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博 士 学 位 论 文

双孢蘑菇耐热分子机理研究及相关基因的
克隆与功能验证

Study on the thermotolerance mechanisms of *Agaricus bisporus*
as well as the cloning and functional verification of related genes

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缩略语

缩写	英文全称	中文全称
2-DE	Two-dimensional electrophoresis	双向电泳
CAT	Catalase	过氧化氢酶
COG	Clusters of Orthologous Groups of proteins	蛋白质直系同源簇
EST	Expression sequence tags	表达序列标签
GERAD	Glycoprotein ER-associated degradation	糖蛋白内质网降解
H2DCFDA	Carboxy- 2,7-dichlorodihydrofluorescein diacetate	2',7'-二氯二氢荧光素二乙酯
<i>Hpb</i>	Hydrophobin gene	疏水蛋白基因
<i>Hph</i>	Hygromycin phosphotransferase gene	潮霉素磷酸转移酶基因
ORF	Open reading frame	开放阅读框
<i>Pab</i>	<i>para</i> -Aminobenzoic acid synthase gene	对氨基苯甲酸合酶基因
PABA	<i>para</i> -Aminobenzoic acid	对氨基苯甲酸
<i>Ppi</i>	Peptidyl-prolyl cis-trans isomerase gene	脯氨酸顺反异构酶基因
PTGS	Post-transcriptional gene silencing	转录后基因沉默
RPKs	Receptor protein kinases	受体蛋白激酶
RACE	Rapid amplification of cDNA ends	cDNA 末端快速扩增
ROS	Reactive oxygen species	活性氧族
SA	Salicylic acid	水杨酸
SAGE	Serial analysis of gene expression	基因表达系列分析
SAR	Systemic Acquired Resistance	系统获得性抗性
SSH	Suppression Subtractive Hybridization	抑制性消减杂交

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摘要

高温胁迫 (Heat stress, HS)指生物体处于超过其适宜温度范围的环境下所受到的一种非生物胁迫 (Abiotic stress), 它能破坏生物体正常的细胞动态平衡, 导致生物体严重的生长、发育停滞, 甚至死亡。随着温室效应的影响和全球变暖的趋势, 高温胁迫以及与之紧密关联的干旱、渗透等胁迫已经成为导致全球性农作物减产的主要原因。本文主要研究高温胁迫对双孢蘑菇的损害, 以及耐温双孢蘑菇菌株 02 对高温的响应和抵御机制; 同时探讨了应用分子生物学和基因工程技术培育耐高温双孢蘑菇品种的可能性。

双孢蘑菇 [*Agaricus bisporus* Lange (Imbach)]是具有高经济价值和生态意义的栽培食用菌。双孢蘑菇属稳温结实性的菌株, 温度是影响其生长的主要因素, 因此培育种性优良的耐高温菌株是促进产业发展的根本。本课题选取同一家系内遗传背景最为接近的两株双孢蘑菇—02 菌株和 8213 菌株 (8213 菌株是常规栽培菌株, 其只能在 16-22℃ 下栽培, 而 02 菌株是显著耐温菌株, 其菌丝体在 33℃ 下仍然能保持正常状态) —作为比较研究的对象, 探索生物体自身对高温胁迫的响应及防御机制。通过转录组学的分析, 我们构建了两个热激转录差减文库, 富集了 197 个耐温相关的差异 EST 片段。经过进一步的序列比对分析和功能注释、聚类, 结果显示差异基因主要为: 分子伴侣类、能量及物质代谢类、信号转导类、损伤修复类等。结合双孢蘑菇热激蛋白质组学的研究结果, 我们描绘了双孢蘑菇耐温菌株 02 自身的高温胁迫抵御网络模型。这个模型揭示了热激信号转导, 核酸损伤修复, 以及蛋白质修复和周转三大系统在生物体高温抵御过程的角色和协同作用, 将热激后单个基因、蛋白质的表达变化与生物体系统整体的胁迫动态平衡有机联系在一起。高温抵御模型的提出不仅丰富了我们的高等真菌耐高温机制和通路的认识, 而且为进一步的机理研究提供了一个清晰的提纲。

在高温抵御模型及耐温基因热激时空转录谱的基础上, 我们克隆了处于网络节点位置的 15 个重要耐温相关 EST 的全基因序列。我们应用逐渐完善的转基因技术, 通过转基因过表达和反向遗传学抑制基因表达的方法在双孢蘑菇及模式物种拟南芥中开展耐高温表型功能验证。结果显示, 有 4 个基因的过表达对高温敏感的双孢蘑菇 8213 的耐温特性起到了明显的促进作用, 它们是对氨基苯甲酸合

成酶基因 (*Pab*)、脯氨酸顺反异构酶基因 (*Ppi*)、疏水蛋白基因 (*Hpb*)和 *Calnexin* 基因, 在反向遗传学研究中, 我们发现抑制疏水蛋白基因 (*Hpb*)表达起到了明显的降低 *O2* 菌株耐高温特性的作用, 反向的结果印证了 *Hpb* 基因在促进双孢蘑菇 *O2* 高温耐受性上的突出作用; 拟南芥中的表型功能验证显示 *Pab*、*Ppi*、*Hpb* 以及一种免疫调节蛋白能够显著增强野生型拟南芥幼苗的基础耐温性。

我们在拟南芥中研究了 PABA 合成酶的耐高温机制。在表型上, 外源直接添加 PABA (1-5 $\mu\text{mol/L}$)能够直接促进拟南芥的耐温性; 同时, 内源过表达 PABA 合成酶的转基因植株 (35S:*AbPabase*)的基础耐温性也明显优于野生型植株。通过深入研究, 我们发现热激能够促进拟南芥 H_2O_2 产生, 同时转基因植株 (35S:*AbPabase*)中的本底 H_2O_2 水平显著高于野生型。此外, 转基因植株 (35S:*AbPabase*)中热休克蛋白表达水平也显著上调, 这一现象同样出现在生长于外源添加 PABA (5 $\mu\text{mol/L}$)的野生型拟南芥小苗中。从以上结果中, 我们认为在担子菌和高等植物中存在一条“PABA- H_2O_2 -Peroxide signaling-HSPs expression- Thermotolerance”的信号转导通路。在这个通路里, PABA 作为一个信号分子介导了 H_2O_2 累积, 适量浓度的 H_2O_2 能作为第二信使, 激活过氧化感受的信号转导通路, 通过蛋白激酶的磷酸化作用逐渐使信号放大, 信号入核促进了热休克蛋白和耐高温相关的功能基因的过表达, 最终提高了生物体的耐温性。PABA 信号通路的提出不仅丰富了我们耐高温信号转导的认识, 而且它也是对水杨酸信号通路最有力的支持和补充。

关键词: 高温胁迫, 高温耐受性, 双孢蘑菇, 抑制性消减杂交, 疏水蛋白, 对氨基苯甲酸合成酶

Abstract

Heat stress is one type of abiotic stress, which caused by high environmental temperature beyond optimum. Heat stress could disturb the cellular homeostasis of organisms, leading to sever stagnation in growth, development, and even death. As the global climate getting warmer, heat stress becomes the main cause for the great reduction in agriculture and breeding. In this dissertation, we investigate the injuries caused by heat stress in the mushroom *Agaricus bisporus*, as well as the innate heat stress response and resistance mechanisms. Additionally, we investigate how to breed thermotolerant mushroom strain with molecular biology and genetic engineering.

Agaricus bisporus is a kind of edible mushroom with high ecological and economical values. Environmental temperature is the key restriction factor in its cultivation. Breeding a thermotolerant strain will eventually benefit its yield. *A. bisporus* 8213 is a common cultivated strain that could only be cultivated under 16-22°C, while its phylogenetically closest strain 02 exhibited evident thermotolerance with healthy mycelium morphology above 33°C, such temperature would be lethal for the strain 8213. To elucidate the thermotolerance mechanism of the strain 02, we compared their differences by integrative transcriptomic and proteomic approaches. A total of 197 differential expression sequence tags (ESTs) were identified to be involved in different cellular and metabolic processes including nucleotide metabolism and recovery, protein turnover, signal transduction and energy metabolism. Among them, the upregulation of the most evident ESTs coincided with the upregulation of their proteins counterparts, suggesting that the transcriptional regulatory mechanism played essential roles. Based on our results, we propose a novel heat stress responsive network in *A. bisporus*. In this network, high temperature stimulates NADPH oxidase-dependent ROS and para-aminobenzoic acid (PABA) synthase-dependent PABA generations, which subsequently triggers the downstream MAPK cascades and defense responses, meanwhile, DNA and protein recoveries are also involved. Such network not only complements our understanding of the

thermotolerant mechanisms and related signalings in higher fungi, but also provides a clear guideline for further functional studies.

Based on the temporal-spatial transcriptional analysis, we cloned 15 typical thermotolerant-related genes and obtained their ORF sequences. The reverse genetics results showed that the overexpression of 4 genes in strain 8213 greatly improve its thermotolerance. They were PABA synthase gene (*Pab*), peptidyl-proline isomerase (*Ppi*), Hydrophobin (*Hpb*) and Calnexin gene. On the other hand, the knockdown of *Hpb* in thermotolerant strain 02 impaired its innate thermotolerance. It should be also noted that the transgenic overexpression of *Pab*, *Ppi*, *Hpb* and a kind of immunomodulatory protein greatly enhanced the baseline thermotolerance of *Arabidopsis* seedlings.

Further, we investigated the thermotolerance-improving mechanisms of mushroom-derived PABA synthase (*AbPabase*) in *Arabidopsis*. In phenotype, exogenous PABA feeding (1-5 $\mu\text{mol/L}$) directly improved the thermotolerance of wild type *Arabidopsis* seedlings; the 35S:*AbPabase* transgenic over-expressed lines also showed high thermotolerance than wild type. Further, we found out that heat stress can induce H_2O_2 generation in *Arabidopsis* and that the basal H_2O_2 level was higher in the 35S:*AbPabase* transgenic lines. Moreover, we found that the HSPs were obviously upregulated in *Arabidopsis* seedlings either by transgenic endogenous overexpression of *AbPabase* or by exogenous PABA feeding. Taken together, we propose a novel thermotolerance-related signaling ‘PABA- H_2O_2 -Peroxide signaling-HSPs expression-Thermotolerance’ in basidiomycetes and plants, during which PABA as a signal molecule mediates the H_2O_2 accumulation, which, in turn, activates the peroxide perceived signal transduction, further up regulates the expression of HSPs and many kinds of thermotolerant genes and improves the thermotolerance of organisms finally. The discovery of this pathway not only improves our concept of thermotolerance signaling transduction, but also is a strong support and supplement for SA signaling.

Keywords: Heat stress, Thermotolerance, *Agricus bisporus*, Suppression Subtractive Hybridization (SSH), Hydrophobin, PABA synthase

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