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·研究论文·

# 甘蓝中催化NMN降解生成NR的5′-核苷酸酶基因克隆和功能分析

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**摘要** 烟酰胺单核苷酸(NMN)具有抗癌和抗衰老等重要生物活性。植物中NMN含量低, 阻断其降解途径是提高NMN含量的有效方式, 而植物中NMN降解途径尚不清晰。从羽衣甘蓝(*Brassica oleracea* var. *acephala*)中克隆到8个5′-核苷酸酶候选基因, 对其编码产物进行生物信息学分析, 利用大肠杆菌表达系统纯化蛋白。系统发育分析表明, 5′-核苷酸酶在植物中保守存在, 暗示其在调控核苷酸代谢中可能发挥重要作用。通过体外酶促反应分析了这些5′-核苷酸酶的催化性能, 结果表明, 羽衣甘蓝中5′-核苷酸酶BolN2、BolN5-X1和BolN6能够催化NMN降解生成烟酰胺核糖(NR)。此外, BolN2、BolN5和BolN6还能够催化烟酸单核苷酸、嘌呤和嘧啶核苷酸水解, 表明其具有广泛的底物适应性。研究揭示了羽衣甘蓝5′-核苷酸酶的催化特性, 为阐明甘蓝中NMN的降解途径, 进而创制高NMN含量的甘蓝新种质奠定了基础。

**关键词** 羽衣甘蓝, 烟酰胺单核苷酸, 5′-核苷酸酶, 功能验证

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羽衣甘蓝(*Brassica oleracea* var. *acephala*)是十字花科芸苔属草本植物, 既可观赏又可食用, 是世界上最健康的蔬菜之一。羽衣甘蓝含有多种天然产物, 如烟酰胺单核苷酸(nicotinamide mononucleotide, NMN)、多酚、维生素和类胡萝卜素, 具有重要的药用价值, 包括抗癌、抗衰老、改善胃溃疡及保护肝脏等(Lučić et al., 2023; 丁琳等, 2023)。因此, 明确可食用羽衣甘蓝中重要天然产物的代谢途径, 对于提高其品质具有重要意义。

NMN是一种天然的维生素B3衍生物。其以α和β两种异构体形式存在, 其中β异构体是其活性形式。作为烟酰胺腺嘌呤二核苷酸(nicotinamide adenine dinucleotide, $NAD^+$)的前体物质, NMN参与生物体内400多种生理代谢反应(Hong et al., 2020)。研究表明, NMN具有抗衰老活性, 常用于治疗因肥胖引发的代谢性疾病、神经退行性疾病(俞韩啸等, 2022)以及急性肾损伤和肝损伤(He et al., 2001; Zong et al., 2021), 还可调节炎症反应(Zheng et al., 2024), 用于防治COVID-19 (Jiang et al., 2022), 并能改善肠道功能(Tang et al., 2024)。除药理活性外, NMN还可增强植物对多种病原菌的抵抗力(Sidiq et al., 2021)。一些蔬菜和水果中富含NMN, 包括大豆(*Glycine max*)、甘蓝类蔬菜、黄瓜(*Cucumis sativus*)、番茄(*Solanum lycopersicum*)、蘑菇(*Agaricus campestris*)和鳄梨(*Persea americana*) (Mills et al., 2016)。通过对可食用羽衣甘蓝中NMN代谢途径进行改造, 进一步提高其NMN含量, 将对提高作物品质和抗逆性具有重要意义。

作为$NAD^+$的前体分子, NMN的合成及降解与$NAD^+$的代谢密切相关。在拟南芥(*Arabidopsis thaliana*)中, NMN由NAD焦磷酸化酶催化$NAD^+$产生(Ogawa et al., 2005)。Yao等(2024)同时激活$NAD^+$合成通路中的4个基因, 将植物内源性NMN含量提高了2–3倍。阻断降解途径是提高NMN含量的另一种有效方式。植物中NMN的降解途径主要有2条, 一是由烟酰胺单核苷酸腺苷转移酶(nicotinamide mononu-

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cleotide adenylyltransferase, NMNAT)催化NMN生成$NAD^+$ (Hashida et al., 2007); 另一途径是由NMN降解生成烟酰胺(nicotinamide, NAM)。Wagner和Wagner (1985)利用同位素示踪法在烟草(*Nicotiana tabacum*)中检测到糖基水解酶活性, 推测植物中NMN生成NAM是由该酶催化的一步反应。Ashihara和Deng (2012)利用同样的方式推测此途径可能通过5′-核苷酸酶和核苷酶的作用, 经过两步反应完成, 即NMN先降解生成NR, 再生成NAM, 然而并未进行验证。因此, NMN在植物中的降解途径仍有待阐明。

5′-核苷酸酶是催化核苷单磷酸去磷酸化并调节细胞内核苷酸和核苷水平的关键酶, 广泛存在于生物体中(Hunsucker et al., 2005)。在大肠杆菌(*Escherichia coli*)中, HAD超家族和SurE家族基因具有5′-核苷酸酶活性, 能够催化嘌呤和嘧啶核苷酸水解(Terakawa et al., 2016)。在酿酒酵母(*Saccharomyces cerevisiae*)中, 5′-核苷酸酶Sdt1和Isn1不仅可以催化嘌呤和嘧啶核苷酸去磷酸化, 还能水解吡啶核苷酸NMN和烟酸单核苷酸(nicotinic acid mononucleotide, NaMN), 表明其具有广泛的底物适应性(Bogan et al., 2009)。植物中有关5′-核苷酸酶编码基因的研究较少。在马铃薯(*Solanum tuberosum*)、甜菜(*Beta vulgaris*)、大豆和豌豆(*Pisum sativum*)中已经分离到5′-核苷酸酶。体外酶学实验表明其能催化嘌呤核苷酸水解(Bideon, 1975; Eastwell and Stumpf, 1982; Christensen and Jochimsen, 1983)。目前已在菜豆(*Phaseolus vulgaris*)中克隆到1个5′-核苷酸酶编码基因*PvNTD1*, 体外酶学实验结果表明PvNTD1对嘌呤和嘧啶核苷酸均具有催化活性(Cabello-Díaz et al., 2015)。但它们是否催化吡啶核苷酸NMN和NaMN尚未见报道。

本研究从可食用羽衣甘蓝中克隆到8个5′-核苷酸酶基因, 利用大肠杆菌表达系统对其进行异源表达并纯化蛋白, 通过体外酶学实验探究5′-核苷酸酶的催化特性, 以期阐明NMN在甘蓝中的降解途径, 并为创制高NMN含量的甘蓝新种质奠定理论基础。

# 1 材料与方法

## 1.1 实验材料及试剂

羽衣甘蓝(*Brassica oleracea* var. *acephala* L. f. tricolor Hort.)种子购自江苏中江种业有限公司。NMN、NaMN、5′-尿苷一磷酸(UMP)、5′-胞苷一磷酸(CMP)、5′-腺苷一磷酸(AMP)、5′-鸟苷一磷酸(GMP)、NR、烟酸核糖(NaR)、尿嘧啶核苷(Uridine)、胞嘧啶核苷(Cytidine)、腺嘌呤核苷(Adenosine)和鸟嘌呤核苷(Guanosine)购自上海源叶生物技术有限公司。

## 1.2 实验方法

### 1.2.1 5'-核苷酸酶基因鉴定及克隆

对大肠杆菌的研究表明, HAD和SurE蛋白家族具有5'-核苷酸酶相关功能。从InterPro网站(https://www.ebi.ac.uk/interpro/)搜索到HAD和SurE蛋白家族的PFAM号分别为PF05761和PF01975。利用HMMER 3.0软件搜索编码HAD和SurE的候选基因。通过InterPro和CDD网站再次验证蛋白保守结构域。

利用*TransZol* Up Plus RNA Kit (TransGen, 北京)试剂盒提取羽衣甘蓝总RNA。参照HiScript II Q RT SuperMix for qPCR (+gDNA wiper) (诺唯赞, 南京)试剂盒说明书合成cDNA。以甘蓝(*Brassica oleracea*)中候选基因为参考序列, 利用SnapGene 6.0.2软件设计引物(表1)。以cDNA为模板, 利用2× Phanta Max Master Mix DNA (诺唯赞, 南京)聚合酶扩增基因片段。

### 1.2.2 5'-核苷酸酶基因家族生物信息学分析

对羽衣甘蓝、拟南芥(*Arabidopsis thaliana*)、油菜(*Brassica napus*)、水稻(*Oryza sativa*)、小麦(*Triticum aestivum*)、玉米(*Zea mays*)、大豆、番茄的HAD超家族和SurE家族5'-核苷酸酶基因进行分析。上述植物的蛋白序列来自NCBI网站(https://www.ncbi.nlm.nih.gov/)。

利用ExPasy (http://web.expasy.org/protparam/)在线软件对5'-核苷酸酶蛋白序列的理化性质进行分析。用WoLF PSORT (http://www.genscript.com/wolf-psort.html)在线软件进行亚细胞定位。通过SOPMA (https://npsa.lyon.inserm.fr/cgibin/npsa_automat.pl?page=/NPSA/npsa_sopma.html)在线软件预测5'-核苷酸酶蛋白的二级结构。用WISS-MODEL (https://swissmodel.expasy.org)在线软件预测其三级结构。使用MUSCLE软件进行多序列比对, 再利用MEGA 7.0软件基于邻接法(neighbor-join-

**表1** 引物序列
**Table 1** Sequence of primers

| Primer name | Sequences (5'–3') | Reference gene |
|---|---|---|
| BolN1-F | AAGAAGGAGATATACATATGAATCTATGCGCCCATCAG | *Bo7g089730* |
| BolN1-R | GTGGTGGTGGTGGTGCTCGAGGTTTTCCATAGCCACATCAG | |
| BolN2-F | AAGAAGGAGATATACATATGCTCCTGTGCGGAGATATG | *Bo2g150410* |
| BolN2-R | GTGGTGGTGGTGGTGCTCGAGGTTTTCCATAGCCGCATCAG | |
| BolN3-F | AAGAAGGAGATATACATATGGTTAGAGGCTTGGAGCTAGA | *Bo6g086480* |
| BolN3-R | GTGGTGGTGGTGGTGCTCGAGGGTTTTGAAAAGGCTTGAAGCC | |
| BolN4-F | AAGAAGGAGATATACATATGACATCATCTCGCCGTCTT | *Bo4g154010* |
| BolN4-R | GTGGTGGTGGTGGTGCTCGAGATGGCATCCTTGGGCAGC | |
| BolN5-X1.2-F | AAGAAGGAGATATACATATGACCTCGAAGAACAACAGCTT | *Bo2g079140* |
| BolN5-X3.4-F | AAGAAGGAGATATACATATGACCTCGAATCTCCAGGAC | |
| BolN5-R | GTGGTGGTGGTGGTGCTCGAGTTGATCAGCATTAAGGGCTT | |
| BolN6-F | AAGAAGGAGATATACATATGGCGATTAACGGCGAAGATCG | *Bo8g039400* |
| BolN6-R | GTGGTGGTGGTGGTGCTCGAGCAAGGATGAAGAAATCTTGGG | |
| BolN7-F | AAGAAGGAGATATACATATGGCGATTAACGGCGAAGATCG | *Bo6g116340* |
| BolN7-R | GTGGTGGTGGTGGTGCTCGAGTTGATCAGAGTTAAGGGCTTTGG | |
| BolN8-F | AAGAAGGAGATATACATATGACCTCAAAAAACAACGGCTTG | *Bo6g089210* |
| BolN8-R | GTGGTGGTGGTGGTGCTCGAGTTGATCAGCGTTGAGGTATTTGT | |
| BolN9-F | AAGAAGGAGATATACATATGCTTCTCAACAAGCGTCATTT | *Bo6g119360* |
| BolN9-R | GTGGTGGTGGTGGTGCTCGAGAAGGGGGAGAAGGTGGAA | |
| BolN10-F | AAGAAGGAGATATACATATGAATCTATGCGCCCATCAG | *Bo6g099860* |
| BolN10-R | GTGGTGGTGGTGGTGCTCGAGGTTTTCCATAGCCACATCAG | |
| pET29a-F | TGATGTCGGCGATATAGGCG | – |
| pET29a-R | GCTTAATGCGCCGCTACA | – |

ing)构建系统发育树，重复次数设为1 000 (高虎虎等, 2017)。

### 1.2.3 5'-核苷酸酶基因的原核表达

#### 1.2.3.1 重组质粒的构建

按照ClonExpress II One Step Cloning Kit-C112 (诺唯赞，南京)试剂盒说明书将pET-29a (+) (用*Nde*I和*Xho*I双酶切)载体与目的基因片段连接，转化至DH5α大肠杆菌感受态细胞中，涂布在LB固体培养基(含50 μg·mL$^{-1}$卡那霉素)上。挑取单克隆，利用pET29a-F/pET29a-R引物(表1)进行菌落PCR鉴定，筛选阳性克隆，并送至生工生物工程(上海)有限公司测序。将测序正确的重组质粒命名为pET29a-*BolN1* (2个转录本分别命名为pET29a-*BolN1-X1*和pET29a-*BolN1-X2*)、pET29a-*BolN2*、pET29a-*BolN3*、pET29a-*BolN4*、pET29a-*BolN5* (4个转录本分别命名为pET29a-*BolN5-X1*、pET29a-*BolN5-X2*、pET29a-*BolN5-X3*和pET29a-*BolN5-X4*)、pET29a-*BolN6*、pET29a-*BolN7*和pET29a-*BolN8* (表2)。

#### 1.2.3.2 重组蛋白的表达

以BolN1-X1为例说明实验步骤。将pET29a-*BolN1-X1*重组质粒转化至大肠杆菌BL21 (DE3)感受态细胞中，涂布在LB固体培养基(含50 μg·mL$^{-1}$卡那霉素)上，37°C培养16小时。挑取单菌落接种到3 mL LB液体培养基(含50 μg·mL$^{-1}$卡那霉素)中，放入37°C摇床过夜培养。将菌液以1:100 (v/v)的比例加至LB液体培养基中扩大培养，相同条件下继续培养至$OD_{600}$值为0.6–0.8，加入异丙基-β-硫代半乳糖苷(IPTG，终浓度为0.3 mmol·L$^{-1}$)诱导蛋白表达，16°C、180 r·min$^{-1}$

**表2** 菌株和质粒

**Table 2** Strains and plasmids

| Strains or plasmids | Characteristics | Sources |
|---|---|---|
| *Escherichia coli* | | |
| DH5a | General cloning host | Tsingke |
| BL21 (DE3) | Host strain for protein expression | Tsingke |
| Plasmids | | |
| pET29a | *Kan*$^r$, protein expression vector | Laboratory preservation |
| pET29a-*BolN1-X1* | *Kan*$^r$, pET29a derivative for the expression of *BolN1-X1* gene | This study |
| pET29a-*BolN1-X2* | *Kan*$^r$, pET29a derivative for the expression of *BolN1-X2* gene | This study |
| pET29a-*BolN2* | *Kan*$^r$, pET29a derivative for the expression of *BolN2* gene | This study |
| pET29a-*BolN3* | *Kan*$^r$, pET29a derivative for the expression of *BolN3* gene | This study |
| pET29a-*BolN4* | *Kan*$^r$, pET29a derivative for the expression of *BolN4* gene | This study |
| pET29a-*BolN5-X1* | *Kan*$^r$, pET29a derivative for the expression of *BolN5-X1* gene | This study |
| pET29a-*BolN5-X2* | *Kan*$^r$, pET29a derivative for the expression of *BolN5-X2* gene | This study |
| pET29a-*BolN5-X3* | *Kan*$^r$, pET29a derivative for the expression of *BolN5-X3* gene | This study |
| pET29a-*BolN5-X4* | *Kan*$^r$, pET29a derivative for the expression of *BolN5-X4* gene | This study |
| pET29a-*BolN6* | *Kan*$^r$, pET29a derivative for the expression of *BolN6* gene | This study |
| pET29a-*BolN7* | *Kan*$^r$, pET29a derivative for the expression of *BolN7* gene | This study |
| pET29a-*BolN8* | *Kan*$^r$, pET29a derivative for the expression of *BolN8* gene | This study |

*Kan*$^r$: 抗卡那霉素基因 *Kan*$^r$: Anti-kanamycin gene

培养12–16小时。BolN1-X2、BolN2、BolN3、BolN4、BolN5、BolN6、BolN7和BolN8的表达实验步骤与BolN1-X1相同。

#### 1.2.3.3 重组蛋白的纯化及定量分析

诱导蛋白表达后, 将培养液在4°C、12 000 ×*g*离心, 收集菌体。用1× PBS磷酸缓冲液(pH7.4)重悬菌体后超声破碎, 离心收集上清液, 即为粗酶液。粗酶液经0.22 μm滤膜过滤后, 加至预平衡后的$Co^{2+}$亲和层析柱(His GraviTrap TALON, Cytiva), 参照说明书, 在4°C下进行蛋白纯化。通过十二烷基硫酸钠-聚丙烯酰胺凝胶电泳(SDS-PAGE)检测蛋白纯化结果。基于BCA法利用蛋白质浓度测定试剂盒(生工生物工程有限公司, 上海)测定蛋白含量。

### 1.2.4 体外酶学反应

酶促反应在体积为500 μL的混合物中进行。混合物含有50 mmol·L$^{-1}$ PBS缓冲液、10 mmol·L$^{-1}$ $MgCl_2$、1 mmol·L$^{-1}$二硫苏糖醇(DTT)、0.1 mmol·L$^{-1}$ NMN (或NaMN、UMP、CMP、AMP和GMP)和5 μmol·L$^{-1}$蛋白。以煮沸失活的蛋白作为阴性对照。将混合物在30°C下反应1小时, 加入等体积三氯甲烷终止酶促反应。反应混合物在12 000 ×*g*下离心5分钟, 收集上层水相进行后续分析。

### 1.2.5 HPLC和LC-MS/MS分析

利用Waters e2695高效液相色谱仪对酶促反应产物进行分析。检测器型号为Waters 2998二极管列阵检测器。色谱条件: ZORBAX SB Aq C18色谱柱(4.6 mm × 250 mm, 5 μm, Agilent), 柱温25°C, 流动相分为A相(0.1%甲酸水溶液)和B相(甲醇), 流速为0.5 mL·min$^{-1}$, 进样量为50 μL。梯度洗脱条件: 0–2分钟, 1% A、99% B; 2–25分钟, 1%–30% A、99%–70% B; 25–35分钟, 30%–100% A、70%–0% B; 35–40分钟, 0%–1% A、100%–99% B。检测波长为254 nm。

LC-MS/MS分析使用Waters Xevo TQ三重四级杆质谱仪。色谱分离条件: ACQUITY UPLC BEH HSS T3柱(2.1 mm × 100 mm, 1.8 μm, Waters), 柱温25°C, 流动相分为A相(0.1%甲酸水溶液)和B相(甲醇), 流速为0.3 mL·min$^{-1}$, 进样量为0.5 μL。梯度洗脱条件: 0–6分钟, 99% A、1% B; 6.1–9分钟, 99%–40% A、1%–60% B; 9.1–13分钟, 40%–99% A、

60%–1% B。质谱条件: 气体温度为500°C, 去溶剂化气体流速为900 L·Hr$^{-1}$, 喷雾器压力为7.0 bar, 毛细管电压为500 V。质谱检测利用电喷雾离子源正离子模式(ESI+)扫描, 多反应监测模式(MRM), NR的监测离子m/z 257.3→123.0, NaR的监测离子m/z 256.2→124.0, Adenosine的监测离子m/z 268.2→136.1, Guanosine的监测离子m/z 284.2→152.1, Cytidine的监测离子m/z 244.2→112.1, Uridine的监测离子m/z 245.2→113.0。所有实验均重复3次。利用MassLynx V4.2软件进行数据处理和分析。

## 2 结果与分析

### 2.1 羽衣甘蓝5'-核苷酸酶基因的克隆

利用隐马尔可夫模型和BLASTP算法对甘蓝全基因组进行搜索, 鉴定到10个5'-核苷酸酶候选基因, 其中属于HAD家族的有6个, 分别为*Bo6g099860*、*Bo7g089730*、*Bo2g150410*、*Bo6g086480*、*Bo4g154010*以及*Bo6g119360*; 属于SurE家族的有4个, 分别为*Bo2g079140*、*Bo8g039400*、*Bo6g116340*以及*Bo6g089210*。以甘蓝10个5'-核苷酸酶基因为参考序列, 从羽衣甘蓝中克隆到8个基因(分别命名为*BolN1*–*BolN8*)。BLAST分析显示, BolN1存在2个转录本, 分别命名为BolN1-X1和BolN1-X2, 与Bo7g089730的同源性分别为96%和99%; BolN2与Bo2g150410的同源性为99%; BolN3与Bo6g086480的同源性为99%; BolN4与Bo4g154010的同源性为98%; BolN5存在4个转录本, 分别命名为BolN5-X1、BolN5-X2、BolN5-X3以及BolN5-X4, 它们与Bo2g079140的同源性分别为96%、96%、96%和95%; BolN6与Bo8g039400的同源性为99%; BolN7与Bo6g116340的同源性为99%; BolN8与Bo6g089210的同源性为97%。上述结果表明从羽衣甘蓝中克隆到正确的5'-核苷酸酶基因。

### 2.2 羽衣甘蓝5'-核苷酸酶蛋白特性及系统发育分析

蛋白质理化性质分析结果显示(表3), 羽衣甘蓝5'-核酸酶的氨基酸序列长度为307 (BolN6)–620 (BolN1-X2) aa; 分子量介于33 476.79 (BolN6)–70 585.80 (BolN1-X1) Da之间; 等电点(pI)为5.01 (BolN7)–7.71 (BolN4)。预测结果表明, BolN3、BolN4、BolN6和BolN8的不稳定系数低于40, 为稳定蛋白, 其余8个5'-核苷酸酶蛋白均为不稳定蛋白。脂肪系数介于74.97–90.16之间, 12个5'-核苷酸酶蛋白的平均亲水系数为负值, 均为亲水性蛋白。

蛋白质二级结构预测结果显示(表4), 12个5'-核苷酸酶蛋白均由α-螺旋、延伸链和无规则卷曲3部分组成, 以α-螺旋和无规则卷曲为主。亚细胞定位预测分析表明, 5'-核苷酸酶蛋白中有5个定位于叶绿体、3个定位于细胞核、2个定位于细胞质、1个定位于内质网、1个定位于细胞骨架, 表明该家族成员可能在细胞的不同位置发挥作用。蛋白质三级结构同源建模预测结果显示(图1), 这些蛋白的空间结构有一定差异, 提示它们很可能具有不同的功能。

为明确羽衣甘蓝与其它植物5'-核苷酸酶基因的亲缘关系, 基于12个羽衣甘蓝、5个拟南芥、23个油菜、6个水稻、13个小麦、6个玉米、16个大豆和8个番茄的5'-核苷酸酶蛋白序列构建系统发育树(陈婷欣等, 2024)。结果显示(图2), HAD和SurE家族分为2个分支, 其中HAD家族可分为HAD-ClassI、HAD-ClassII以及HAD-ClassIII三个亚家族, 分别包含18、11和18个成员; SurE家族可分为SurE-ClassI和SurE-ClassII两个亚家族, 分别包含13和29个成员。上述8个物种的HAD和SurE基因都出现在这5个亚家族中, 特别是HAD-ClassII和SurE-ClassI亚家族, 其中6个物种仅有1个基因包含其中。以上结果提示这5个亚家族在植物中可能具有重要作用, 它们在长期的进化过程中被保留下来。此外, 油菜、拟南芥和羽衣甘蓝均为十字花科植物, 与其它物种相比它们的HAD和SurE基因之间亲缘关系更近。

### 2.3 羽衣甘蓝5'-核苷酸酶编码基因的原核表达

将从羽衣甘蓝中克隆到的12个序列与pET29a连接(图3A), 并转化大肠杆菌BL21 (DE3)。在大肠杆菌中诱导表达后, BolN2、BolN5-X1、BolN5-X2、BolN5-X3、BolN5-X4、BolN6、BolN7以及BolN8在SDS-PAGE中可见明显的特异性条带, 并且与预期的蛋白分子量(69.4、41.2、39.6、40.1、40.2、34.5、41.3和41.7 kDa)一致(图3B)。综上表明, 通过与*N*-His$_6$标签结合, 在大肠杆菌中成功表达BolN2、BolN5、BolN6、BolN7和BolN8的可溶性蛋白。

**表3** 5'-核苷酸酶蛋白基本信息

**Table 3** Basic information of 5'-nucleotidase protein

| Protein name | Amino acid number (aa) | Molecular weight (Da) | Isoelectric point | Acidic amino acid | Basic amino acid | Instability index | Aliphatic index | GRAVY |
|---|---|---|---|---|---|---|---|---|
| BolN1-X1 | 619 | 70585.80 | 5.95 | 84 | 74 | 44.71 | 83.34 | –0.338 |
| BolN1-X2 | 620 | 70579.90 | 5.91 | 85 | 75 | 44.38 | 85.71 | –0.307 |
| BolN2 | 613 | 69374.34 | 5.73 | 85 | 73 | 43.79 | 85.30 | –0.310 |
| BolN3 | 433 | 50351.39 | 6.48 | 63 | 59 | 39.38 | 82.77 | –0.552 |
| BolN4 | 346 | 40495.22 | 7.71 | 43 | 44 | 34.57 | 74.97 | –0.402 |
| BolN5-X1 | 380 | 40007.05 | 5.23 | 46 | 37 | 42.58 | 84.24 | –0.170 |
| BolN5-X2 | 381 | 40135.18 | 5.23 | 46 | 37 | 42.49 | 84.02 | –0.179 |
| BolN5-X3 | 370 | 39063.98 | 5.15 | 46 | 36 | 41.08 | 83.08 | –0.189 |
| BolN5-X4 | 371 | 39179.07 | 5.09 | 47 | 36 | 41.42 | 82.59 | –0.204 |
| BolN6 | 307 | 33476.79 | 5.11 | 38 | 27 | 37.19 | 90.16 | –0.156 |
| BolN7 | 379 | 40218.03 | 5.01 | 51 | 37 | 47.43 | 81.06 | –0.271 |
| BolN8 | 381 | 40611.46 | 5.04 | 50 | 38 | 39.86 | 85.75 | –0.273 |

GRAVY: 亲水性平均系数 GRAVY: Grand average of hydropathicity

**表4** 5'-核苷酸酶蛋白二级结构分析

**Table 4** The secondary structure analysis of 5'-nucleotidase protein

| Protein name | Alpha helix (%) | Extended strand (%) | Random coil (%) | Subcellular location |
|---|---|---|---|---|
| BolN1-X1 | 43.62 | 12.12 | 44.26 | Cytoplasm |
| BolN1-X2 | 47.74 | 9.35 | 42.90 | Nucleus |
| BolN2 | 45.35 | 10.11 | 44.54 | Chloroplast |
| BolN3 | 49.19 | 11.09 | 39.72 | Cytoplasm |
| BolN4 | 54.34 | 8.09 | 37.57 | Endoplasmic reticulum |
| BolN5-X1 | 29.74 | 16.32 | 53.95 | Chloroplast |
| BolN5-X2 | 28.61 | 14.17 | 57.22 | Chloroplast |
| BolN5-X3 | 31.89 | 15.41 | 52.70 | Nucleus |
| BolN5-X4 | 30.46 | 16.17 | 53.37 | Nucleus |
| BolN6 | 28.99 | 15.31 | 55.70 | Cytoskeleton |
| BolN7 | 28.50 | 14.51 | 56.99 | Chloroplast |
| BolN8 | 28.87 | 15.49 | 55.64 | Chloroplast |

## 2.4 羽衣甘蓝5'-核苷酸酶的体外功能验证

### 2.4.1 5'-核苷酸酶对吡啶核苷酸NMN和NaMN的水解活性

为验证羽衣甘蓝中5'-核苷酸酶对NMN的催化功能，将重组蛋白与NMN、$MgCl_2$和DTT共同孵育，并利用HPLC和LC-MS/MS分析反应混合物。HPLC分析结果表明，在BolN2、BolN5-X1和BolN6催化的反应中产生了一种新物质，其保留时间与NR标准品一致(图4A)。收集各产物并进行LC-MS/MS分析，发现产物与标准品NR的分子量相同(附图1A)。上述结果表明，羽衣甘蓝中5'-核苷酸酶BolN2、BolN5-X1和BolN6可在体外催化NMN产生NR。

Bogan等(2019)研究发现，酵母中5'-核苷酸酶能催化吡啶核苷酸NaMN的水解。我们分析了BolN2、BolN5、BolN6、BolN7和BolN8对NaMN的催化活性。HPLC分析结果显示，BolN2、BolN5和BolN6反应混合物中各产生1个新峰，其保留时间与NaR标准品一致(图4B)。LC-MS/MS分析显示，产物的分子量与标准品相同(附图1B)。以上结果表明，BolN2、BolN5和BolN6可水解NaMN生成NaR。

### 2.4.2 5'-核苷酸酶对嘌呤和嘧啶核苷酸的催化活性

研究表明，细菌、酵母和其它植物中的5'-核苷酸酶可以催化嘌呤和嘧啶核苷酸。因此，我们进一步探究羽衣甘蓝中5'-核苷酸酶对AMP、GMP、CMP和UMP的催化作用。HPLC和LC-MS/MS分析结果显示(图5，图6，附图2，附图3)，BolN2、BolN5-X1、BolN5-X2、BolN5-X3和BolN6可催化AMP、GMP、CMP和UMP

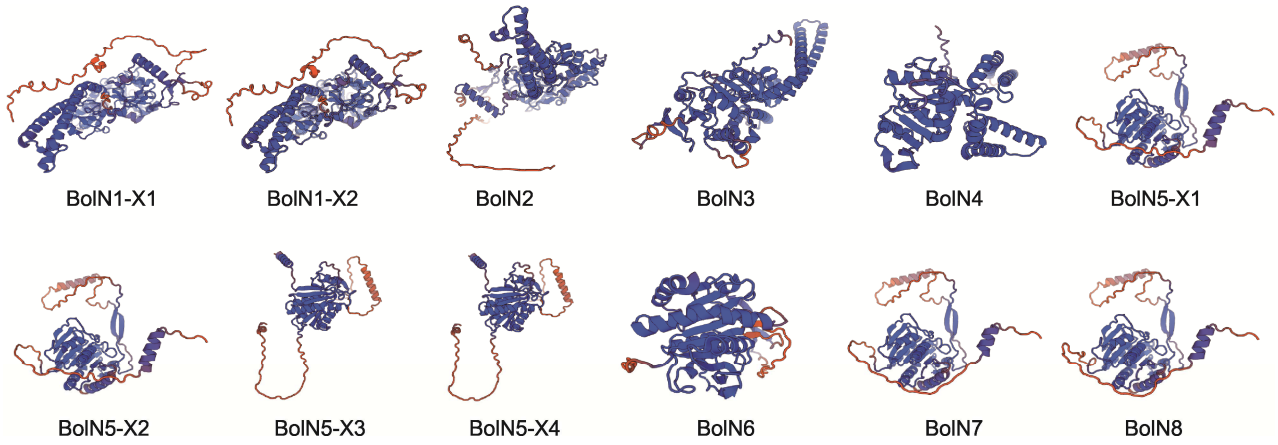



**图1** 5'-核苷酸酶蛋白三级结构预测

**Figure 1** Prediction of tertiary structure of 5'-nucleotidase protein

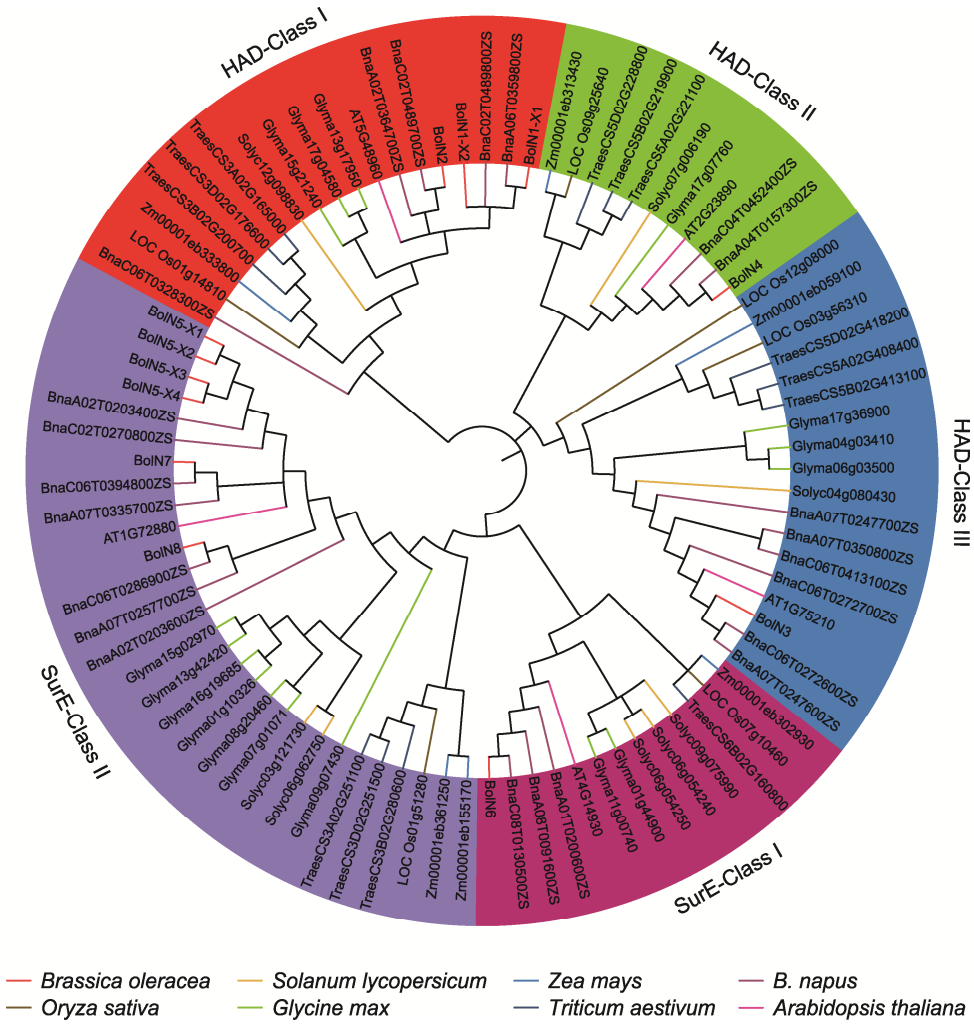



**图2** 8种植物HAD和SurE系统进化树

**Figure 2** Phylogenetic tree of HAD and SurE in 8 plants

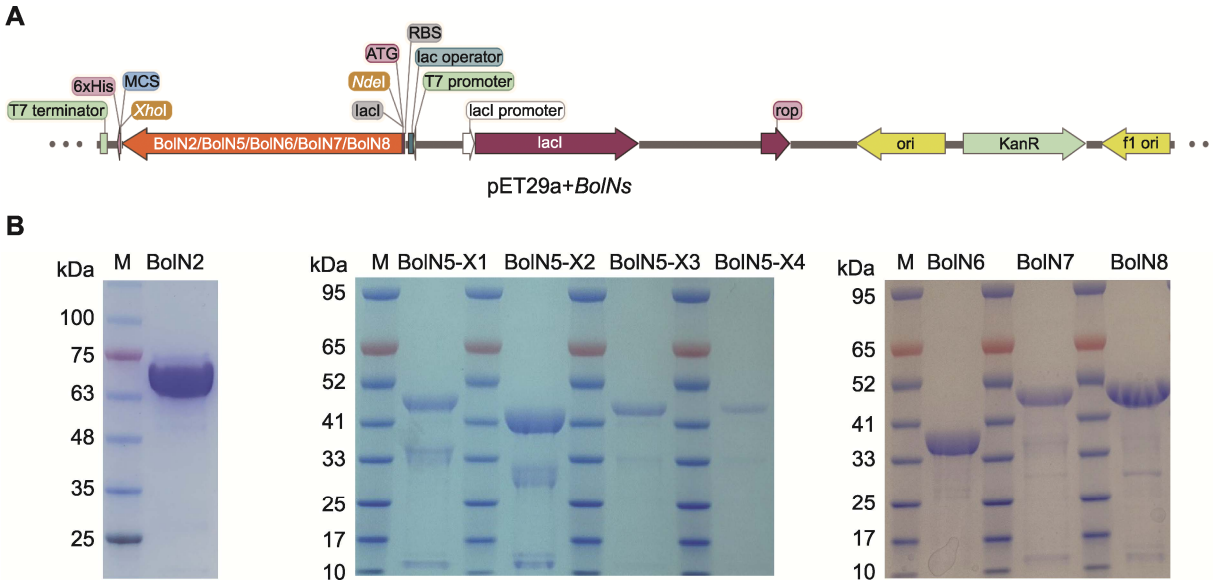



**图3** pET29a+*BolNs*重组质粒构建**(A)**和5'-核苷酸酶*N*-$His_6$标签纯化蛋白**(B)**

M: 蛋白分子量标准。BolN2、BolN5、BolN6、BolN7和BolN8均为与*N*-$His_6$标签结合的纯化蛋白。

**Figure 3** Schematic diagram of construction of recombinant plasmid pET29a+*BolNs* **(A)** and 5'-nucleotidase proteins purified by *N*-$His_6$ tag **(B)**

M: Protein marker. BolN2, BolN5, BolN6, BolN7, and BolN8 are purified proteins bound to *N*-$His_6$ tag.

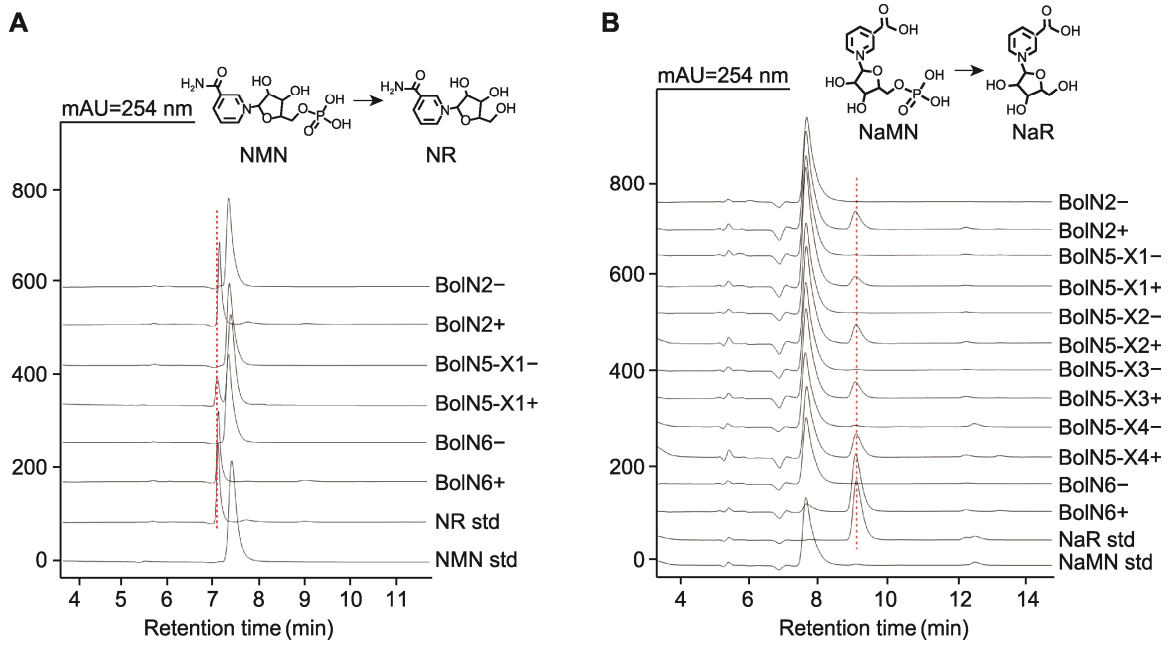



**图4** BolN2、BolN5和BolN6酶学反应产物的HPLC分析

**(A)** 以烟酰胺单核苷酸(NMN)为底物; **(B)** 以烟酸单核苷酸(NaMN)为底物。+: 酶催化反应; –: 阴性对照; NMN std: NMN标准品; NR std: NR标准品; NaMN std: NaMN标准品; NaR std: NaR标准品

**Figure 4** HPLC analysis of the products produced in the BolN2, BolN5, and BolN6 catalyzed reaction

**(A)** Using nicotinamide mononucleotide (NMN) as substrate; **(B)** Using nicotinic acid mononucleotide (NaMN) as substrate. +: Enzyme-catalyzed reaction; –: Negative control; NMN std: NMN standard; NR std: NR standard; NaMN std: NaMN standard; NaR std: NaR standard

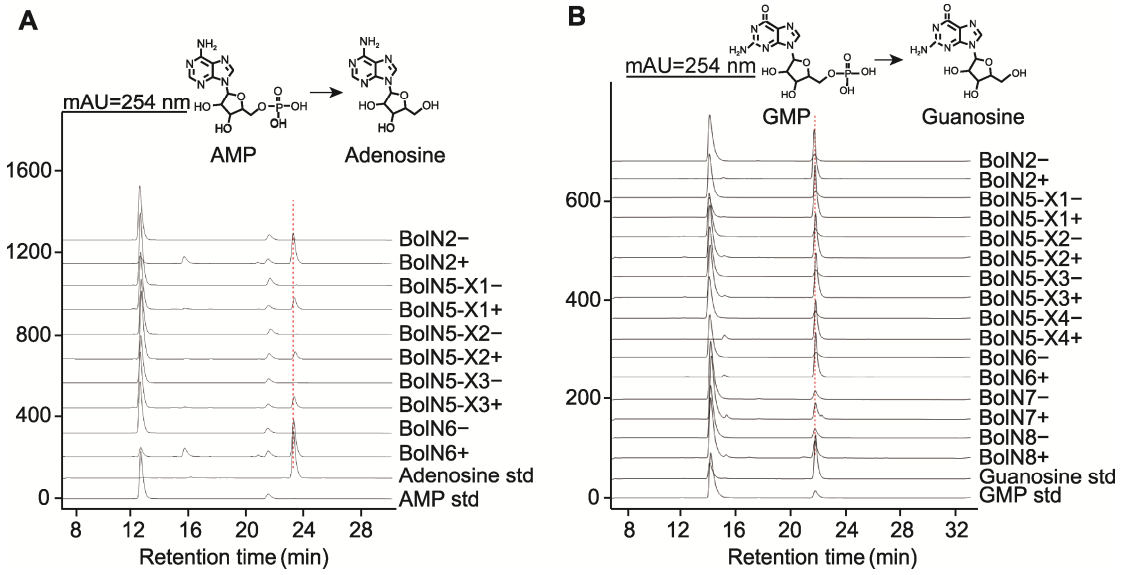



**图5** BolN2、BolN5、BolN6、BolN7和BolN8酶学反应产物的HPLC分析

**(A)** 以腺嘌呤核苷酸(AMP)为底物; **(B)** 以鸟嘌呤核苷酸(GMP)为底物。+: 酶催化反应; –: 阴性对照; AMP std: AMP标准品; Adenosine std: Adenosine标准品; GMP std: GMP标准品; Guanosine std: Guanosine标准品

**Figure 5** HPLC analysis of the products produced in the BolN2, BolN5, BolN6, BolN7, and BolN8 catalyzed reaction

**(A)** Using adenosine monophosphate (AMP) as substrate; **(B)** Using guanosine monophosphate (GMP) as substrate. +: Enzyme-catalyzed reaction; –: Negative control; AMP std: AMP standard; Adenosine std: Adenosine standard; GMP std: GMP standard; Guanosine std: Guanosine standard

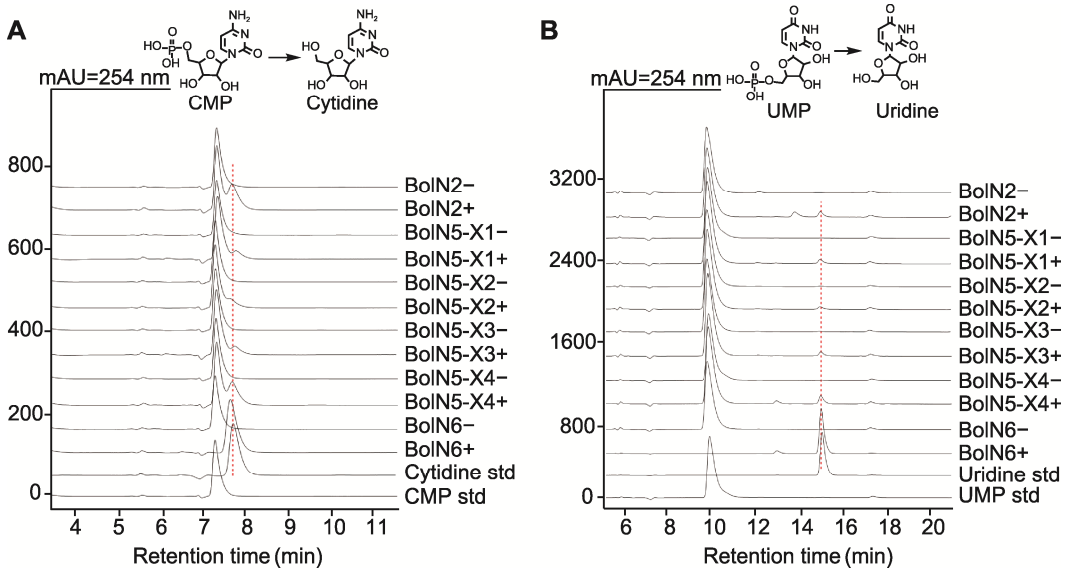



**图6** BolN2、BolN5和BolN6酶学反应产物的HPLC分析

**(A)** 以胞嘧啶核苷酸(CMP)为底物; **(B)** 以尿嘧啶核苷酸(UMP)为底物。+: 酶催化反应; –: 阴性对照; CMP std: CMP标准品; Cytidine std: Cytidine标准品; UMP std: UMP标准品; Uridine std: Uridine标准品

**Figure 6** HPLC analysis of the products produced in the BolN2, BolN5, and BolN6 catalyzed reaction

**(A)** Using cytidine monophosphate (CMP) as substrate; **(B)** Using uridine monophosphate (UMP) as substrate. +: Enzyme-catalyzed reaction; –: Negative control; CMP std: CMP standard; Cytidine std: Cytidine standard; UMP std: UMP standard; Uridine std: Uridine standard

水解生成对应产物。BolN5-X4可水解GMP、CMP和UMP，但不能以AMP作为底物；BolN7和BolN8特异性催化GMP水解。上述结果表明，羽衣甘蓝中5'-核苷酸酶能够催化嘌呤和嘧啶核苷酸，且其底物谱较广。

## 3 讨论

NMN具有抗癌、抗衰老及提高作物抗逆性等重要生物活性。可食用羽衣甘蓝具有较高的营养价值，解析甘蓝中NMN的代谢途径，通过阻断降解途径提高NMN含量具有重要意义。植物中NMN的降解与$NAD^+$补救合成(吡啶核苷酸循环)途径紧密相连(Ashihara et al., 2005)。与细菌和哺乳动物相比，植物中$NAD^+$补救合成途径的研究主要采用同位素示踪法，缺乏对具体基因的功能分析，且仅在少数植物(如拟南芥、烟草和马铃薯)中有相关研究报道(Wang and Pichersky, 2007; Zheng et al., 2008; Katahira and Ashihara, 2009)。同时，$NAD^+$补救合成途径在不同物种甚至不同器官之间差异较大(Noctor et al., 2006)。本研究从甘蓝基因组中检索到10个5′-核苷酸酶基因，以其为参考序列，最终从羽衣甘蓝中成功克隆到8个5′-核苷酸酶候选基因，为进一步揭示NMN的降解途径奠定了基础。系统发育分析显示，植物中5'-核苷酸酶较保守，表明其在植物核苷酸代谢中可能发挥重要作用。

在酿酒酵母中已报道5′-核苷酸酶能够催化NMN的去磷酸化(Bogan et al., 2009)。在植物中已分离到5′-核苷酸酶，但它们能否催化NMN的降解尚不清楚。本研究利用大肠杆菌表达系统在体外探究羽衣甘蓝中5′-核苷酸酶的催化特性，结果表明BolN2、BolN5-X1和BolN6能够催化NMN去磷酸化生成NR，在植物中验证了5′-核苷酸酶催化NMN的降解。此外，BolN2、BolN5和BolN6还能催化吡啶核苷酸NaMN以及嘌呤和嘧啶核苷酸(包括AMP、GMP、CMP和UMP)的水解，表明它们具有广泛的底物适应性；而BolN7和BolN8则仅对GMP具有微弱的催化活性。

核苷酸是生物体中重要的含氮化合物，是多种维生素(如核黄素、硫胺素、叶酸、黄素腺嘌呤二核苷酸(FAD)和S-腺苷甲硫氨酸)合成的前体物质(Crozier et al., 2000; Herz et al., 2000; Terakawa et al., 2016)。其中，嘌呤和嘧啶核苷酸不仅是体内核酸合成的重要前体，还参与生物大分子的合成。吡啶核苷酸NMN参与体内多种生化反应，$NAD^+$和$NADP^+$作为辅酶参与多种氧化还原反应。本研究从羽衣甘蓝中克隆到5′-核苷酸酶基因，体外酶学实验表明其具有催化嘌呤、嘧啶和吡啶核苷酸的活性，但它们在调节体内不同核苷酸代谢中的作用机制有待进一步证实。

## 4 结论

本研究克隆了羽衣甘蓝中HAD家族和SurE家族的5′-核苷酸酶基因，系统发育分析表明其在植物中保守。通过构建原核表达载体，在大肠杆菌中表达并纯化了5′-核苷酸酶。体外酶学反应表明，BolN2、BolN5-X1和BolN6能够催化NMN降解生成NR。此外，BolN2、BolN5和BolN6对NaNM、嘌呤和嘧啶核苷酸具有催化作用。本研究将增进人们对羽衣甘蓝中NMN代谢途径的认识，并为创制高NMN含量的食用羽衣甘蓝新种质提供理论依据。

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### 作者贡献声明

刘茹：完成实验、分析数据及撰写论文；李阳：协助完成实验及撰写论文；唐兆成：论文修改润色；郝婷婷：构思实验，监督、审阅及修改论文；张保龙：监督、审阅及修改论文。

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# Cloning and Functional Analysis of the 5'-nucleotidase Genes Catalyze NMN Degradation to NR in *Brassica oleracea* var. *acephala*

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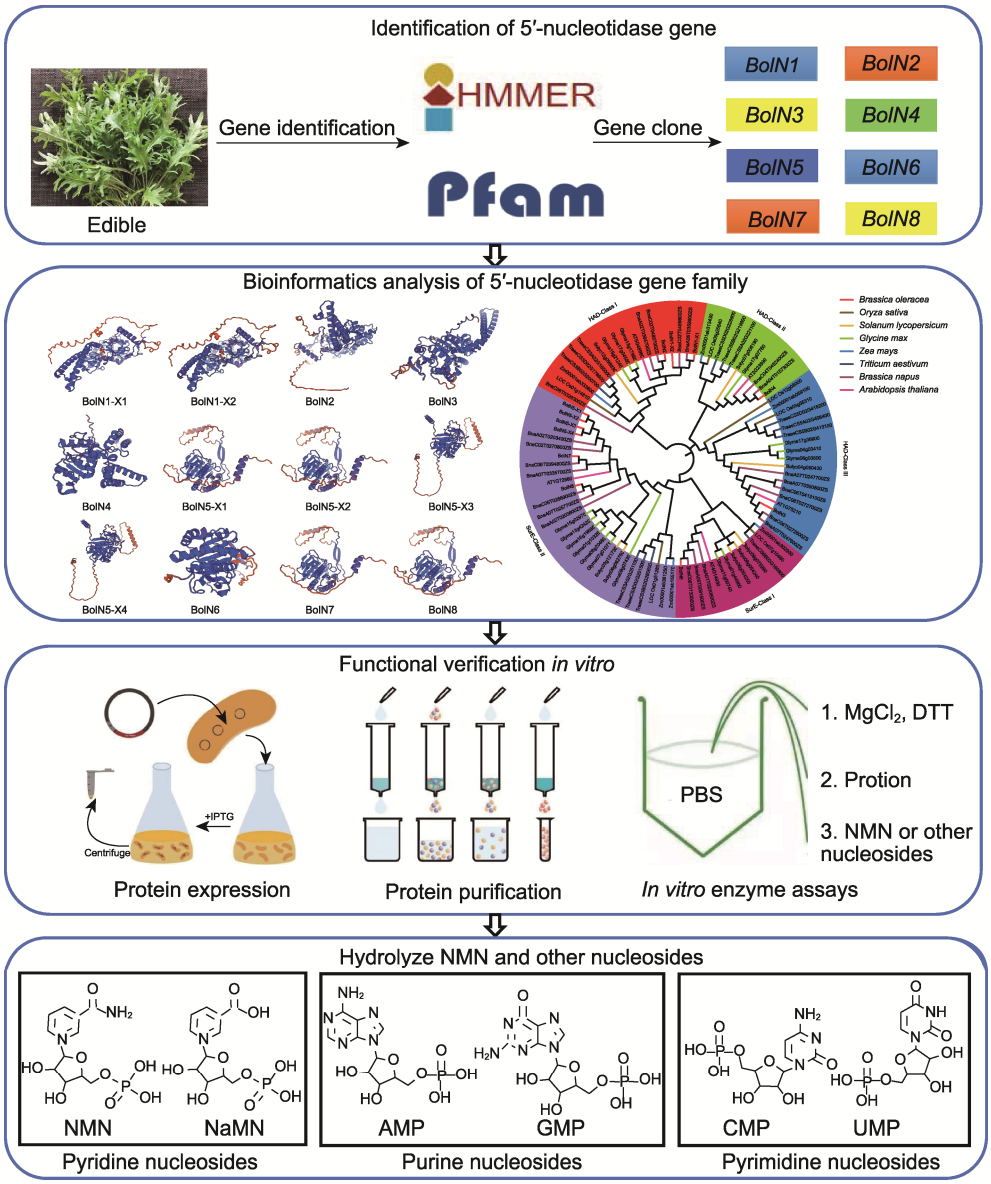
**INTRODUCTION:** Nicotinamide mononucleotide (NMN) has important biological activities such as anti-cancer, anti-aging and improving crop stress resistance, and its importance as a nutritional health product has been established. However, the content of NMN in plants is low, and the metabolic pathway of NMN degradation is poorly understood. It has been reported that 5'-nucleotidases can catalyze the dephosphorylation of NMN in *Saccharomyces cerevisiae*. At present, 5'-nucleotidases have been isolated in plants, but whether they can catalyze the degradation of NMN remains unclear. Edible kale (*Brassica oleracea* var. *acephala*) has high nutritional value. It is important to analyze the metabolic pathway of NMN in kale and increase the content of NMN by blocking the degradation pathway.

**RATIONALE:** The degradation of NMN in plants is closely related to the $NAD^+$ remediation synthesis (pyridine nucleotide cycle) pathway. Compared with bacteria and mammals, studies on the biosynthetic pathway of $NAD^+$ remediation in plants mainly use isotope tracer method, lack specific gene and function analysis, and only a few related studies have been reported in plants. Eight 5'-nucleotidase genes were cloned from *B. oleracea* var. *acephala*, heterologous expression of them was performed by *Escherichia coli* expression system, and the catalytic properties of 5'-nucleotidase were investigated by enzymological means *in vitro*.

**RESULTS:** In this study, ten 5'-nucleotidase genes were retrieved from the genome of *B. oleracea*. Based on these sequences, eight 5'-nucleotidase candidate genes were successfully cloned from *B. oleracea* var. *acephala*, which laid a foundation for further revealing the degradation pathway of NMN. Phylogenetic analysis revealed that 5'-nucleotidase is conserved in plants, suggesting that it may play an important role in plant nucleotide metabolism. The catalytic properties of 5'-nucleotides in kale were investigated by using the expression system of *E. coli*. *In vitro* enzymatic experiments showed that 5'-nucleotides can catalyze purine, pyrimidine and pyridine nucleotides, and have a wide range of substrate adaptability. Specifically, BolN2, BolN5-X1 and BolN6 can catalyze the dephosphorylation of NMN to the generation of NR, which proves that 5'-nucleotidase can catalyze the degradation of NMN in plants. In addition, BolN2, BolN5 and

BolN6 can catalyze the hydrolysis of pyridine nucleotides NaMN, purine and pyrimidine nucleotides (including AMP, GMP, CMP and UMP). However, BolN7 and BolN8 had only weak catalytic activity against GMP.

**CONCLUSION:** The 5'-nucleotidase gene from the HAD and SurE families of *B. oleracea* var. *acephala* was cloned and phylogenetic analysis showed that it was conserved in plants. The results of enzymatic reaction *in vitro* showed that BolN2, BolN5-X1 and BolN6 could catalyze the degradation of NMN to produce NR. In addition, BolN2, BolN5 and BolN6 have catalytic effects on NaNM, purine and pyrimidine nucleotides. This study further enhanced our understanding of the NMN metabolic pathway in kale, and provided a theoretical basis for creating edible kale new germplasm with high NMN content.




**Cloning and functional analysis of 5'-nucleotidase gene catalyzing NMN degradation to NR in *Brassica oleracea* var. *acephala*.** The 5'-nucleotidase gene of *B. oleracea* var. *acephala* was successfully cloned and phylogenetic analysis showed that it was conserved in plants. By constructing prokaryotic expression vector, 5'-nucleotidase was expressed and purified in *Escherichia coli*. *In vitro* enzymatic experiments showed that 5'-nucleotidase could catalyze the dephosphorylation of NMN to NR, and had catalytic effects on NaMN, purine nucleotides (AMP, GMP) and pyrimidine nucleotides (CMP, UMP).

**Key words** *Brassica oleracea* var. *acephala*, nicotinamide mononucleotide, 5′-nucleotidase, functional verification

**Liu R, Li Y, Tang ZC, Hao TT, Zhang BL** (2025). Cloning and functional analysis of the 5'-nucleotidase genes catalyze NMN degradation to NR in *Brassica oleracea* var. *acephala*. *Chin Bull Bot* **60**, 363–376.

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**附图1** 以NMN **(A)**和NaMN **(B)**为底物, BolN2、BolN5和BolN6酶学反应产物的LC-MS/MS分析
**Appendix figure 1** LC-MS/MS analysis of enzymatic reaction products of BolN2, BolN5, and BolN6 with NMN **(A)** and NaMN **(B)** as substrates

**附图2** 以AMP **(A)**和GMP **(B)**为底物, BolN2、BolN5、BolN6、BolN7和BolN8酶学反应产物的LC-MS/MS分析
**Appendix figure 2** LC-MS/MS analysis of enzymatic reaction products of BolN2, BolN5, BolN6, BolN7, and BolN8 with AMP **(A)** and GMP **(B)** as substrates

**附图3** 以CMP **(A)**和UMP **(B)**为底物, BolN2、BolN5和BolN6酶学反应产物的LC-MS/MS分析
**Appendix figure 3** LC-MS/MS analysis of enzymatic reaction products of BolN2, BolN5, and BolN6 with CMP **(A)** and UMP **(B)** as substrates

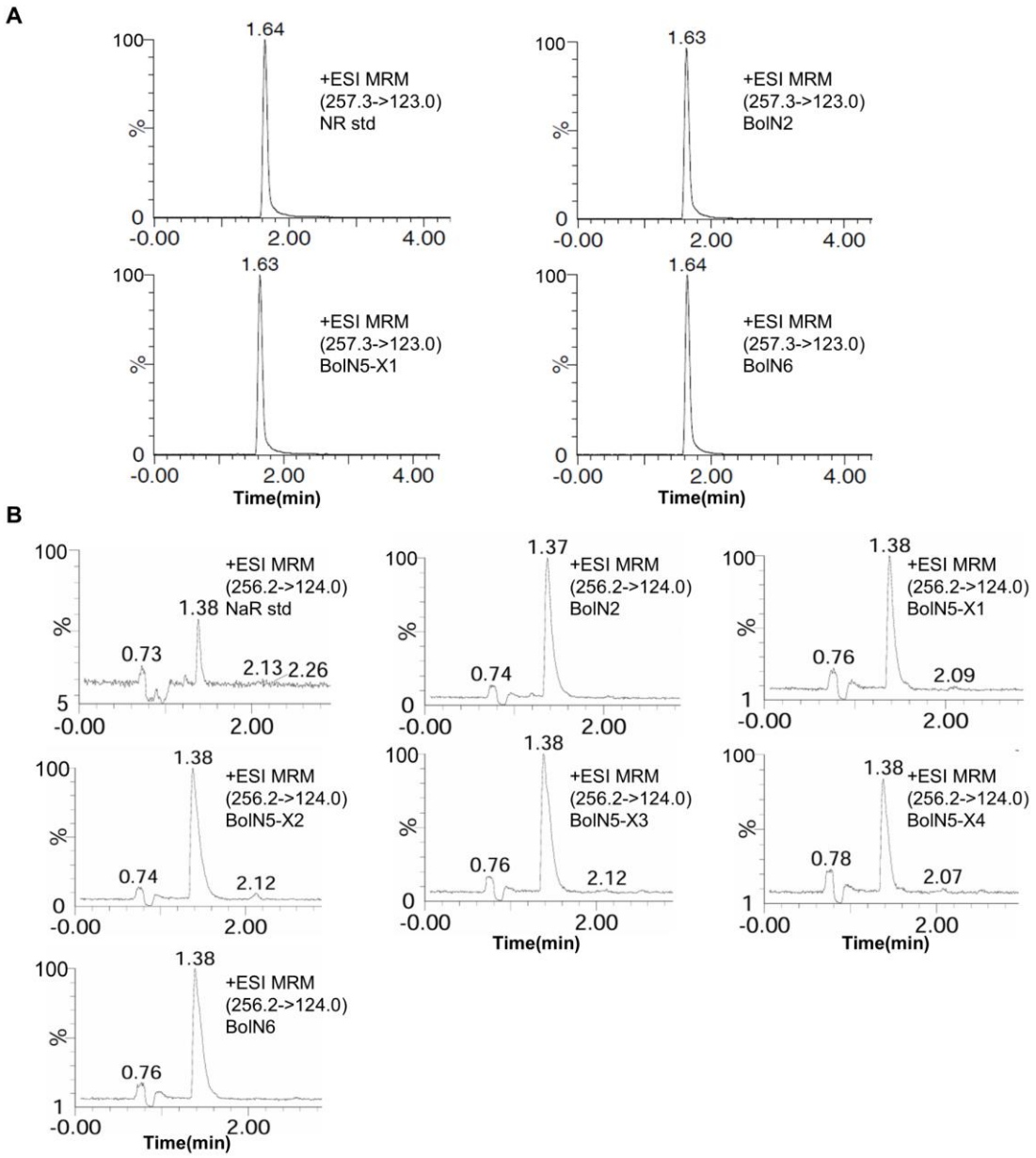
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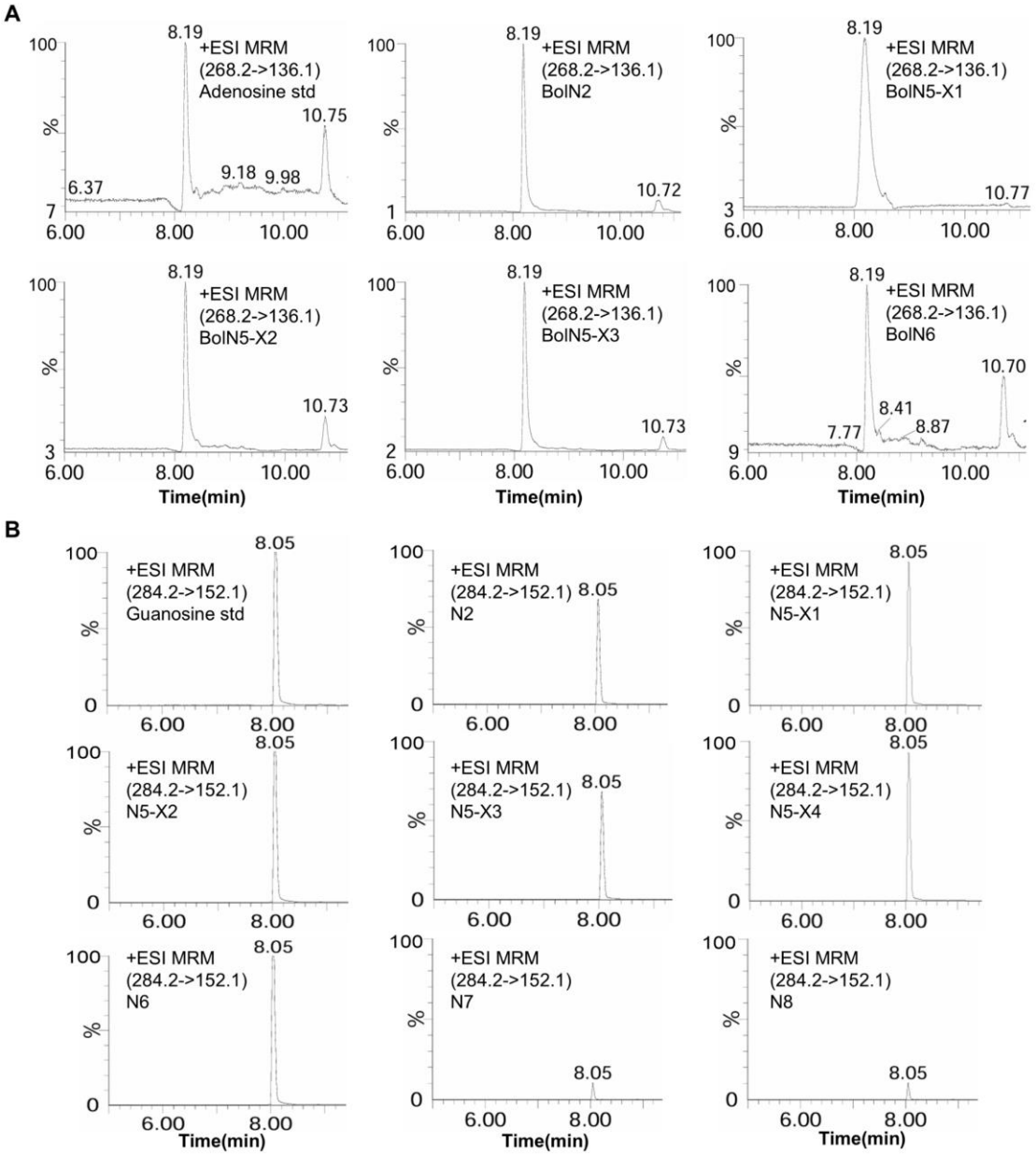
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**附图1** 以NMN **(A)**和NaMN **(B)**为底物, BolN2、BolN5和BolN6酶学反应产物的LC-MS/MS分析
NR std: NR 标准品; NaR std: NaR 标准品

**Appendix figure 1** LC-MS/MS analysis of enzymatic reaction products of BolN2, BolN5, and BolN6 with NMN **(A)** and NaMN **(B)** as substrates
NR std: NR standard; NaR std: NaR standard

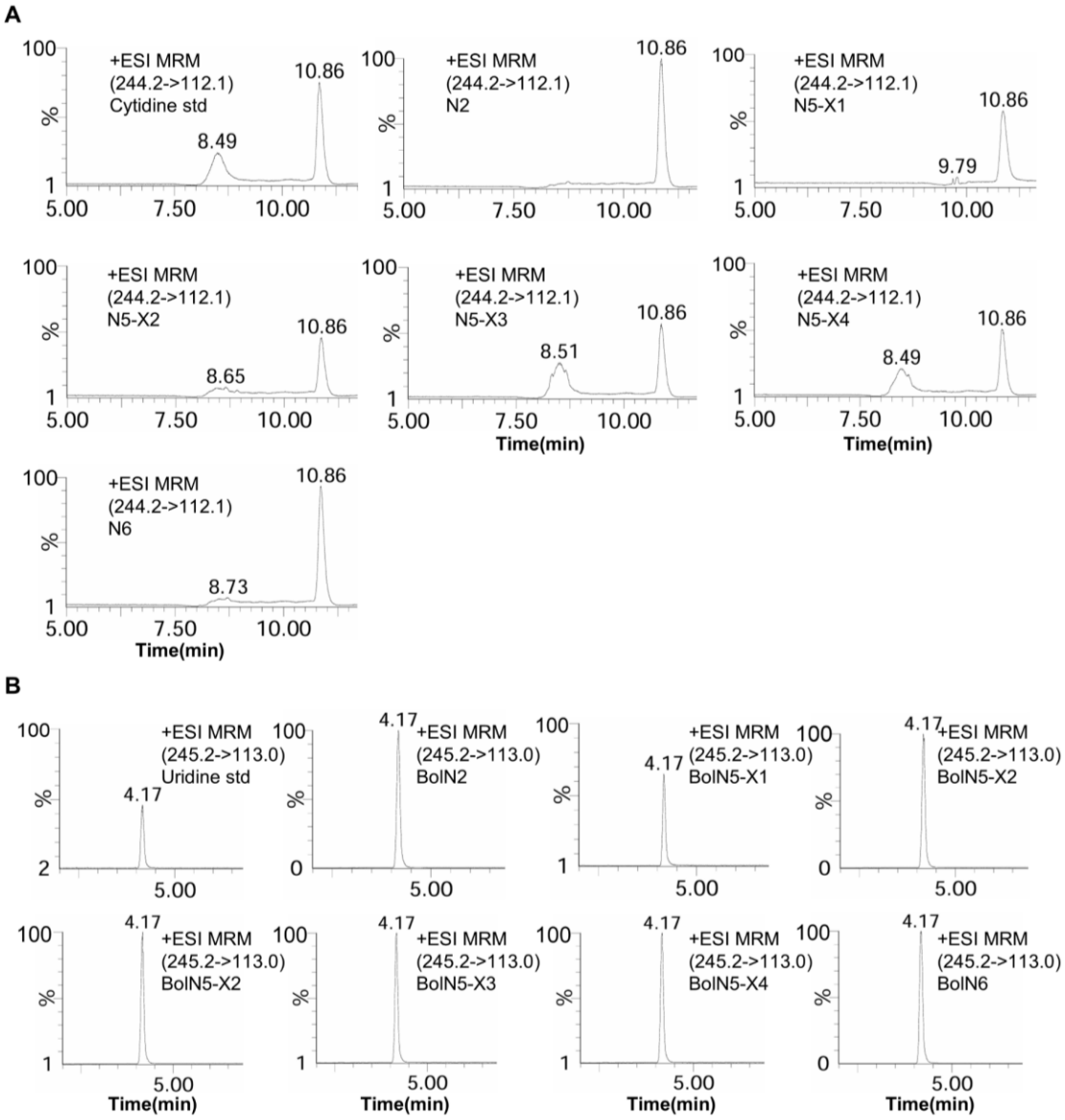



**附图2**　以AMP **(A)**和GMP **(B)**为底物, BolN2、BolN5、BolN6、BolN7和BolN8酶学反应产物的LC-MS/MS分析

AMP std: NR 标准品; NaR std: NaR 标准品

**Appendix figure 2**　LC-MS/MS analysis of enzymatic reaction products of BolN2, BolN5, BolN6, BolN7, and BolN8 with AMP **(A)** and GMP **(B)** as substrates

Adenosine std: Adenosine standard; Guanosine std: Guanosine standard




**附图3** 以CMP **(A)**和UMP **(B)**为底物, BolN2、BolN5和BolN6酶学反应产物的LC-MS/MS分析
Cytidine std: Cytidine 标准品; Uridine std: Uridine 标准品

**Appendix figure 3** LC-MS/MS analysis of enzymatic reaction products of BolN2, BolN5, and BolN6 with CMP **(A)** and UMP **(B)** as substrates
Cytidine std: Cytidine standard; Uridine std: Uridine standard