apoptosis. This would then cause an accumulation of genetic lesions that eventually compromise cellular function and viability leading to neurodegeneration [109]. Recently, ATM has also emerged in the general response to reactive oxygen species [110–113].

Arabidopsis also possesses ATM orthologs [114]. *AtATM* is expressed ubiquitously at low levels, slightly higher in flower buds than in other tissues [114]. *atm* knockout mutants are particularly sensitive to DNA DSB induced by γ radiation, X-ray treatment, and radiomimetic agents (Table 1) [115, 116] and are defective in the transcriptional induction of genes involved in DNA metabolism, repair, chromatin, and chromosome structure in response to γ irradiation [115, 117, 118]. While ATM function appears to be conserved in plants and humans, the signal transduction pathways in these organisms are not precisely the same. Plants lack apoptotic counterparts of downstream regulators. Transmission of signal from ATM depends on a plant-specific transcription factor SOG1 (suppressor of gamma response 1) [119, 120]. Recent reports have demonstrated that Arabidopsis root and shoot stem cells undergo cell death as a downstream response to DNA damage mediated by *ATM* [116, 121].

Taken together, the above-cited studies demonstrate that the mechanisms connecting DNA damage to downstream effectors in plants do not mirror those in human cells. In fact, plants are continuously exposed to environmental mutagens; thus plants have evolved different strategies to sustain growth under genotoxic stress.

3. Mitochondrial Disorders

3.1. Friedreich's Ataxia. Friedreich's ataxia (FA) is an autosomal recesive disease in humans [122–124]. FA causes progressive cardio- and neurodegeneration as well as skeletal muscle abnormalities, increased risk of diabetes, and sometimes liver and renal failure [123, 125–128].

This disorder is caused by a GAA triplet expansion, and/or a point mutation in the FA gene, resulting in a deficiency in the expression of frataxin [122, 129, 130]. Frataxin is a nuclear-encoded mitochondrial protein highly conserved across the evolution and with homologues found in prokaryotes and eukaryotes (Figure 1). This protein is

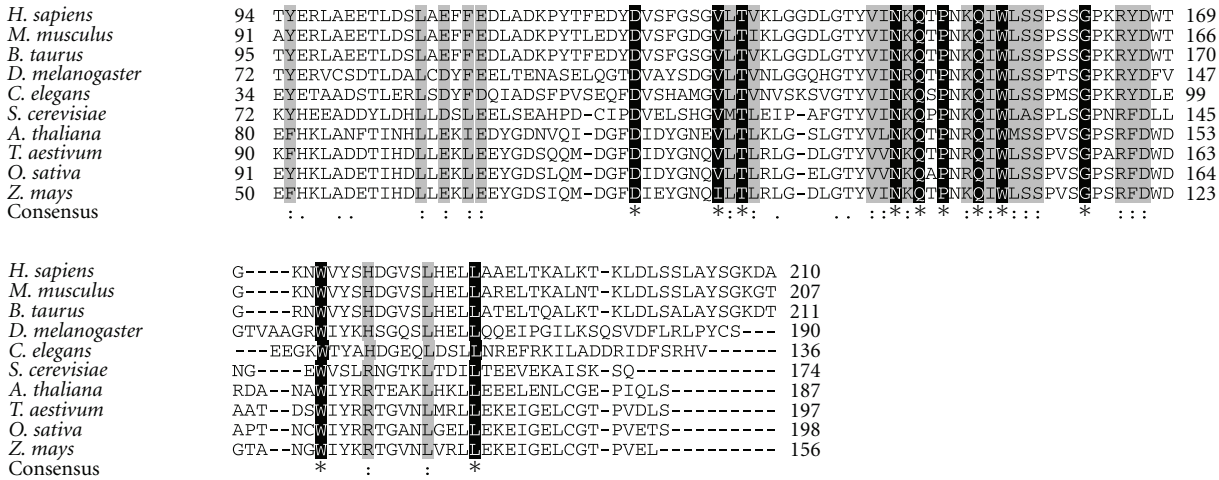

Figure 1: Sequence alignment of frataxin homologues from different organisms. The amino acid sequence of *Homo sapiens* (accession no. Q16595), *Mus musculus* (accession no. O35943), *Bos taurus* (accession number NP_001074196.1), *Drosophila melanogaster* (accession no. Q9w385), *Caenorhabditis elegans* (accession no. Q9TY03), *Saccharomyces cerevisiae* (accession no. Q07540), *Arabidopsis thaliana* (accession no. NP_192233.2), *Triticum aestivum* (accession no. CN010373), *Oryza sativa*, (accession no. BE040598), and *Zea mays* (accession no. CA830057) is shown. Alignment was performed by using the CLUSTALW2 method (Protein Weight Matrix Blosum, clustering NJ) (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Identical residues (*) are marked in black, and conserved substitutions (:) are shaded in gray.

predominantly expressed in tissues with a high energetic demand such as neurons and cardiac muscle [131, 132]. In addition, frataxin is highly expressed in flowers, a high energy demand tissue in plants [133]. The function of frataxin has not been established yet, but its deficiency was associated with oxidative stress, iron accumulation, decrease activities of several Fe-S containing proteins and a deficiency in oxidative phosphorylation [129, 134–140]. In addition, it was recently described that frataxin would participate in heme metabolism [141–143].

The high conservation of the structure of frataxin allowed the development of models using different organisms such as *Saccharomyces cerevisiae*, mouse, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Candida albicans*, *Escherichia coli*, and *Salmonella enterica* [126, 129, 144–148]. In addition, our group has developed two different Arabidopsis lines with reduced expression of frataxin; (i) *atfh-1*, a homozygous mutant line carrying the T-DNA in the 5′UTR region of the *AtFH* gene, which shows a decrease of about 50% in AtFH expression [140] and (ii) *as-AtFH*, an antisense line showing a decrease of about 70% in frataxin expression [142].

One of the proposed roles of frataxin is its involvement in the maturation of cellular Fe-S proteins. It has been described that the synthesis of Fe-S clusters requires a complex machinery and the participation of several genes [149, 150]. Most of these genes are conserved in bacteria, mammals, and yeasts. In addition, The presence of homologue gene sequences in photosynthetic organisms was recently identified, especially in mitochondria and plastids of Arabidopsis.

Several studies reported the role of yeast frataxin (Yfh1) in the assembly of Fe-S clusters, and the deficiency of frataxin results in decreased activity of Fe-S proteins such as aconitase and succinate dehydrogenase [135, 136]. Similar results were found in other models of frataxin deficiency such as knock out mice and cultured human cells [126]. Moreover, we recently found that the frataxin-deficient Arabidopsis lines *atfh-1* and *as-AtFH* also have less than 5% of total aconitase activity and also a decrease of about 40% in succinate dehydrogenase (SDH) activity. In addition, these plants show also increased ROS and Fe levels and upregulation of transcripts involved in ROS stress responses [140]. We have also reported that AtFH can participate in heme formation in plants. We found that as-AtFH line shows a decrease level of total heme and also shows downregulation of genes involved in heme metabolism such as HEMA1 and 2, GSA1 and 2, HEMB1 and 2, and AtFC-1 and 2. Furthermore, the deficiency of catalase activity was rescued with the addition of hemin [142]. These results are in agreement with those reported in neuronal cells about the hemin rescue of adrenodoxin, heme A levels, and cytochrome oxidase activity [151]. Taking together, these results allow us to propose Arabidopsis AtFH-deficient lines as interesting models to investigate the biogenesis of Fe-S clusters, Fe-S- and heme-containing proteins, as well as for better understanding the FA human disease.

3.2. Mitochondrial Respiratory Chain Diseases. CI Subunit Mutations. The mitochondrial respiratory chain is composed by about 90 proteins encoded by the nuclear genome and 13 proteins encoded by the mitochondrial DNA. These proteins are organized into five macromolecular complexes (CI to V) and play a central role in energy production, generating most of the cellular ATP [152, 153].

Most of the about 1500 mitochondrial proteins are nuclear encoded and participates in several pathways such as oxidative phosphorylation, Krebs cycle, fatty acid oxidation, heme and Fe-S groups synthesis, Fe and Ca homeostasis,

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                                  *
H. sapiens          107 WENPLMGWASTADPLSN 123
M. musculus         116 WENPLMGWASTADPLSN 132
B. taurus           107 WENPLMGWASTADPLSN 123
D. melanogaster     114 WENPLMGWASSGDPLSN 130
A. thaliana          84 WENPLMGWTSTGDPYAN 100
P. trichocarpa       88 WENPLMGWTSTGDPYAH 104
H. vulgare           86 WENPLMGWTSTGDPYAN 102
Z. mays              86 WENPLMGWTSTGDPYAN 102
O. sativa            88 WENPLMGWTSTGDPYAN 104
G. max               75 WENPLMGWTSTGDPYSH 89
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Figure 2: Sequence alignment of NDUFS4 homologues from different organisms showing the high conservation in the flanking region of D119. The amino acid sequence of *Homo sapiens* (accession no. NP_002486.1), *Mus musculus* (accession no. NP_035017.2), *Bos taurus* (accession no. DAA17925.1), *Drosophila melanogaster* (accession no. NP_573385), *Arabidopsis thaliana* (accession no. Q9FJW4), *Populus trichocarpa* (accession no. XP_002310893), *Hordeum* vulgare (accession no. BAK01929), *Zea mays* (accession no. NP_001132398), *Oryza sativa* (accession no. NP_001060126) and *Glycine max* (accession no. NP_001235335) are shown. Alignment was performed by using the CLUSTALW2 method (Protein Weight Matrix Blosum, clustering NJ) (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The conserved Asp residue is shown in grey.

aging and cell death, among others [152]. Defects in any of the different mitochondrial pathways can cause mitochondrial diseases. In most of these diseases, the muscle and cerebral function is affected, and because of this, disorders are known as mitochondrial encephalomyopaties [154].

In addition, it has been described that many mutations on the mitochondrial genome can cause a wide variety of clinical syndromes such as LHON (Leber's hereditary neuropathy), LS (Leigh's syndrome), MELAS (mitochondrial encephalomyopathy), sporadic anemia, encephalomyopathy, among others [154].

Due to the evolutionary conservation of several human proteins that are part of the respiratory complexes, it was possible to develop different models such as yeast mutants [155, 156] and *C. elegans* mitochondrial mutants for CI, CII, and CIII [157] to study the pathogenesis of mitochondrial dysfunction in humans. In addition, the presence of homologous proteins in Arabidopsis plants allows us to propose this as a model plant for the study of several mitochondrial diseases related to the mitochondrial respiratory chain dysfunction.

Dysfunction of CI of the mitochondrial respiratory chain is the most common enzyme defect of mitochondrial disorders, and it is involved in more than 30% of the hereditary mitochondrial encephalopaties, including Leigh's syndrome. [158, 159]. CI (NADH: ubiquinone oxidoreductase) is the largest complex of the OXPHOS system. Human CI contains at least 45 protein subunits, 7 encoded by the mitochondrial genome, and 28 by the nuclear DNA [160]. This complex is located on the mitochondrial inner membrane and catalyzes the oxidation of NADH by ubiquinone.

One of the most studied OXPHOS deficiencies is the mutation in the *NDUFS4* gene, a small 18 kD protein of CI highly conserved in different organisms. Mutations in *NDUFS4* result in a loss of the last 10–15 amino acids of its final fifth exon and lead to mitochondrial diseases such as Leber's hereditary optic neuropathy (LHON), Leigh's syndrome (LS), and mitochondrial encephalomyopathy, lactic acidosis, and stroke (MELAS). The mutation affects OXPHOS and metabolism by limiting respiratory substrates such as NADH due to the deficiency in CI function [161].

The first mutation found in *NDUFS4* was a 5-bp duplication in the ORF of the gene that impairs the phosphorylation of the protein leading to an inactivation of the complex [162, 163]. Other mutations in patients with Leigh syndrome were found in *NDUFS4* such as a homozygous G-A transition at nucleotide +44 of the coding sequence [164]. The G44A mutation results in the change of a TGG codon, which encodes for a tryptophan residue, to a TAA stop codon, which causes the premature termination of the protein, thus obtaining a truncated form of NDUFS4. A third mutation in *NDUSF4* was reported, a single-base deletion at position 289/290 [165]. Recently, two novel mutations in NDUFS4 causing Leigh syndrome has been reported [166]. One of these mutations, D119H, is in a conserved region of the protein. Interestingly, the D119 is highly conserved within human, mammals, nematodes, and plant species (Figure 2). All the mentioned mutations were found to be associated with a defect of the assembly of a functional complex in the inner mitochondrial membrane. These data suggest that NDUFS4 has an essential role in the structure and function of CI.

Recently, Meyer et al. [167] reported the characterization of an *ndufs4* mutant of *A. thaliana*. As mentioned above, AtNDUFS4 is highly conserved, showing a 41% identity respect to the human homolog. NDUFS4-deficient plants show low phosphorylation efficiency, sucrose-sensitive germination, delayed growth, a modified respiration pathway, and altered stress responses. The lack of CI has no major influence on the mitochondrial proteome or transcriptome but leads to a lowering of growth-related nuclear transcripts and clearly influences central metabolism [167]. In addition, the deletion of *NDUFS4* prevents the assembly of CI and alters the adenylate control of cellular metabolism without pleiotropic effects on other respiratory components [167]. Taken togheter, these observations show the essential role of the *NDUFS4* gene in the structure and function of CI. Moreover, due to evolutionary conservation of this protein, it is possible to use different models, including Arabidopsis, in order to better understand the mechanism of assembly of this respiratory complex, whose dysfunction is responsible for many mitochondrial human diseases.

4. Concluding Remarks

Plants have preserved most of the pathways essential for life and then represent complementary resources within human disease research. As described in this paper, observations demonstrate that plants encode orthologs of human proteins, which function in mechanisms reminiscent of those in other eukaryotes. Thus, plant research opens new areas regarding drug development and disease therapy, which are crucial to human health.

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Research Article

Difference between Pb and Cd Accumulation in 19 Elite Maize Inbred Lines and Application Prospects

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In the last two decades, the accumulation of heavy metal in crop grains has become the study hotspot. In this study, 19 representative elite maize inbred lines and 3 hybrid varieties were investigated at the seedling stage, which can accumulate Pb and Cd in the stems and leaves, respectively. The results demonstrated that significant differences are among inbred lines for accumulation of heavy metals, implying that the Cd accumulation is significant correlation between the male parents and their hybrids and some inbred lines have been selected for cross-breeding with low Pb or Cd accumulation, such as S37, 9782, and ES40; Moreover, some inbred lines could be suitable for phytoremediation species for soil bioremediation with high levels of Pb and Cd accumulation, including 178, R08, 48-2, and Mo17ht.

1. Introduction

Heavy metal pollution has become a major global problem, which threatens the environment and human life by its toxicity [1]. And it is becoming more seriously along with urbanization, industrialization, and modern agricultural activities. Heavy metals do not easily degrade or volatilize owing to their stable physical and chemical properties. Therefore, it has led to more serious and possibly irreversible pollution resulting from increasing heavy metals accumulation in soil every year [2]. Among these heavy metals, lead (Pb) and cadmium (Cd) are the most harmful and ubiquitous in everywhere. According to the recent statistics, the area of farming land (contaminated with Cd and Pb) is nearly 2.0×10^7 hm^2 in China, which accounts for about 1/5 of the total area of that in China [3]. Pb and Cd can enter into soil, and then transferred to crops by the food chain, which will impose a threat to the health of humans and livestock eaten by them [4–7].

The Chinese Academy of Sciences and Southwest Agricultural University have conducted an investigation of wheat grains in the Liangfeng irrigated area, Beijing, and the results showed that although the concentration of heavy metal accumulation in grains was much lower than that in roots and leaves, the overstandard concentration of Pb and Zn (Zinc) in wheat grains was still 28.16% and 4.18%, respectively. The concentrations of Hg (mercury) and Cd met the standard, and some of the samples have been close to the upper limits of national food hygiene standard [8]. Lucho-Constantino et al. [9] found that crops showed higher concentrations of Cd and Pb that had been irrigated with wastewater for 20 years than the standard concentrations of those in The Netherlands and Germany. Currently, foods, such as vegetables and fruits, produced in industrial and

mining areas of China, which contain high levels of Pb and exceed standard values and even near to the critical values, so it has imposed a great threat to the safety of agricultural products [10–14]. In order to reduce the hazard of foods produced in soil with Pb and Cd concentration, a large number of studies have been conducted worldwide to restore the soil by treatment with hyperaccumulation plants. However, most hyper-accumulation plants are characterized by low biomass, long remediation cycle, and narrow biological adaptability, and insufficient to meet the demands of large-scale applications.

Compared with low-biomass plants, maize is more advantageous for restoring soil with heavy metals pollution [6, 15]. Maize is one of the most important economically food crops in china, and the uptake of Pb or Cd among the maize cultivars can affect the quality of food and fodder. 19 elite maize inbred lines were used as materials in this experiment, which has represented the different heterotic groups and been widely used in commercial breeding in China, including PA, Lancaster, BSSS (Reid), Sipingtou, Luda Red Cob, and PB, of which, 3 hybrid maize varieties that have been widely grown in southwest China. And then, differences of Cd and Pb accumulation and correlations between the Cd concentration of the hybrid varieties and their male parents were analyzed in different maize germplasms. At last, several maize germplasms were selected for future breeding that accumulate low levels of Cd or Pb and identified for new phytoremediation species with higher biomass and better ecoadaption for bioremediation of soil with Pb and Cd pollution that accumulate high levels of Cd and Pb.

2. Materials and Methods

2.1. Soil Preparation. Soil samples were taken from Duoying district, Ya'an, and treated by air drying and then digested with HNO_3-HF-H_2O_2 acids system. The Pb concentration in soil was 60.92 mg·kg^{-1}, which was within the range of grade II of the National Soil Environmental Quality Standard (GB15618-1995). Meanwhile, the Cd concentration was 3.23 mg·kg^{-1}, which was higher than grade III standard limits. Measurements of Pb and Cd were conducted by a flame atomic absorption spectrophotometer (SHIMADZU AA-6600, Japan) in soil samples.

2.2. Maize Collection. 19 maize inbred lines and three hybrid varieties were selected for the experiment; all inbred lines and hybrid varieties were provided by the Maize Research Institute of Sichuan Agricultural University (Table 1).

2.3. Experimental Methods. Soil was put into some plastic pots and each pot with 15 kg soil, with a diameter of 22 cm and a depth of 20 cm, and then divided into four groups: (1) high Pb, (2) high Cd, (3) low Pb, and (4) low Cd. High Pb soil contained Pb concentration (750 mg·kg^{-1}) and high Cd soil contained Cd concentration (30 mg·kg^{-1}), respectively, which was created by evenly mixing a certain amount of $Pb(NO_3)_2$ or $CdCl_2$ $2.5H_2O$ solution with the soil. Low Pb and Cd soil were untreated. Each group was repeated three times, for a total of 171 pots. And three concentration levels were set for each hybrid varieties: (1) Cd 15 mg·kg^{-1}, (2) 30 mg·kg^{-1} and (3) 50 mg·kg^{-1}. Each level was repeated three times, for a total of 27 pots.

The seeds of inbred lines and hybrid varieties were planted in seedling trays and fully saturated with water. When each seed sprouted and grown 2-3 leaves, then the seedlings with similar growth trend will be transferred into plastic pots, with three plants in each pot and arranged in a triangular pattern. Soil was watered and loosened at regular intervals to keep it wet and breathing freely. In this experiment, 12 g of compound fertilizer was applied four times to each pot according to weather conditions and the growth condition of the plants, which contains N, P, and K concentrations of 13.5%, 11.0%, and 9.5%, respectively.

2.4. Sampling and Analysis. Samples were taken after 70 days and washed thoroughly with tap water to remove soil and dirt, then washed with demonized water three times. Plantlets were divided into roots and aboveground parts. Samples were first dried for 3 days at room temperature, then in an oven at 80°C until the weight remained unchanged. Finally, the dried samples were crushed and put into sealed bags for analysis.

During the experiment, all containers were cleaned with tap water, 5% HNO_3, and deionized water. Then, 0.5 g of each sample was taken and digested with HNO_3-$HCLO_4$ (guaranteed reagent, 4 : 1) and subjected to flame atomic absorption spectrophotometry (SHIMADZU AA-6600, Japan) to determine the concentrations of Pb and Cd.

Microsoft Excel 2003 and SPSS 13.0 were used for data analysis.

3. Results and Analysis

3.1. The Concentrations of Pb and Cd in Roots and Aboveground Parts of Inbred Lines. As shown in Table 2, the Pb concentration range and the average value at low-Pb conditions in roots were 0–11.5 mg·kg^{-1} and 3.63 mg·kg^{-1}, respectively, and 0–54.0 mg·kg^{-1} and 28.1 mg·kg^{-1} in stems and leaves, respectively. Of which, 3 low-accumulation inbred lines (S37, 9782, and ES40) and 4 high-accumulation inbred lines (178, R08, 48-2, and Mo17ht) were obtained. Moreover, the range and average value of Pb concentration in roots were 51.6–312.8 mg·kg^{-1} and 161.5 mg·kg^{-1}, and 0–222.3 mg·kg^{-1} and 89.1 mg·kg^{-1}, in stems and leaves under high-Pb conditions, respectively. Of those inbred lines, S37 was the lowest-accumulation inbred line obtained under high-Pb conditions, but no high-accumulation inbred line was obtained. It may be noted that the translocation coefficients of most of inbred lines were greater than 1 at low-Pb conditions, which indicates that the Pb translocation of maize plants is efficient under such a pollution level (Table 3). As shown in Tables 2 and 3, although the Pb concentration in roots, stems, and leaves were increasingly rapidly, the translocation efficiency decreased considerably, it is probably that it is caused by the self-protection mechanism of maize inbred lines under high-Pb conditions.

Table 1: Inbred lines and hybrid varieties.

S/N	Description	Remarks	Heterotic groups
1	S37	Inbred line	PA
2	Shen 137	Inbred line	PB
3	Zheng 58	Inbred line	BSSS (Reid)
4	9782	Inbred line	BSSS (Reid)
5	Qi 319	Inbred line	PB
6	Golden 96C	Inbred line	Lancaster
7	178	Inbred line	PB
8	ES40	Inbred line	PA
9	R15	Inbred line	Lancaster
10	P138	Inbred line	PB
11	R08	Inbred line	BSSS (Reid)
12	Moqun 17	Inbred line	Lancaster
13	RP125	Inbred line	BSSS (Reid)
14	Huangzao 4	Inbred line	Sipingtou
15	Mo17ht	Inbred line	Lancaster
16	48-2	Inbred line	PA
17	R18	Inbred line	PB
18	Chang 7-2	Inbred line	Sipingtou
19	Zong 31	Inbred line	Luda red cob
20	Chuandan no. 14	Hybrid variety (R08 × 21-es)	
21	Chuandan no. 10	Hybrid variety (48-2 × R18)	
22	Yayu no. 2	Hybrid variety (S37 × 7922)	

Under low-Cd conditions, the Cd concentration range and average value of inbred lines were 0–12.0 $mg \cdot kg^{-1}$ and 2.83 $mg \cdot kg^{-1}$ in roots, and 0–1140 $mg \cdot kg^{-1}$ and 1.34 $mg \cdot kg^{-1}$, in stems and leaves respectively. However, no Cd was detected in the stems and leaves, indicating that the aboveground parts of maize are less polluted by Cd under such conditions. At high-Cd conditions, the Cd concentration range and average value were 21.7–175.1 $mg \cdot kg^{-1}$ and 83.2 $mg \cdot kg^{-1}$ in roots and 0–614 $mg \cdot kg^{-1}$ and 22.4 $mg \cdot kg^{-1}$ in stems and leaves, respectively. Three low-accumulation inbred lines (Golden 96C, R08, and Huangzao 4) and 2 high-accumulation inbred lines (Zheng 58 and 9782) were obtained (Tables 1 and 2).

3.2. Cd Concentrations in Roots and Aboveground Parts of Hybrid Varieties. Cd has been recognized as a trace toxic heavy metal and should be monitored closely. In order to investigate whether low Cd accumulation in inbred lines is related to the parents, we conducted a comparison of (Chuandan no. 14, Chuandan no. 10, and Yayu no. 2) to their male parents (R08, 48-2, and S37, resp.). It is shown that the Cd concentrations in stems and leaves of Chuandan no. 14 were lower than those of the other two hybrid varieties under the three treatment levels (listed in Table 4).

4. Discussion

The accumulation and translocation coefficient of Pb in three inbred lines was as follows in descending order: R08, S37, and 48-2. It is similarly in the stems and leaves of varieties, such as Chuandan no. 14, Yayu no. 2, and Chuandan no. 10, It is demonstrated that a significant correlation of Cd concentrations was between hybrid varieties and their male parents.

However, Pb could be maintained at low levels in grains of maize under high-Pb conditions (595.6 $mg \cdot kg^{-1}$), and the concentrations were much lower than nutritional organs [16]. It is demonstrated that the stems and leaves of maize plants are easily polluted with Pb; it will pose a hazard for livestock, especially cattle as as silage fodder. Therefore, the selection of maize germplasms with low Pb accumulation in stems and leaves is very important. In this experiment, we obtained three low-Pb inbred lines under low-Pb conditions, S37, 9782, and ES40. Under high-Pb conditions, the Pb concentration in stems and leaves of the other inbred lines was increasing greatly; with the exception of S37, it remained at 0.00 $mg \cdot kg^{-1}$, indicating that the Pb translocation of S37 is inefficient and stable. Therefore, S37 can be assumed to be a satisfactory inbred line. Due to the highly homozygous genome and high combining ability of maize inbred lines, S37, 9782, and ES40 may be used to cross-breed for low-Pb varieties in stems and leaves.

We obtained three inbred lines (Golden 96C, R08, and Huangzao 4)with low-Cd accumulation in stems and leaves under high-Cd conditions,. The Cd concentration in the stems and leaves of Golden 96C and R08 was 0.00 $mg \cdot kg^{-1}$ respectively, but 0.26 $mg \cdot kg^{-1}$ in Huangzao 4. Therefore, these three inbred lines may be satisfactory as

Table 2: Pb and Cd concentrations in roots and aboveground parts of inbred lines ($mg \cdot kg^{-1}$).

Inbred line number	Cd				Pb			
	Roots		Stems and leaves		Roots		Stems and leaves	
	Low	High	Low	High	Low	High	Low	High
1	0.00 ± 0.00^{f}	175.09 ± 9.54^{a}	0.00 ± 0.00^{e}	8.42 ± 0.16^{l}	11.55 ± 0.03^{a}	150.18 ± 0.22^{de}	0.00 ± 0.00^{i}	0.00 ± 0.00^{g}
2	0.00 ± 0.00^{f}	86.43 ± 1.77^{cde}	0.00 ± 0.00^{e}	61.38 ± 0.63^{a}	7.16 ± 0.29^{b}	211.84 ± 0.09^{bc}	46.88 ± 1.59^{b}	115.63 ± 5.35^{bc}
3	11.68 ± 0.21^{a}	47.34 ± 1.94^{ghi}	11.40 ± 0.66^{a}	24.86 ± 1.23^{h}	0.00 ± 0.00^{f}	102.26 ± 0.10^{fg}	5.78 ± 0.28^{h}	13.47 ± 2.79^{efg}
4	6.99 ± 0.33^{c}	43.21 ± 0.18^{hi}	4.09 ± 0.12^{c}	46.26 ± 0.50^{c}	1.92 ± 0.08^{e}	136.34 ± 3.02^{ef}	0.00 ± 0.00^{i}	7.72 ± 1.60^{fg}
5	2.85 ± 0.45^{d}	75.02 ± 0.86^{defg}	0.00 ± 0.00^{e}	51.04 ± 0.05^{b}	5.77 ± 0.26^{c}	109.22 ± 4.47^{fg}	13.43 ± 0.45^{g}	40.47 ± 0.87^{defg}
6	0.00 ± 0.00^{f}	118.86 ± 1.27^{b}	0.00 ± 0.00^{e}	0.00 ± 0.00^{o}	3.86 ± 0.41^{d}	51.64 ± 1.47^{h}	7.84 ± 0.19^{h}	67.17 ± 1.79^{cdef}
7	11.05 ± 0.93^{a}	60.15 ± 12.17^{efgh}	8.74 ± 0.31^{b}	17.84 ± 0.26^{j}	0.00 ± 0.00^{f}	212.03 ± 8.62^{bc}	53.31 ± 2.49^{a}	127.19 ± 4.40^{bc}
8	12.03 ± 0.03^{a}	122.86 ± 8.85^{b}	0.00 ± 0.00^{e}	25.58 ± 0.70^{gh}	5.76 ± 0.37^{c}	82.04 ± 5.55^{fg}	0.00 ± 0.00^{i}	94.52 ± 4.56^{bcd}
9	0.00 ± 0.00^{f}	97.59 ± 0.60^{bcd}	0.00 ± 0.00^{e}	12.08 ± 0.48^{k}	3.72 ± 0.16^{d}	199.19 ± 1.78^{bc}	37.06 ± 3.53^{cd}	94.71 ± 3.57^{bcd}
10	0.00 ± 0.00^{f}	57.29 ± 11.82^{efgh}	1.18 ± 0.26^{d}	26.78 ± 0.43^{g}	0.00 ± 0.00^{f}	217.71 ± 1.20^{b}	20.41 ± 0.01^{f}	126.43 ± 0.73^{bc}
11	0.00 ± 0.00^{f}	79.53 ± 2.05^{cde}	0.00 ± 0.00^{e}	0.00 ± 0.00^{o}	0.00 ± 0.00^{f}	186.20 ± 9.09^{bc}	48.02 ± 0.31^{b}	72.44 ± 0.51^{cde}
12	9.24 ± 0.06^{b}	21.73 ± 0.27^{i}	0.00 ± 0.00^{f}	22.57 ± 0.79^{i}	11.07 ± 0.09^{a}	109.46 ± 8.61^{fg}	31.48 ± 3.94^{e}	77.77 ± 12.34^{bcd}
13	0.00 ± 0.00^{f}	50.75 ± 0.29^{fgh}	0.00 ± 0.00^{f}	34.20 ± 0.14^{e}	0.00 ± 0.00^{f}	186.27 ± 0.68^{bc}	31.41 ± 0.15^{e}	104.31 ± 13.15^{bcd}
14	0.00 ± 0.00^{f}	124.02 ± 18.88^{b}	0.00 ± 0.00^{f}	0.26 ± 0.03^{no}	0.00 ± 0.00^{f}	312.80 ± 6.54^{a}	33.48 ± 2.26^{de}	141.31 ± 3.50^{b}
15	0.00 ± 0.00^{f}	49.09 ± 3.33^{ghi}	0.09 ± 0.01^{e}	38.97 ± 0.59^{d}	0.00 ± 0.00^{f}	148.95 ± 10.17^{de}	54.02 ± 0.48^{a}	79.92 ± 4.74^{bcd}
16	0.00 ± 0.00^{f}	101.94 ± 6.20^{bcd}	0.00 ± 0.00^{f}	17.88 ± 0.56^{j}	0.00 ± 0.00^{f}	201.17 ± 11.31^{bc}	53.99 ± 0.28^{a}	127.71 ± 0.24^{bc}
17	0.00 ± 0.00^{f}	103.67 ± 7.45^{bc}	0.00 ± 0.00^{f}	32.62 ± 0.37^{f}	0.00 ± 0.00^{f}	124.80 ± 8.47^{ef}	38.81 ± 3.09^{c}	72.73 ± 12.80^{cde}
18	0.00 ± 0.00^{f}	87.75 ± 1.38^{cde}	0.00 ± 0.00^{f}	1.79 ± 0.51^{n}	6.80 ± 0.03^{b}	180.72 ± 13.20^{cd}	37.22 ± 0.08^{cd}	107.89 ± 11.01^{bc}
19	0.00 ± 0.00^{f}	77.86 ± 0.47^{cdef}	0.00 ± 0.00^{f}	3.58 ± 0.39^{m}	11.41 ± 0.92^{a}	144.99 ± 1.12^{de}	19.98 ± 0.42^{f}	222.28 ± 16.63^{a}
AVG $mg \cdot kg^{-1}$	2.83	83.17	1.34	22.43	3.63	161.46	28.06	89.14
C.V. (%)	162.91	44.04	239.30	81.95	118.18	37.39	68.63	59.15

Values followed by same letters are not significantly different at ($P < 0.01$). Means ±SE, $n = 3$.

Table 3: Accumulation coefficient and translocation coefficient of inbred lines.

Variety number	Cd-stressed		Pb-stressed		Pb-contrast	
	Accumulation coefficient	Translocation coefficient	Accumulation coefficient	Translocation coefficient	Accumulation coefficient	Translocation coefficient
1	0.28	0.05	0.00	0.00	0.00	0.00
2	2.05	0.71	0.15	0.54	0.77	6.54
3	0.83	0.53	0.02	0.13	0.09	∞
4	1.54	1.07	0.01	0.06	0.00	0.00
5	1.70	0.68	0.05	0.37	0.22	2.33
6	0.00	0.00	0.09	1.30	0.13	2.03
7	0.59	0.30	0.17	0.60	0.88	∞
8	0.85	0.21	0.13	1.15	0.00	0.00
9	0.40	0.12	0.13	0.48	0.61	9.96
10	0.89	0.47	0.17	0.58	0.34	∞
11	0.00	0.00	0.10	0.39	0.79	∞
12	0.75	1.04	0.10	0.71	0.52	2.84
13	1.14	0.67	0.14	0.56	0.52	∞
14	0.01	0.00	0.19	0.45	0.55	∞
15	1.30	0.80	0.11	0.54	0.89	∞
16	0.60	0.18	0.17	0.63	0.89	∞
17	1.09	0.31	0.10	0.58	0.64	∞
18	0.06	0.02	0.14	0.60	0.61	5.47
19	0.12	0.04	0.30	1.53	0.33	1.75
AVG	0.75	0.38	0.12	0.59	0.46	—

∞: Pb was nonreadout in roots; Cd-contrast was not listed for nonreadout of Cd in roots and aboveground parts of most inbred lines under contrast condition.

Table 4: Cd Concentrations in roots and aboveground parts of hybrid varieties ($mg \cdot kg^{-1}$).

Hybrid varieties	Treatment ($mg \cdot kg^{-1}$)	Concentration roots ($mg \cdot kg^{-1}$)	Concentration stems and leaves ($mg \cdot kg^{-1}$)
Chuandan no. 14	15	18.96 ± 0.66	0.17 ± 0.005
Chuandan no. 10		31.16 ± 4.81	8.19 ± 1.03
Yayu no. 2		8.79 ± 1.48	5.89 ± 0.56
Chuandan no. 14	30	35.24 ± 1.74	11.98 ± 0.66
Chuandan no. 10		139.36 ± 6.40	19.40 ± 0.76
Yayu no. 2		4.89 ± 1.65	15.10 ± 2.02
Chuandan no. 14	50	72.94 ± 5.26	9.95 ± 0.61
Chuandan no. 10		63.48 ± 6.35	24.82 ± 3.53
Yayu no. 2		97.80 ± 7.52	23.89 ± 3.80

Means ±SE; $n = 3$.

parents for low-Cd breeding. Moreover, Chuandan no. 14 is undoubtedly suitable for planting in Cd-polluted soil as a low-Cd variety.

Plant rehabilitation technology is widely advocated all over the world. It is reported that hyper-accumulation plants have high accumulation coefficient and translocation coefficient, but limitation of the capacities of heavy metal absorption as their low biomass, slow growth, and weak ecoadaption. So such plants have not been applied on a large scale for breeding. At present, attention has been focused on those plants with wider biological adaptability and higher aboveground biomass. As shown in Table 3, though Pb concentration was low in soil, maize inbred lines have also shown a strong Pb translocation ability and the Pb translocation coefficients. The Pb accumulation coefficients of 178, R08, 48-2, and Mo17ht on growth day 70 were 0.86, 0.79, 0.89, and 0.89, respectively, and the growing period of maize was generally 140 days, implying that the accumulation coefficients would be greater than 1 at maturity. Therefore, the 4 inbred lines obtained the two major features of hyper-accumulation plants [17]; making them suitable phytoremediation species for restoring soil slightly polluted by Pb. Under high Pb conditions, the absorption and translocation ability of maize plants decreased largely, probably due to the self-protection mechanism of maize inbred lines. The accumulation coefficients of 19 inbred lines were 0.00–0.30, and the translocation coefficients were also less than 1, with only several exceptions. Therefore, we concluded that maize was incompetent for restoring soil that was severely polluted with Pb. Similarly, Zheng 58 and 9782 might be useful phytoremediation species for restoring soils that were either slightly or severely polluted with Cd. Although the accumulation coefficient and translocation coefficient of maize were lower than those of some reported small hyper-accumulation plants, maize possesses other advantages, such as higher biomass in aboveground parts, better ecoadaption, short growing period, easy cultivation,

and accumulation of a minimum of two heavy metals (Pb and Cd), making maize a suitable phytoremediation plant for restoring Cd-polluted soil and also soil slightly polluted with Pb.

5. Conclusion

There are significant differences of Pb and Cd accumulation in interspecific hybrid of zea mays, so it is feasible to select maize germplasms of low-Pb and low-Cd accumulation in stems and leaves. And three low-Pb inbred lines are obtained under low-Pb conditions, such as S37, 9782, and ES40 in this research and may be used to cross-breed for low-Pb varieties in stems and leaves. On the contrary, those, with high-target metal concentrations, can play an important role in the treatment of polluted soils. However, future studies are required to identify the stability of heredity of the traits in maize germplasms, S37 can be assumed to be a satisfactory inbred line.

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Review Article

Metabolic Engineering of Yeast and Plants for the Production of the Biologically Active Hydroxystilbene, Resveratrol

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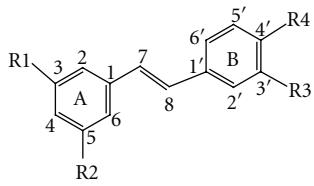
Resveratrol, a stilbenic compound deriving from the phenyalanine/polymalonate route, being stilbene synthase the last and key enzyme of this pathway, recently has become the focus of a number of studies in medicine and plant physiology. Increased demand for this molecule for nutraceutical, cosmetic and possibly pharmaceutic uses, makes its production a necessity. In this context, the use of biotechnology through recombinant microorganisms and plants is particularly promising. Interesting results can indeed arise from the potential of genetically modified microorganisms as an alternative mechanism for producing resveratrol. Strategies used to tailoring yeast as they do not possess the genes that encode for the resveratrol pathway, will be described. On the other hand, most interest has centered in recent years, on *STS* gene transfer experiments from various origins to the genome of numerous plants. This work also presents a comprehensive review on plant molecular engineering with the *STS* gene, resulting in disease resistance against microorganisms and the enhancement of the antioxidant activities of several fruits in transgenic lines.

1. Introduction

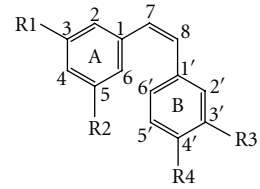
Hydroxystilbenes (hereafter referred to as stilbenes) are natural phenolic compounds occurring in a number of plant families including Vitaceae, Dipterocarpaceae, Gnetaceae, Pinaceae, Poaceae, Fabaceae, Leguminoseae, and Cyperaceae [1]. Although polyphenolic compounds display an enormous chemical diversity, stilbenes seem to constitute a rather restricted group of molecules, the skeleton of which is based on resveratrol especially in Vitaceae and Fabaceae (Figure 1) [2]. Resveratrol is one of the most extensively studied natural products, doubtless for its large spectrum of biological activities in human health as a cardioprotective, an antitumor, a neuroprotective, and an antioxidant agent. Some of the resveratrol's properties have been associated with the benefits of a moderate consumption of red wine. Many roles have also been ascribed to resveratrol and its derivatives (Figure 1) in plants; namely, they constitute antimicrobial, deterrent or repellent compounds acting as allelochemicals or phytoalexins, protecting them from attacks by fungi, bacteria, nematodes, or herbivores [3–5].

According to these potential activities in plants and humans, the interest for resveratrol has increased. Currently, rising demand for resveratrol and derivatives for nutraceutical, cosmetic, and putatively pharmaceutical uses makes their production a necessity. Metabolic engineering for resveratrol production thus has significant commercial value. As all the genes encoding enzymes responsible for resveratrol biosynthesis have been cloned and characterized in detail, this makes molecular engineering of this compound relatively straightforward.

Most interest has now centered upon metabolic engineering of resveratrol in plants with the objectives of increasing tolerance of the latter to pathogenic microorganisms and



	R1	R2	R3	R4
1	OH	OH	H	OH
2	OH	GlcO	H	OH



	R1	R2	R3	R4
3	OH	OH	H	OH
4	OH	GlcO	H	OH

Figure 1: Molecular structures of *trans*- and *cis*-resveratrol 1 and 3, and *trans*- and *cis*-piceid, 2 and 4, respectively (GlcO = β-D-glucose).

improving the nutritional quality of food products through the expression of pharmaceutically active compounds in plants incapable of synthesizing resveratrol.

Microorganisms can also be used to heterologously express the resveratrol biosynthetic pathway to obtain this compound in valuable amounts. In this paper, we will discuss the potential of tailored microorganisms, specifically yeast, and plants for resveratrol production.

2. Resveratrol in Health and Disease

Resveratrol was identified in 1940 as a constitutive compound of the roots of white hellebore (*Veratrum grandiflorum*) [6]. Resveratrol is also present in high amounts in the roots of *Polygonum cuspidatum* used in traditional Chinese and Japanese medicine, and this compound was acclaimed for its wondrous effects for the treatment of human fungal diseases (suppurative dermatitis, gonorrhea favus, and athlete's foot), hyperlipidemia, atherosclerosis, or inflammations. Moreover, resveratrol recently has been shown to be a potent therapeutic agent [7–10].

First, there is considerable evidence that resveratrol acts both as a free radical scavenger [11, 12] and a potent antioxidant doubtless for its ability to promote the activities of numerous antioxidative enzymes [12, 13]. Resveratrol inhibits lipid peroxidation which is an indicator of possible free radical damage to cellular membranes [14, 15]. Resveratrol may operate in a number of antioxidant mechanisms leading to the development of atherosclerosis, protecting, for example, LDL molecules against peroxidation [16–18] or reactive oxygen species production by blood platelets [19]. There is however some evidence that resveratrol exhibits prooxidant activity under certain experimental conditions (in the presence of copper ions) [20], causing oxidative DNA damage that may lead to cell cycle arrest or apoptosis [21].

Resveratrol is also considered as an antiproliferative agent for cancer [22], exerting an antitumor activity either as a cytostatic or as a cytotoxic agent in various types of cancer. Resveratrol induces cell death through positive induction of death receptor-mediated apoptosis [23–25], mitchondria-mediated apotosis [26], and nuclear (transcription) factor-mediated apotosis [27, 28].

Refractory disease and poor prognosis in many tumors have been related with high expression of antiapoptotic molecules such as survivin; resveratrol was proven to act at this level through survivin depletion [29]. Similarly, numerous works showed that resveratrol is a potent inhibitor of cell cycle progression, causing G1 phase arrest [30], inhibiting G0-G1 transition in human lymphocyte [31] or perturbing progression through S and G2 phases in cultured bovine artery endothelial cell proliferation [32]. Since the pioneering work of the group of Pezzuto [33], affording for the very first time evidence for the cancer chemoprotective activity of resveratrol on HL-60 and Hepa 1clc7 cells, many other models of human cancers were used, confirming these findings. The most frequently described mode of action for resveratrol concerns apoptosis (see above), the response depending on the expression of the tumor suppressor gene p53. A number of related factors can be modulated by resveratrol, such as activation of caspases, decreases in the antiapoptotic proteins Bcl-2 and Bcl-x^L [34, 35], increases in the proapoptotic proteins Bax [36], inhibition of cyclins and cyclin-dependent kinases, that is, proteins implicated in cell cycle progression [32, 37], and interference with nuclear transcription factor kappa B (NF-κB)—and activator protein 1 (AP-1)—mediated cascades.

Most importantly, there are several works concerning both the antitumor effects and the antitumor mechanisms of resveratrol *in vivo*. In several studies, it was shown, namely, that resveratrol inhibits the development of skin cancer *in vivo* by topical applications, causing a significant reduction of the tumor diameter and the tumor incidence [33, 38, 39]. Moreover, resveratrol significantly inhibits the UV-B-mediated increase in skin thickness and skin edema [40]. On the other hand, resveratrol was proven not to be very effective in inhibiting the progression of leukemia *in vivo*, even when high doses were used [41], despite its antileukemic activity *in vitro* and though resveratrol can be converted to the known antileukemic related stilbene, piceatannol [42]. In regard to breast cancer, resveratrol was shown to reduce N-methyl-N-nitrosourea-induced mammary tumorigenesis in female rats at high doses (100 mg/kg body weight) though being uneffective at lower doses (10 mg/kg body weight) [43]. Otherwise, some authors have shown that resveratrol glucosides, namely, piceid (a 3-O-β-D-resveratrol glucoside), administered orally or intraperitoneally to mice, reduced tumor volume, tumor weight, and metastasis in lung carcinoma [44, 45]. A few investigations about the antitumor effects

of resveratrol and derivatives against liver cancers have been reported to date, showing that this compound administered orally or intraperitoneally to rats caused a 25% reduction in tumor cell numbers and restrained hepatoma cell invasion but not proliferation [46, 47]. Finally, orally administration of resveratrol to mice was shown to prevent colonic tumor formation and reduce small intestinal tumors by 70% [48].

Neurologic benefits of resveratrol described experimentally concern the following diseases: cerebral ischemia in rats [49, 50], amyotrophic lateral sclerosis [51], Parkinson's disease in rats [52], spinal cord lesions in rabbits [53], brain edema and tumors in human cells [54], seizure in rats [55–57], pain and cognitive impairment in rats [58, 59]. According to Doré [60], the neuroprotective effects of resveratrol could be mediated by regulation of the heme oxygenase antioxidant systems in neurons, especially in case of age-related vascular dementia and Alzheimer's disease [61].

There are several mechanisms to support resveratrol as a cardioprotective agent such as inhibition of LDL peroxidation, reduction of the degree of neointimal hyperplasia and restenosis, inhibition of platelet aggregation together with chemoprevention of atherosclerogenesis. Resveratrol was proven to inhibit LDL peroxidation *via* antioxidant and free scavenging activities in *ex vivo* rat heart studies [62] and circumstantial evidence of its potent role in preventing atherosclerogenesis could thus be given [63, 64]. Resveratrol was shown to inhibit neointimal hyperplasia in several animal studies and vascular smooth muscle cell proliferation in a rabbit model of restenosis [65, 66] and to block platelet aggregation from high-cholesterol-fed rabbits [67].

Resveratrol exerts some activities against a range of bacteria affecting humans (*Chlamydia pneumonia*, the cause of human acute respiratory tract infections [68], and *Helicobacter pylori*, the main agent responsible for chronic gastritis and peptic ulcer disease [69, 70]). Resveratrol also inhibited the growth of several bacteria known to be major agents of human skin infections such as *Staphylococcus aureus*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa* [71]. Similarly, resveratrol completely inhibits the growth of *Neisseria gonorrhoeae* (responsible for the sexually transmitted disease gonorrhoea) [72]. Stilbenes generally also have biocidal activities against plant fungal pathogens (namely, *Botrytis cinerea*, the causal organism for gray mold, *Pyricularia oryzae*, the agent of pyriculariosis, *Plasmopara viticola*, the causal agent for downy mildew [73–77], fungi associated with esca of grapevine [78]), and human fungal pathogens such as *Candida albicans*, an agent of candidiasis [79].

Finally, resveratrol has been shown to extend the lifespan of lower organisms, yeast [80], and metazoans [81], *via* the sirtuin/Sir2 family. More recently, a study has confirmed the conservation of these preventive and protective mechanisms controlling lifespan extension in higher organisms such as mice [82]. Using a similar approach, other works reached the same conclusions with regard to the protective effects of resveratrol against diet-induced obesity and insulin resistance [7, 82].

Otherwise, DNA-damaging products can induce premature senescence in cancer cells, limiting tumor development. However, senescent cancer cells may reenter the cell cycle and lead to tumor relapse. Recently, resveratrol was remarked by its ability to induce DNA damage in cancer cells. In fact, resveratrol suppressed viability and induced DNA damage in human head and neck squamous carcinoma cells [83]. Similarly, human squamous cancer cells treated with resveratrol were shown to express oxidative stress-mediated DNA damage [84].

3. Biosynthesis of Resveratrol

Stilbene phytoalexins, as flavonoid-type phytoalexins, are formed on the phenylalanine/polymalonate route, being the last step of this biosynthesis pathway catalyzed by stilbene synthase (STS) (Figure 2). *Trans*-resveratrol can be synthesized either starting with phenylalanine or from tyrosine, both pathways giving rise to *para*-coumaric acid through, respectively, the phenylalanine ammonia lyase (PAL) and the cinnamate 4 hydroxylase (C4H), or directly through the tyrosine ammonia lyase (TAL). A *para*-coumaric-acid coenzyme A ligase (4CL) transforms the *para*-coumaric acid into *para*-coumaroyl-CoA, which then leads to *trans*-resveratrol after condensation with three molecules of malonyl-CoA through STS activity [85]. STS belongs to the so-called type III of the polyketide synthase enzyme superfamily, a class of enzymes which carry out iterative condensation reactions with malonyl-CoA. [86, 87]. Resveratrol synthesis is induced in plants as a response to fungal infection [73, 74, 88], abiotic stresses (UV irradiation, metallic salts, methyl jasmonate) [89–93] as well as to natural compounds eliciting plant defense responses [94, 95] or nonpathogenic rhizobacteria [96, 97].

STS is encoded by a multigene family mainly comprising the resveratrol-forming *STS* genes from grapevine (*pSV21*, *pSV25*, *pSV696*, *pSV368*, and *StSy*) [98], (*Vst1*, *Vst2*, *Vst3*) [99], the *AhRS* gene from *Arachis hypogea* [100], and an STS-encoding gene from *Parthenocissus henryana* [101]. Other *STS* genes have also been isolated from pine [102–104], together with a stilbene synthase gene from *Vitis riparia* cv Gloire de Montpellier [105]. The *Sb STS1* gene isolated from *Sorghum* is at present the only one *STS* gene described in a Monocotyledonous plant [106]. Knowledge of the resveratrol biosynthetic route thus paves the way for metabolic engineering in microorganisms or plants.

4. Tailoring Yeast for Resveratrol Biosynthesis

Microorganisms are widely used biological systems whose engineering may be useful for the production of numerous valuable molecules and can lead to interesting applications for example in the wine industry [107–109]. Engineering bacteria or yeast for resveratrol might thus represent a valuable means of its production in large quantities. Their tailoring is necessary because microorganisms do not possess the genes that encode for the resveratrol pathway. In this paper, we will focus only on yeast tailoring for resveratrol production. Two main strategies have already been used to that end: (i) introducing the entire biosynthetic pathway

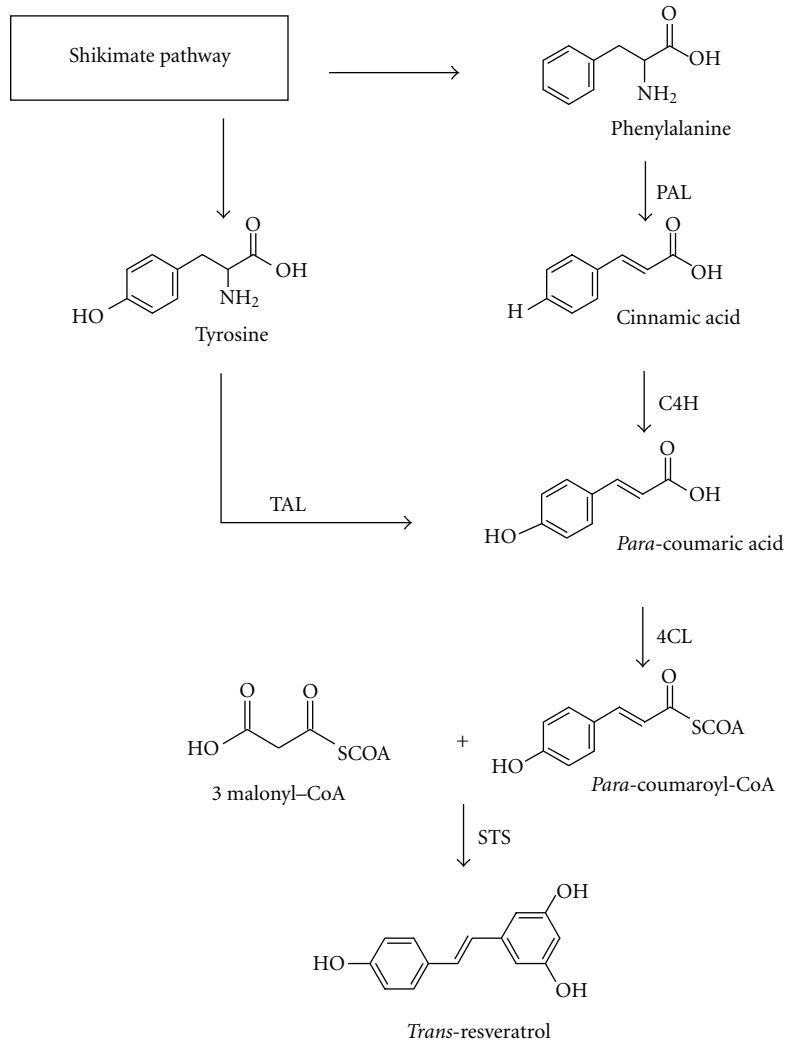


Figure 2: Biosynthesis of resveratrol via the phenylalanine/polymalonate pathway. PAL, phenylalanine ammonia lyase; TAL, tyrosine ammonia lyase; C4H, cinnamate-4-hydroxylase; 4CL, *para*-coumaric acid: coenzyme A ligase; STS, stilbene synthase.

using aromatic amino acids as substrates (L-phenylalanine or L-tyrosine) [110–113] and (ii) introducing specific genes, such as *para*-coumaroyl-CoA ligase and stilbene synthase, starting with *para*-coumaric acid as a substrate (Figure 2) [114–120].

4.1. Engineering the Entire Pathway. To obtain resveratrol from its precursors L-phenylalanine or L-tyrosine on the phenylpropanoid route appears to be the most promising option in terms of production cost. The entire resveratrol pathway has been introduced successfully into the oleaginous yeast *Yarrowia lipolytica* (ATCC 20362 strain) with the genes encoding for phenylalanine/tyrosine ammonia lyase (PAL/TAL), cinnamate-4-hydroxylase (4CH), *para*-coumaroyl-CoA ligase (4CL), and stilbene synthase (STS) activities [110] (Table 1). Metabolic engineering was completed by constitutive expression of malonyl CoA, a precursor for both naringenin-chalcone, the first C15 intermediate in the flavonoid route, and stilbene synthesis. Genes that encode for each required enzyme from different plant species (grapevine, peanuts) have been tested. In the best performing yeast, genes that encode for the PAL and TAL from *Rhodotorula glutinis*, the 4CL from *Streptomyces coelicolor*, and an STS from *Vitis* sp. have been used, and the resulting production of resveratrol reached in this system 1.46 mg/L [110].

Current efforts to increase resveratrol bioproduction in yeast have focused, so far not surprisingly, on heterologous expression of the genes encoding for the enzymes involved in the phenylpropanoid pathway and for STS, in baker yeast *Saccharomyces cerevisiae* [111, 113–120]. Namely, the entire resveratrol pathway has been introduced in *S. cerevisiae* (CEN.PK113-5D strain), but also in molds such as *Aspergillus niger* (FGSC A913 strain) and *A. oryzae* (MG1363 strain). In these cases, pathway expression started with the PAL enzyme. The ability of tailored *S. cerevisiae* to produce resveratrol was characterized, but it was not synthesized in measurable amounts in this system. [111]. More recently,

Table 1: Metabolic engineering of resveratrol in yeast.

Microorganisms/species	Introduced gene(s)	Origin of genes	Resveratrol quantity	References
Yeast *Yarrowia lipolytica*	*PAL/TAL, C4H*, *4CL*, *STS*	*Rhodotorula glutinis* (*PAL/TAL*), *Streptomyces coelicolor* (*4CL*), *Vitis* sp. (*STS*)	1.46 mg/L	[110]
Yeast *Saccharomyces cerevisiae*	*PAL, C4H*, *4CL*, *STS*	*Arabidopsis thaliana* (*PAL, C4H, 4CL*), *Rheum tataricum* (*STS*)	not detectable	[111]
Yeast *Saccharomyces cerevisiae*	*TAL, 4CL*::*STS fusion protein*	*Rhodobacter sphaeroides* (*TAL*), *Arabidopsis thaliana* (*4CL*), *Vitis vinifera* (*STS*)	5.25 mg/L	[116]
Yeast *Saccharomyces cerevisiae*	*4CL*, *STS*	*Populus trichocarpa*×*Populus deltoides* (*4CL*), *Vitis vinifera* (*STS*)	1.45 mg/L	[114]
Yeast *Saccharomyces cerevisiae*	*4CL*, *STS*	*Nicotiana tabacum* (*4CL*), *Vitis vinifera* (*STS*)	5.8 mg/L	[115]
Yeast *Saccharomyces cerevisiae*	*4CL1*, *STS*	*Arabidopsis thaliana* (*4CL*), *Vitis vinifera* (*STS*)	262–391 mg/L	[117]
Yeast *Saccharomyces cerevisiae* with phenylalanine	*PAL, CPR, C4H, 4CL*, *STS*	*Populus trichocarpa*×*P. deltoides* (PAL, CPR[a]) *Glycine max* (*C4H, 4CL*), *Vitis vinifera* (*STS*)	0.29 mg/L	[113]
Yeast *Saccharomyces cerevisiae* with *para*-coumaric acid	*PAL, CPR, C4H, 4CL*, *STS*	*Populus trichocarpa*×*P. deltoides* (*PAL, CPR*) *Glycine max* (*C4H, 4CL*), *Vitis vinifera* (*STS*)	0.31 mg/L	[113]
Yeast *Saccharomyces cerevisiae*	*TAL, 4CL*::*STS fusion protein, araE transporter*	*Rhodobacter sphaeroides* (*TAL*), *Escherichia coli* (*araE*), *Arabidopsis thaliana* (*4CL*), *Vitis vinifera* (*STS*)	3.1 mg/L 1.27 mg/L (without the araE transporter)	[119]
Yeast *Saccharomyces cerevisiae*	*TAL, 4CL*::*STS fusion protein*	*Rhodobacter sphaeroides* (*TAL*), *Arabidopsis thaliana* (*4CL*), *Vitis vinifera* (*STS*)	14.4 mg/L	[120]

[a] CPR: Cytochrome P450 Reductase.

Trantas et al. [113] constructed the complete resveratrol biosynthetic pathway in *S. cerevisiae* to produce resveratrol from phenylalanine. When the medium was supplemented with 10 mM of phenylalanine, the strain produced 0.29 mg/L *trans*-resveratrol after about 120 h of cultivation. One can thus admit that the introduction of the complete resveratrol pathway in yeast leads to a low production of this compound when compared to some other data obtained in engineered bacteria where resveratrol synthesis can reach up to 100–170 mg/L [121, 122].

4.2. Introducing Selective Genes. An alternative strategy to engineering the entire pathway is directed towards transforming microorganisms with selective genes [114–120] (Table 1). The yeast strain *S. cerevisiae* FY23 was transformed with both the *4CL* and *STS* genes under utilization of *para*-coumaric acid as a precursor (added to the culture medium). However, in this system, resveratrol production remained low (1.45 mg/L) [114]. Interestingly, in another study, authors reported that transformation of *S. cerevisiae* (CEN.PK113-3b strain) with the *4CL* gene from tobacco and the *STS* gene from grapevine enabled it to produce resveratrol in higher quantities (5.8 mg/L) [115]. A *S. cerevisiae* (WAT11 strain) cotransformed with 4CL and STS constructs and fed with *para*-coumaric acid only produced 0.65 mg/L resveratrol, but, when the 4CL and the STS-encoding genes that were added to the yeast genome were submitted to protein fusion, yeast expressing 4CL::STS fusion protein exhibited a 10-fold increase in resveratrol production (5.25 mg/L resveratrol) compared to the coexpression of 4CL and STS [116]. This underlines the importance of the spatial localization of these two related enzymes [116]. In another study of the same group [120], authors have utilized a synthetic scaffold to recruit the 4CL1 and STS enzymes of the resveratrol pathway to improve resveratrol production in *S. cerevisiae*. A 5-fold improvement of the resveratrol production was obtained over the nonscaffolded control, and a 2.7-fold increase (14.4 mg/L within 96 h incubation) was finally observed over the previous reported study with protein fusion [116]. This work clearly demonstrated that synthetic scaffolds can be used for the optimization of engineered metabolic pathway.

Most importantly, it has recently been shown that yeast cells expressing *4CL*, *STS* genes together with the *araE* gene encoding for a high-capacity *Escherichia coli* transporter, but with no affinity for resveratrol itself, could enhance resveratrol accumulation. Yeast cells carrying the *araE* gene produced up to 3.1 mg/L, that is, 2.44-fold higher resveratrol than the control cells [119]. Such an engineered yeast was also proven to increase the resveratrol content in a white wine during the fermentation process [119].

It should be noted that the efficacy of recombinant microorganisms for resveratrol production depends on various factors, such as the species and the strain, the origin of the transferred genes, culture conditions as well as other parameters such as plasmids or precursors used (Table 1). A recent study [117] has shown that fermenting yeast expressing the *para*-coumaroyl-coenzyme A ligase (4CL1) from *Arabidopsis thaliana* and the stilbene synthase from *Vitis vinifera* in a rich medium could considerably improve resveratrol production, rising from a few milligrams per liter to 262 mg/L in rich medium using a laboratory strain.

Moreover, resveratrol amounts reached up to 391 mg/L when fermentation was achieved with an industrial Brazilian sugar cane yeast [117].

Taken together, these data indicate that the levels of resveratrol that can be produced by microorganisms remain low, although optimization of the processes might be possible.

5. Tailoring Plants for Resveratrol Biosynthesis

As resveratrol is a potent phytoalexin against plant pathogens and can enhance plant resistance to microbial disease, earlier applications of resveratrol engineering focused primarily on this antimicrobial potential [123, 124]. Tailoring plants for resveratrol synthesis thus constitutes the second aspect of this paper. Particular attention will be given in this section to the choice of the promoters and the enhancer elements used to improve STS transcriptional activity in the transgenes as well as the biological benefits of resveratrol production in terms of enhancement of antioxidant activity in fruits and legumes.

We have seen that tailoring yeast needs the introduction of the entire resveratrol pathway (requiring at least 4 or 5 genes) or the introduction of selective genes (requiring at least 2 genes) depending on the precursor used, phenylalanine or *para*-coumaric acid. Tailoring plants for resveratrol synthesis appear to be very simple, since STS is a key enzyme of resveratrol synthesis utilizing as substrates precursor molecules that are present throughout the plant kingdom. The introduction of a single gene is thus sufficient to synthesize resveratrol in heterologous plant species. A lot of transformations were then operated to investigate the potential of stilbene biosynthetic genes to confer resistance to pathogens or to increase their nutritional values [123, 124].

5.1. Production of Resveratrol in Transgenic Plants: Gene and Promoter Options. The first gene transfer experiment was performed by the group of Kindl with a complete *STS* gene from *Arachis hypogea* introduced into tobacco [100], leading to resveratrol accumulation in response to short-wavelength ultraviolet light. This experiment was then continued by the same group by the transfer of two grapevine *STS* genes, *Vst1* and *Vst2*, in tobacco, conferring to the plant a higher resistance to *Botrytis cinerea* infection. This work constitutes the first report of a disease resistance resulting from foreign phytoalexin expression in a novel plant [125]. Since this pioneering work, *STS* genes have been transferred to a number of plants, including rice [126], barley and wheat [127–130], alfalfa [131], kiwifruit [132], grapevine [133, 134] apple [135, 136], aspen [137], papaya [138], white poplar [139], oilseed rape [140], banana [141], *Rehmannia* [142] tomato [143–147], *Arabidopsis* [148], lettuce [101], pea [149], and hop [150] (Table 2).

In grapevine, genome sequencing has revealed a large array of *STS* genes, with 43 genes identified and 20 of these being shown to be expressed [151]. But to date, only a few *STS* genes from grapevine are used for the metabolic engineering of plants, being *Vst1* and *Stsy* the most commonly genes chosen (Table 2). STS-encoding genes from other plants have also been used, notably the *AhRS* gene from *Arachis hypogea* [100, 131], the *SbSTS1* gene from *Sorghum bicolor* [106, 148], and an STS-encoding gene from *Parthenocissus henryana* [101]. Chimeric genes or a combination of two STS encoding genes, based on *Vst1* and *Vst2*, can increase significantly resveratrol production in the transformed lines [128, 152].

On a practical point of view, the modulation of gene expression is mainly controlled by the promoter chosen to drive the transgene. By now, a limited number of promoters (the constitutive promoter pCaMV35S, its own stress-responsive promoter pVst1, the fungus-inducible promoter pPR10.1 or the tissue specific promoter p-nap) have been used for the expression of STS-encoding genes upon plant transformation (Table 2 and references therein). As expected, the pCaMV35S promoter, which is the most commonly used to overexpress a transgene in plants [152], triggered strong and constitutive stilbene accumulation (Table 2). In this case, stilbene synthesis is higher than that observed with inducible promoters, but, as a consequence, causes a drastic depletion of the endogenous pools of precursors. In grapevine, the pVst1 promoter which is induced either by biotic factors (pathogens, elicitors) or abiotic stresses (wounding, UV light), allows high stilbene production without interfering with secondary biosynthetic pathways. Generally, it appears thus preferable to transform plants with a construct having a pathogen-inducible promoter in order to avoid depletion of other pathways.

Expression of the *STS* gene may be optimized by the utilization of enhancer elements [127, 130] and/or heterologous promoters [131–135, 140, 152] to improve STS transcription. Combination of the pVst1 promoter with the 35S enhancer element [127, 130] or use of a chimeric promoter resulting, for example, from a fusion between the alfalfa pPR10.1 promoter and the pVst1 promoter can lead to higher expression of the transgene without affecting promoter inducibility or specific expression patterns [127], or to an increased production of resveratrol upon fungal infection (reaching 5–100-fold the levels found in nontransgenic leaves [133]). Finally, tissue-specific promoters such as the p-nap seed-specific napin promoter can be used to induce stilbene production [140].

At this stage of the discussion, it appears important to underline that the choice of the promoter for *STS* gene expression should be performed depending on the expected results: when the enhancement of plant resistance against pathogens through resveratrol production is searched, thus one can recommend the choice of a strong constitutive promoter or a pathogen-inducible promoter (see above). As far as may concern this review, when the improvement of food products is sought through, for example the increase of the antioxidant activity of the transformed plants, a tissue-specific [140, 147] or inducible promoter would be a better option.

STS genes were transferred to plants by means of *Agrobacterium* spp. in tomato [143], grapevine [133], kiwifruits [132] or particle bombardment in barley and wheat [127–129]. However, as epigenetic modifications may occur and lead to expression variability, decreasing the ability

Table 2: Metabolic engineering of stilbene synthase in plants, and resulting effects on stilbene levels, resistance to pathogens, and antioxidant activities.

Plant/species	Introduced gene(s)	Promoter	Produced stilbene(s)	Stilbene quantity (mg/kg of FW)	Biological activity	References
Tobacco (*Nicotiana tabacum L.*)	*Arachis hypogea STS*	Stress-induced promoter	Resveratrol	—	—	[100]
	Grapevine Vst1 and Vst2	Stress responsive pVst1	Resveratrol	400	Resistance to *Botrytis cinerea*	[125]
	Chimeric *STS* gene	Constitutive CaMV 35S	Resveratrol	50 to 290	Altered flower morphology, male sterility	[152]
Rice (*Oryza sativa L.*)	*Grapevine Vst1*	Stress responsive pVst1	—	—	Resistance to *Pyricularia oryzae* ?	[126]
Wheat (*Triticum aestivum L.*)	*Grapevine Vst1*	Combination pVst1 +35S enhancer	—	—	Resistance to *Botrytis cinerea*	[127, 129]
	Chimeric *STS* gene	Maize ubiquitin promoter	Resveratrol	2	—	[128]
	Grapevine Vst1 and *Vst2*	Combination pVst1 +35S enhancer	Unknown derivative stilbene compounds	35 to 190	Resistance to *Puccinia recondita* and *Septoria nodorum*	[130]
Barley (*Hordeum vulgare L.*)	*Grapevine Vst1*	Combination pVst1 +35S enhancer	—	—	Resistance to *Botrytis cinerea*	[127]
Alfalfa (*Medicago sativa L.*)	*Arachis hypogea* STS gene *(AhRS)*	Constitutive CaMV 35S	Piceid	0.5 to 20	Resistance to *Phoma medicaginis*	[131]
Arabidopsis thaliana L.	*Sorghum SbSTS1*	Constitutive CaMV 35S	Piceid	584	—	[106, 148]
Kiwi (*Actinidia deliciosa*)	*pSV25*	Constitutive CaMV 35S	Piceid	20 to 182	No resistance to *Botrytis cinerea*	[132]
Grapevine (*Vitis vinifera L.*)	*Grapevine Vst1*	Fungus inducible *ms* PR 10.1	Resveratrol		*In vitro* resistance to *Botrytis cinerea*	[133]
	Vitis pseudoreticulata STS	Constitutive CaMV 35S	Resveratrol	2.586	Not determined	[134]
Apple (*Malus domestica*)	*Grapevine Vst1*	Stress responsive pVst1	Unknown resveratrol-glycoside	—	—	[135]
	Grapevine Vst1	Stress responsive pVst1	Piceid	3 to 7 for non-UV-irradiated fruit and 23 to 62 for UV-irradiated fruit	No influence on other phenolic compounds	[136]
Tomato (*Lycopersicon esculentum Mill.*)	*Grapevine Vst1* and *Vst2*	Stress responsive pVst1	Resveratrol	—	Resistance to *Phytophthora infestans* No resistance to *Botrytis cinerea* and *Alternaria solani*	[143]
	Grapevine StSy	Constitutive pCaMV 35S	Resveratrol and piceid	4 to 53	Antioxidant primary metabolism and increase in total antioxidant activity	[144]

Table 2: Continued.

Plant/species	Introduced gene(s)	Promoter	Produced stilbene(s)	Stilbene quantity (mg/kg of FW)	Biological activity	References
	Grapevine StSy	Constitutive pCaMV 35S	Resveratrol and piceid	0.1 to 1.2	Enhancement of natural antiradical properties	[145]
	Grapevine StSy	Constitutive pCaMV 35S	Resveratrol and piceid	0.42 to 126 depending on the stage of ripening and fruit samples	Differences in rutin, naringenin, and chlorogenic acid contents	[146]
	Grapevine StSy	Fruit-specific promoter TomLoxB	Resveratrol and piceid		Increases in total antioxidant capability and ascorbic acid content	[147]
Rehmannia glutinosa Libosch.	*Arachis hypogea AhRS3*	Constitutive pCaMV 35S	Resveratrol and piceid	22 to 116 up to 650 with stress treatment	Antioxidant capabilities Resistance to *Fusarium oxysporum*	[142]
Lettuce (*Lactuca sativa L.*)	*Parthenocissus henryanaSTS*	Constitutive pCaMV 35S	Resveratrol	56.4	Effect on Hela cell morphology	[101]
Pea (*Pisum. sativum L.*)	*Grapevine Vst1*	Stress responsive pVst1	Occurrence of two resveratrol-glucoside compounds	0.53 to 5.2	—	[149]
White poplar (*Populus alba L.*)	*Grapevine StSy*	Constitutive pCaMV 35S	Piceid	309 to 615	No *in vitro* resistance to *Melampsora pulcherrima*	[137, 139]
Papaya (*Carica papaya L.*)	*Grapevine Vst1*	Stress responsive pVst1	Resveratrol glucoside	54	Resistance to *Phytophthora palmivora*	[138]
Oilseed rape (*Brassica napus L.*)	*Grapevine Vst1*	Tissue specific p-nap	Resveratrol glucoside	361 to 616	Food quality improvement: high piceid rate content and reduction of sinapate esters	[140]
Hop (*Humulus lupulus L.*)	*Grapevine Vst1*	Constitutive pCaMV 35S	Piceid, unknown stilbene astringin, resveratrol	490 to 560	Higher amounts of flavonoids and acids	[150]

of the transgenes for stilbene synthesis, the selection of plants with a single gene insertion will be more appropriate. As a consequence, the use of *Agrobacterium*-mediated transformation, which leads to lower transgene insertion numbers, should preferentially be chosen [124].

5.2. Stilbene Production and Biological Benefits of Resveratrol Synthesis in STS Engineered Plants. Expression of *STS* genes resulted in resveratrol accumulation in transgenic lines. The obtained stilbene amounts are generally higher than those reached in engineered yeast, ranging from a few mg/kg fresh weight to hundreds of mg/kg fresh weight (see Table 2). Both free resveratrol and its glycosylated forms can be recovered in plant extracts [144]: piceid, a 3-O-β-D-resveratrol glucoside, occurred in different plant species transformed with *STS* genes (Table 2) [106, 132, 136, 139, 140, 142, 145, 146, 148, 150]. However, levels of accumulated stilbenes depend on the plant species (probably because of different endogenous pools of enzymes or precursors, as well as differences in secondary metabolism pathways), the promoter used (constitutive or inducible promoters), the ripening stage in case of transgenic fruits [136, 144] and the age of the organs [125, 132].

From a practical viewpoint, the ectopic production of resveratrol observed in transformed plants can lead to broad-spectrum resistance against fungi in transgenic plants, but

disparate effects were observed (Table 2). Some works indeed reported transformations improving the resistance of rice to *Pyricularia oryzae* [126], tomato to *Phytophthora infestans* [143], barley and wheat to *B. cinerea* [127, 129], wheat to *Oidium tuckeri* [127, 129], alfalfa to *Phoma medicaginis* [131] and papaya to *Phytophthora palmivora* [138]. Conversely, in some others, no resistance was observed after transforming the plants with stilbene synthase genes. For example, transformation of white poplar (*Populus alba*) with *STS*, leading to the accumulation of piceid, does not confer any increased resistance to rust disease (*Melaspora pulcherrima*) [137, 139]. Similarly, no increased resistance against *B. cinerea* has been observed in STS transgenic kiwi plants [132].

With regard to the improvement of the nutritional value of agricultural crops and fruits, there are already several studies reporting an increase of the antioxidant activities in transgenic tomatoes and apples overexpressing STS-encoding genes [136, 144, 147]. In tomato, for example, resveratrol accumulation in transgenic fruit increased their global antioxidant activities, as well as their contents in other well-known antioxidants such as ascorbic acid and glutathione [144]. Antioxidant activity, as a consequence of resveratrol accumulation, was also shown to suffer a two-fold increase in transgenic tomato fruit *versus* controls [145]. Moreover, a correlation was found between resveratrol concentrations and antioxidant activities in ripe and unripe fruits [145]. More recently [147], tomato plants expressing a stilbene synthase gene *(StSy)* under the control of a fruit-specific promoter (promoter TomLoxB) were shown to accumulate resveratrol and piceid in the skin of the mature fruits, being the resveratrol content of the plants transformed with the specific promoter TomLoxB 20-fold lower than that of plants previously transformed with the constitutive pCaMV35S promoter [144]. However, both the total antioxidant capability and the ascorbic acid content were increased in the transformed fruits. These results explain the higher capability of transgenic fruits to counteract the proinflammatory effects of phorbol ester in monocyte–macrophages *via* the inhibition of induced cyclooxygenase-2 enzyme [147]. This last example constitutes a nice illustration of what can be expected from molecular engineering of resveratrol in plants in terms of improvement of the nutritional value of fruits or food products.

6. Conclusions

Increased demand for resveratrol for nutraceutical, cosmetic and possibly pharmaceutic uses makes its production from sustainable sourcing a necessity. In this context, the use of biotechnology through recombinant microorganisms and plants is particularly promising [5, 112, 118]. Interesting results can indeed arise from the potential of genetically modified microorganisms as an alternative mechanism for producing resveratrol, as this compound can be synthesized directly in recombinant yeast (the subject of the present review) but also in bacteria, such as *Escherichia coli* [112, 118]. Use of recombinant bacteria or yeast is of interest for the food industry, which could produce resveratrol in large quantities in biofermentators. Tailoring yeast can also receive direct applications in winemaking, as for example, fermentation engineering to produce resveratrol in wine (or to increase the wine resveratrol content) [119]. Otherwise, a transgenic yeast expressing a gene for a glycosyl hydrolase capable of liberating free resveratrol from its glucoside form has been reported as well to increase resveratrol amounts in wine [153] or the wine-related antioxidant content [114]. Beside the fact that disease resistance can be obtained following expression of STS-encoding genes, molecular engineering of plants with resveratrol may also lead to food products comprising edible legumes, cereals, or fruits, which can be ingested with their potential clinical benefits, by humans. Taken together, these results suggest the overall relevance of metabolic engineering of resveratrol.

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