

· 研究报告 ·

## 拟南芥 *AtR8* lncRNA 对盐胁迫响应及其对种子萌发的调节作用

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**摘要** 长链非编码RNA (lncRNA) 是一类长度大于200个核苷酸且不编码蛋白质的非编码RNA, 主要由RNA聚合酶II转录生成, 大量存在于生物体内并具有多种生物学功能。*AtR8* lncRNA是拟南芥(*Arabidopsis thaliana*)中RNA聚合酶III转录的长链非编码RNA。前期研究发现, 水杨酸(SA)处理诱导萌发种子中*AtR8* lncRNA的表达, *AtR8* lncRNA缺失抑制SA胁迫下的种子萌发。进一步研究发现, *AtR8* lncRNA转录区域内存在保守的盐胁迫响应元件(TCTTCTTCTTTA); NaCl处理抑制萌发种子中*AtR8* lncRNA的表达; 与野生型相比, 高浓度NaCl处理明显抑制了*atr8* (*AtR8* lncRNA部分缺失型拟南芥)种子萌发。研究表明, *AtR8* lncRNA在拟南芥种子萌发期的盐胁迫中起重要作用。

**关键词** 长链非编码RNA, 拟南芥, 种子萌发, 盐胁迫

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种子是植物特有的繁殖器官。种子萌发是指有活力的种子吸胀后, 重新开始物质合成与代谢活动, 促使胚根露出种皮的过程。种子萌发极易受到光照、温度、水分和盐分等外部环境因素的影响, 同时受多基因调控。例如, 脱落酸(abscisic acid, ABA)胁迫下, WRKY41通过直接调节*ABI3* (*ABA-insensitive 3*)的表达影响拟南芥(*Arabidopsis thaliana*)种子萌发(Ding et al., 2014); WRKY2通过影响ABA信号通路中*ABI3*、*ABI5* (*ABA-insensitive 5*)、*EM1*和*EM6* (*early methionine-labeled 1 and 6*)的表达, 抑制拟南芥种子萌发(Jiang and Yu, 2009); WRKY6通过影响RAV1 (related to *ABA-insensitive3/viviparous1*)及其下游*ABI3*、*ABI4*和*ABI5*的表达调节拟南芥的种子萌发(Huang et al., 2016)。此外, 还有研究表明, RSM1 (*radialis-like sant/MYB 1*)与HY5 (*elongated hypocotyl 5*)/HYH (*HY5 homolog*)结合后聚集在*ABI5*启动子上,

调节*ABI5*及其下游ABA应答基因的表达, 从而改变拟南芥萌发种子对ABA、NaCl以及甘露醇的敏感性(Yang et al., 2018)。CAMTA6 (*calmodulin-binding transcription activator 6*)通过调节Na<sup>+</sup>稳态和盐胁迫耐受性相关基因的表达, 影响拟南芥萌发种子对ABA的敏感性和盐胁迫的耐受性(Shkolnik et al., 2019)。尽管如此, 种子萌发的详细分子机制仍不十分清楚。

非编码RNA (non-coding RNA, ncRNAs)不编码蛋白质, 但却大量存在于生物体中发挥重要作用。长度大于200个核苷酸的非编码RNA为长链非编码RNA (long non-coding RNA, lncRNA), 它们通过充当诱饵、支架和增强子的方式调节许多基因的表达, 在剂量补偿(dosage compensation)、基因组印记(genomic imprinting)、X染色体失活(X chromosome inactivation)、发育和环境胁迫等生物过程中发挥重要作用(黄小庆等, 2015)。现已发现植物中Pol II转录

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的一些功能性lncRNAs, 并对其作用机制进行了解析。*AtIPS1* (*induced by phosphate starvation 1*)通过模仿*miR399*的靶基因*PHO2*抑制*miR399*活性, 影响磷酸盐饥饿条件下拟南芥根的生长(Franco-Zorrilla et al., 2007; 黄小庆等, 2015); *COOLAIR* (*cold induced antisense intragenic RNA*)通过启动子转录干扰方式抑制开花调控基因*FLC*的表达, 进而调节植物开花时间(Swiezewski et al., 2009; Heo and Sung, 2011; 黄小庆等, 2015); *COLD AIR* (*cold assisted intronic noncoding RNA*)能引起*FLC*基因位点的组蛋白修饰, 导致*FLC*表观遗传沉默, 从而诱导植物快速开花(Kim and Sung, 2012; 黄小庆等, 2015); 水稻(*Oryza sativa*) *LDMAR* (*long-day-specific male-fertility-associated RNA*)序列中C碱基突变成G导致其启动子区域甲基化, 抑制其表达, 引起NK58S雄性不育(Ding et al., 2012; 黄小庆等, 2015)。热胁迫下, RNA测序分析发现了白菜(*Brassica pekinensis*)中4 594个lncRNA, 其中lncRNA (*TCONS\_00048391*)通过模拟*miR164a*的靶基因*NAC1*, 影响白菜的耐热性(Wang et al., 2019)。Wu等(2019b)通过RNA-seq分析了梅花(*Armeniaca mume*)雌蕊发育中lncRNA的表达谱, 发现一些lncRNAs及其靶基因在雌蕊分化和花发育中发挥作用, 其中*XR\_514690.2*和*TCONS\_00032517*两个lncRNA可能在梅花多个雌蕊形成过程中发挥作用。Zhang等(2018)通过高通量测序, 发现*LNC1*和*LNC2*两个lncRNA可以作为*miR156a*和*miR828a*的靶基因, 分别抑制*SPL9*和诱导*MYB114*的表达, 调节沙棘(*Hippophae rhamnoides*)果实中花青素的合成。Qin等(2017)通过转录组分析发现, *DRIR* (*drought induced lncRNA*)能够增强拟南芥对干旱和盐胁迫的耐受性及对ABA的敏感性。Zhao等(2018)研究表明, *MAS* (*MAF4* (*mads affecting flowering 4*)的反义转录lncRNA)通过与*WDR5a*相互作用激活*MAF4*的转录, 从而调节拟南芥的开花时间。Liu等(2019)研究发现, *T5120* lncRNA过表达促进拟南芥硝酸盐同化并提高氮的利用率, 进而促进根生长。Zhang等(2019)研究表明, lncRNA973通过调节一系列盐胁迫相关基因的表达进而调控棉花(*Gossypium* spp.)对盐胁迫的响应。

近年来, 萌发相关ncRNA逐渐被发现并被解析, 如*miR156*、*miR159*、*miR167*和*miR9678*。拟南芥

*miR156*基因下调*SPL13* (*SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE13*)的表达, 延迟萌发后幼苗的发育(Martin et al., 2010a, 2010b)。*miR159*通过影响*MYB101*和*MYB33*的表达介导拟南芥的种子萌发(Reyes and Chua, 2007)。*miR167*通过调节*ARF*基因的表达影响生长素的信号转导, 在种子萌发以及胚发育过程中起重要作用(Rhoades et al., 2002)。*miR9678*通过调节GA/ABA信号转导影响小麦(*Triticum aestivum*)的种子萌发(Guo et al., 2018)。近几年, 参与种子萌发的长链非编码RNA也被发现。Xu等(2018)研究表明, 萌发的蓖麻(*Ricinus communis*)种子中多个DNA甲基化相关的lncRNA在胚乳和胚胎发育中起重要作用。Zhu等(2017)在玉米(*Zea mays*)种子中鉴定出753个lncRNA, 其中7个新lncRNA可能参与了玉米种子的发育和代谢过程。Yin等(2018)从发育的牡丹(*Paeonia suffruticosa*)种子中鉴定出22 430个lncRNA, 并预测39个lncRNA可能参与种子的脂肪酸合成和脂质代谢过程。*BoNR8* lncRNA是甘蓝(*B. oleracea*)中RNA聚合酶III转录的长链非编码RNA, 拟南芥中*BoNR8*过表达影响ABA信号通路中重要基因的表达, 并抑制根生长和果实发育, 降低萌发种子对ABA的敏感性和盐胁迫的耐受性(Wu et al., 2019a)。

前期研究中, 我们根据RNA聚合酶III的转录活性及其转录的非编码RNA基因结构特征, 发现了拟南芥中RNA聚合酶III转录的*AtR8* lncRNA (259 nt), 其在幼苗根端细胞质中大量表达, 并响应低氧胁迫(Wu et al., 2012); 且水杨酸(salicylic acid, SA)处理诱导萌发种子中*AtR8* lncRNA的表达, *AtR8* lncRNA缺失降低SA胁迫下的种子萌发(Li et al., 2016)。我们进一步研究发现, *AtR8* lncRNA转录区域内包含保守的盐胁迫响应元件, 盐胁迫处理影响萌发种子中*AtR8* lncRNA的表达, 且*AtR8* lncRNA缺失降低了萌发种子对盐胁迫的耐受性, 表明*AtR8* lncRNA在拟南芥种子萌发期盐胁迫中起重要作用。

## 1 材料与方法

### 1.1 植物材料

实验材料为野生型拟南芥(*Arabidopsis thaliana* L.)和*AtR8* lncRNA部分缺失型拟南芥突变体(*atr8*, FLAG-

410H04), 后者购自凡尔赛拟南芥储备中心(versailles *Arabidopsis* stock center (<http://publiclines.versailles.inra.fr/>)) (Li et al., 2016)。

## 1.2 各种应激应答处理及萌发率、鲜重和干重统计

将相同批次拟南芥野生型和 *atr8* 各50粒种子经70% (v/v) 乙醇溶液和5% (v/v) 次氯酸钠溶液表面消毒后, 播种在1/2MS固体培养基和分别含有50、100、150和200 mmol·L<sup>-1</sup> NaCl的培养基上。4℃吸胀处理72小时后, 于22℃ (16小时光照/8小时黑暗) 培养箱中培养; 统计1、2、3、4、5、6和7天的种子萌发率(以胚根突破种皮为萌发标准)。称量萌发7天种子的鲜重, 70℃烘箱处理2天后, 称量萌发种子的干重。每个实验重复3次, 然后进行统计学分析。

## 1.3 RNA提取方法

RNA提取参照Martin等(2005)和刘春晓等(2019)文献所述方法。称取0.1 g拟南芥干种子或萌发种子于液氮中彻底研磨后, 加入1 mL RNA提取液(45.5% (v/v) 苯酚, 9% (v/v) 氯仿, 0.45% (w/v) SDS, 41 mmol·L<sup>-1</sup> LiCl, 2 mmol·L<sup>-1</sup> EDTA, 5.9 mmol·L<sup>-1</sup> β-巯基乙醇, 82 mmol·L<sup>-1</sup> Tris-HCl), 混匀后离心。吸取上清, 加入等体积的PCI溶液(苯酚:氯仿:异戊醇=25:24:1, v/v/v); 离心后取上层溶液, 加入等体积的氯仿, 室温孵育; 离心取上层溶液加入1/3体积的8 mol·L<sup>-1</sup> LiCl, 于-20℃静置过夜。次日, 离心取上清并加入1/4体积的异丙醇, 于-20℃静置30分钟; 离心取上清并加入3/5体积的异丙醇, -20℃静置30分钟; 离心后得RNA沉淀, 使用75%乙醇漂洗后加入适量的焦碳酸二乙酯(diethyl pyrocarbonate, DEPC)水, 于-80℃冰箱保存。

## 1.4 Northern分析

将经6%聚丙烯酰胺凝胶电泳分离的RNA转移至尼龙膜上, UV交联固定后, 于42℃杂交箱预杂交1小时; 加入变性地高辛标记 *AtR8* lncRNA 特异性RNA探针, 于42℃过夜杂交; 杂交后的膜于2× SSC (含0.1% SDS)及0.2× SSC (含0.1% SDS)洗液中清洗2次; 室温封闭1小时后, 与稀释2 000倍的Anti-Digoxigenin-AP抗体反应2小时; 马来酸洗液洗膜3次; CDP-Star 暗处反应15分钟后, 使用LAS-4 000化学发光系统

检测信号。

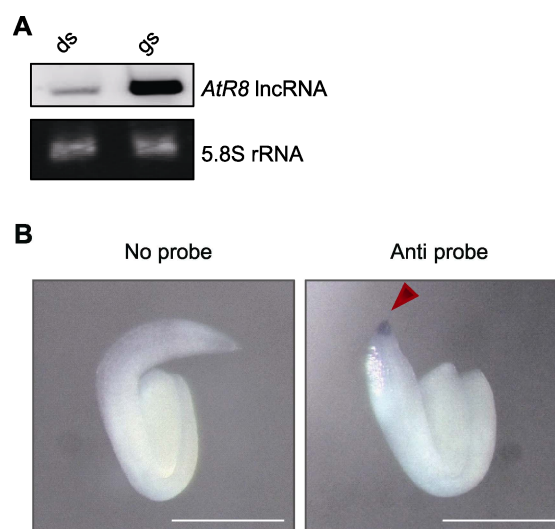
## 1.5 整体原位杂交(*in situ*)

将萌发2天的拟南芥种子经固定液(4% (w/v) 多聚甲醛, 15% (v/v) DMSO, 0.1% (v/v) tween 20, 0.08 mol·L<sup>-1</sup> EGTA (pH8.0)) 4℃固定3天后, 进行脱水、水饱和及蛋白酶K处理。与地高辛标记 *AtR8* lncRNA 特异性探针于42℃过夜杂交; 杂交后的样品经水洗加入blocking封闭2小时, 于4℃与稀释1 500倍的Anti-Digoxigenin-AP抗体反应过夜, 经BM Purple 检测信号。

## 2 结果与讨论

### 2.1 种子萌发过程中 *AtR8* lncRNA 的表达特性分析

为确定拟南芥种子萌发过程中 *AtR8* lncRNA 的表达特性, 提取其干种子和萌发48小时种子的RNA进行



**图1** 拟南芥种子萌发过程中 *AtR8* lncRNA 的表达特性分析 (A) 拟南芥种子萌发过程中 *AtR8* lncRNA 表达特性的Northern 分析(以5.8S rRNA作为上样对照); (B) 拟南芥种子萌发过程中 *AtR8* lncRNA 组织表达特性的整体原位杂交(箭头指示 *AtR8* lncRNA 信号) (Bars=200 μm)。

**Figure 1** Analysis of *AtR8* lncRNA expression during seed germination in *Arabidopsis thaliana*

(A) Northern blotting analysis of *AtR8* lncRNA expression during seed germination (expression of 5.8S rRNA serves as loading controls); (B) *In situ* hybridization of *AtR8* lncRNA expression during seed germination (arrow indicates *AtR8* lncRNA signal) (Bars=200 μm).

Northern分析,发现*AtR8* lncRNA在萌发种子中大量表达(图1A)。整体原位杂交实验进一步确认*AtR8*

lncRNA于萌发种子的根尖端大量表达(图1B),表明拟南芥萌发种子中*AtR8* lncRNA的表达具有高度的

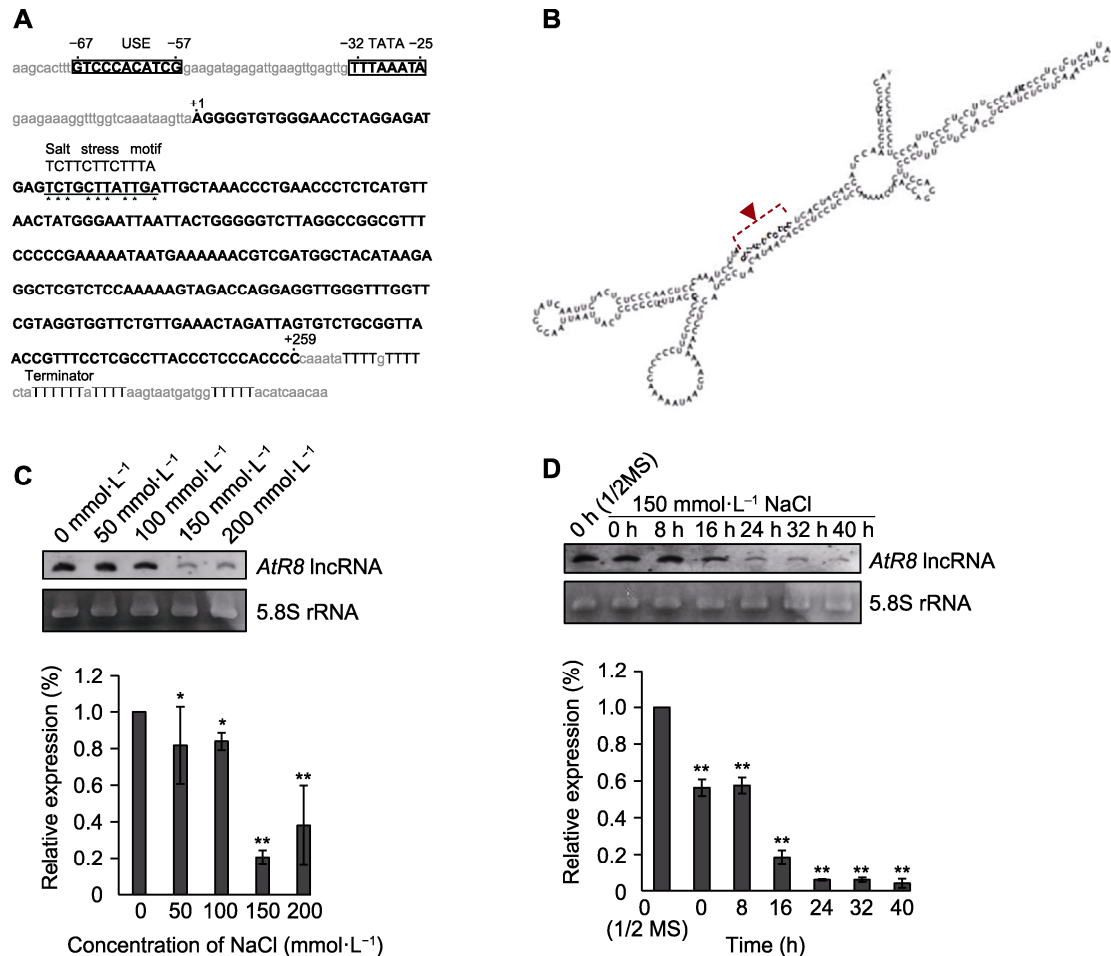


图2 拟南芥种子萌发过程中*AtR8* lncRNA表达响应NaCl逆境胁迫

(A) *AtR8* lncRNA与UCC盐胁迫响应元件的序列比较分析(USE、TATA启动子序列为大写、加粗并加框, *AtR8* lncRNA转录区域为大写并加粗, 保守的盐胁迫响应元件用星号标注)。(B) *AtR8* lncRNA二级结构中盐胁迫响应元件存在位置的RNAlogo预测(盐胁迫响应元件为大写、加粗并用箭头指出)。(C) 拟南芥种子萌发过程中不同浓度NaCl处理下*AtR8* lncRNA表达特性的Northern分析,以5.8S rRNA作为上样对照。下方为Northern半定量分析,2个独立的实验给出了相似的结果,并显示了1个代表性的例子。值为平均值±标准误(*t*-检验, \**P*<0.05, \*\**P*<0.01)。(D) 拟南芥种子萌发过程中150 mmol·L<sup>-1</sup> NaCl处理不同时间*AtR8* lncRNA表达特性的Northern分析,以5.8S rRNA作为上样对照。下方为Northern半定量分析,2个独立的实验给出了相似的结果,并显示了1个代表性的例子。值为平均值±标准误(*t*-检验, \*\**P*<0.01)。

Figure 2 *AtR8* lncRNA expression during seed germination of *Arabidopsis thaliana* after NaCl treatment

(A) Sequence comparison between *AtR8* lncRNA and UCC salt stress-responsive element (the USE and TATA promoter sequences are capitalized, bloded and framed; the *AtR8* lncRNA transcriptional region is capitalized and bolded; and the conserved salt stress-responsive element is marked with asterisk). (B) The location of the salt stress-responsive element in the secondary structure of *AtR8* lncRNA predicted by RNAlogo (the salt stress-responsive motif is capitalized, bloded and indicated with an arrow). (C) Northern blotting analysis of *AtR8* lncRNA expression in germinating seeds under different NaCl treatments, 5.8S rRNA was used as a loading control. The lower panel shows semi-quantitative analysis of the Northern blotting signals. Two independent experiments gave similar results, and a representative example is shown. Values are means ± SE (*t*-test, \**P*<0.05, \*\**P*<0.01). (D) Northern blotting analysis of *AtR8* lncRNA in germinating seeds under different periods of 150 mmol·L<sup>-1</sup> NaCl treatment, 5.8S rRNA was used as a loading control. The lower panel shows semi-quantitative analysis of the Northern blotting signals. Two independent experiments gave similar results, and a representative example is shown. Values are means ± SE (*t*-test, \*\**P*<0.01).

组织特异性。

## 2.2 种子萌发过程中 *AtR8* lncRNA 表达响应盐胁迫

UCC 盐胁迫响应元件(TCTTCTTCTTTA)是盐应答基

因中高度保守的序列。Di等(2014)证明长链非编码 RNA 中存在该保守UCC元件。因此, 我们比较了 *AtR8* lncRNA 序列与UCC元件, 发现 *AtR8* lncRNA 转录区域内存在UCC盐胁迫响应元件, 序列相似性达75% (9 bp/12 bp) (图2A), 这表明 *AtR8* lncRNA 可能响应

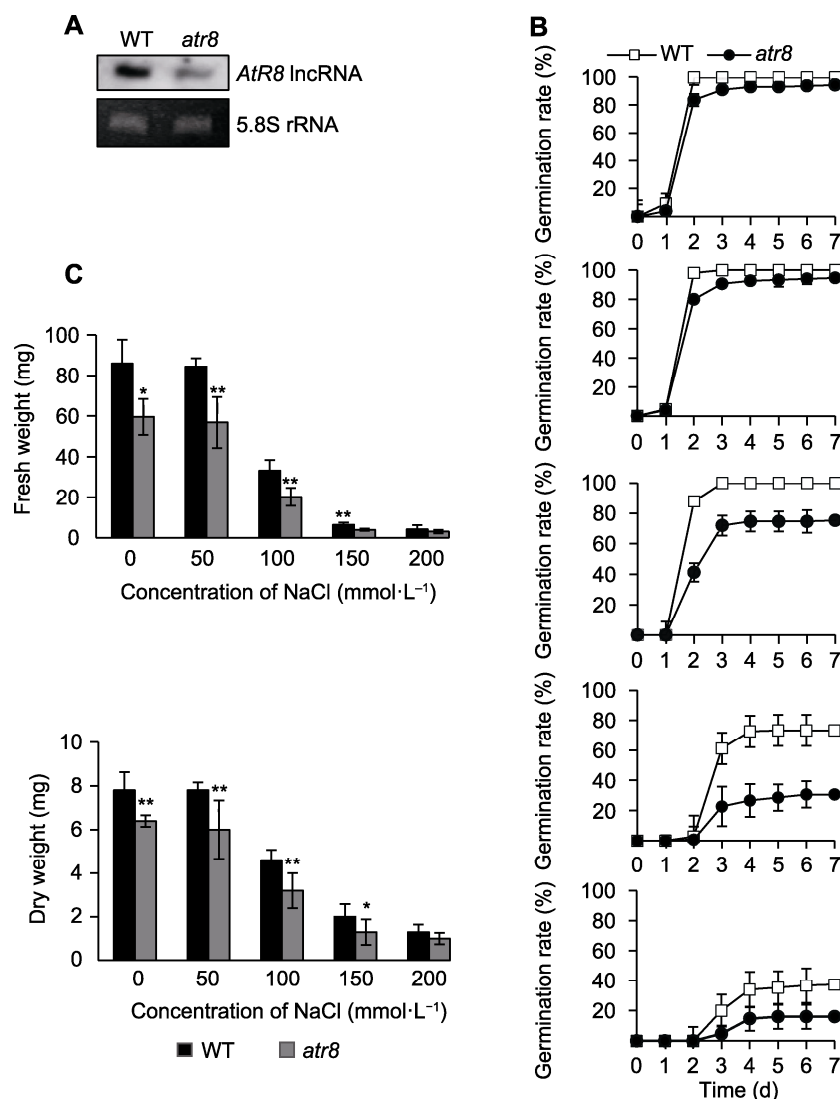


图3 盐胁迫下 *AtR8* lncRNA 缺失抑制拟南芥种子萌发

(A) Northern分析鉴定 *AtR8* lncRNA 缺失突变体 *atr8* (以5.8S rRNA作为上样对照); (B) 不同浓度NaCl处理下, 野生型和 *atr8* 种子生长状况及萌发率统计分析(数据为3次独立试验的平均值, 误差为标准误); (C) 不同浓度NaCl处理下, 野生型和 *atr8* 萌发7天种子的鲜重及干重(数值为3次独立试验的平均值, 误差为标准误, 星号表示 *atr8* 与野生型的显著性差异( $t$ -检验,  $*P < 0.05$ ,  $**P < 0.01$ )). WT: 野生型

Figure 3 Loss of *AtR8* lncRNA inhibits *Arabidopsis thaliana* seed germination under salt stress

(A) Northern blotting analysis of the *AtR8* lncRNA loss-of-function mutant *atr8* (5.8S rRNA was used as the loading control); (B) Statistical analysis of growth and germination rate of the wild-type and *atr8* seeds under different concentrations of NaCl (data are average of three independent experiments, and bars indicate standard error); (C) Fresh and dry weight of the wild-type and *atr8* seeds after 7 d of germination under NaCl treatment (the values are average of three independent experiments, and bars indicate standard error (the asterisk indicate significant differences between *atr8* and wild type ( $t$ -test,  $*P < 0.05$ ,  $**P < 0.01$ )). WT: Wild type

盐胁迫。RNAlogo (<http://rnaplogo.mbc.nctu.edu.tw/index.php>)预测到UCC元件存在于*AtR8* lncRNA二级结构的茎环相连位置(图2B)。

此外,实验结果表明,50、100、150和200 mmol·L<sup>-1</sup> NaCl处理均抑制*AtR8* lncRNA的表达(图2C),且150 mmol·L<sup>-1</sup> NaCl的抑制作用最明显。150 mmol·L<sup>-1</sup> NaCl分别处理0、8、16、24、32和40小时的Northern分析结果进一步表明,处理时间越长,*AtR8* lncRNA表达受抑制越明显(图2D)。

### 2.3 盐胁迫下*AtR8* lncRNA缺失抑制种子萌发

乔慧萍等(2007)研究表明,盐胁迫对种子萌发具有抑制作用。我们使用*AtR8* lncRNA部分缺失型突变体(*atr8*),调查了盐胁迫下*AtR8* lncRNA缺失是否影响种子萌发(图3A)(Li et al., 2016)。结果表明,正常培养条件下,*atr8*种子萌发率低于野生型。50 mmol·L<sup>-1</sup> NaCl处理时,野生型和*atr8*种子萌发率与正常培养条件类似。100、150和200 mmol·L<sup>-1</sup> NaCl处理时,野生型和*atr8*种子萌发均受到抑制,但对*atr8*种子萌发的抑制作用更明显(图3B)。对萌发7天的野生型与*atr8*种子鲜重和干重的分析结果表明,正常培养条件下野生型的鲜重和干重均大于*atr8*。随着NaCl浓度的增加,二者的鲜重和干重均明显降低,但*atr8*的鲜重和干重仍明显低于野生型(图3C),表明NaCl胁迫下,*AtR8* lncRNA缺失进一步抑制了拟南芥的种子萌发。

### 2.4 讨论

高盐环境会对植物造成离子毒害、渗透胁迫和矿质营养缺失,使其生理代谢紊乱,导致种子萌发和植株生长受到阻碍,严重降低植物的产量和品质(韩志平等, 2015)。植物耐盐性是多基因控制的数量性状,是多种耐盐生理性状的综合体现(孙兰菊等, 2001; 陈洁和林栖凤, 2003; 陆玉建等, 2012)。苏永全和吕迎春(2007)研究表明,盐胁迫通过增效、负效和完全阻抑效应影响种子的萌发。低盐可增强种子的呼吸作用,提高蛋白酶和脂肪酶活性,促进贮藏物质的转化,进而促进种子萌发和生长;高盐条件下,由于盐形成的渗透势阻碍种子吸水(盐浓度越高,阻碍作用越强),严重影响了种子内蛋白质等大分子物质的分解和合成进程,降低种子的发芽率、发芽指数和活力指数(苏永全和吕迎春, 2007; 郝雪峰等, 2013)。盐胁迫下,γ-

氨基丁酸(GABA)能够增强淀粉酶的活性,使种子获得更多营养和能量,改善种子的萌发质量(王泳超, 2016)。NaHS显著缓解了盐处理对种子萌发过程中水解酶活性的抑制作用(窦伟, 2010)。拟南芥U-Box泛素连接酶*AtPUB18*与*AtPUB19*双突变后降低了种子对高盐的敏感性(Bergler and Hoth, 2011; 张新宇等, 2014)。NAC57过表达拟南芥的种子发芽率、超氧化物歧化酶和过氧化物酶活性较高,种子的耐盐性增强(Yao et al., 2018)。目前,盐胁迫调节种子萌发的详细分子机制尚不十分清楚。

前期研究表明, RNA聚合酶III转录的*AtR8* lncRNA同源物*BoNR8* lncRNA大量存在于甘蓝萌发种子的根部(Wu et al., 2019a)。本研究发现, *AtR8* lncRNA在拟南芥萌发种子的根尖端特异表达,表明*AtR8* lncRNA与*BoNR8* lncRNA均在种子萌发阶段特异性表达,这表明种子萌发过程中RNA聚合酶III具有较高的转录活性,可以转录与萌发相关的特殊非编码RNA。*BoNR8* lncRNA和*AtR8* lncRNA转录区域内都存在盐胁迫响应元件。盐胁迫下, *BoNR8* lncRNA被诱导表达, *AtR8* lncRNA的表达则被抑制,表明*AtR8* lncRNA与*BoNR8* lncRNA均响应盐胁迫但表达趋势不同。拟南芥中*BoNR8* lncRNA过表达影响ABA信号中RAV1、ABI3、ABI5、EM1和EM6等重要基因的表达,抑制正常培养条件下的种子萌发、角果发育和幼苗根生长,高盐胁迫进一步抑制种子萌发。*AtR8* lncRNA缺失抑制正常培养条件下的种子萌发,高盐胁迫也进一步抑制种子萌发,但不影响根生长,表明*BoNR8* lncRNA与*AtR8* lncRNA均参与拟南芥的生长发育和盐胁迫响应过程,但它们的生物学功能存在差异。后续,我们将从分子水平及遗传学角度详细分析种子萌发过程中*AtR8* lncRNA参与盐胁迫的作用机制,为培育优质丰产的耐盐作物新品种提供理论依据。

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## Response of *AtR8* lncRNA to Salt Stress and Its Regulation on Seed Germination in *Arabidopsis*

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**Abstract** Long non-coding RNA (lncRNA) is a type of non-coding RNA that is longer than 200 nucleotides and does not encode proteins. lncRNAs are mainly produced by the transcription of RNA polymerase II and are abundant in the organism and have various biological functions. *AtR8* lncRNA is transcribed by RNA polymerase III in *Arabidopsis thaliana*. Previous studies revealed that Salicylic acid (SA) induces *AtR8* lncRNA expression in germinated seeds and that the deletion of *AtR8* lncRNA decreases seed germination under SA stress. In this study, we found a conserved salt-stress-responsive element (TCTTCTTCTTTA) in the transcriptional region of *AtR8* lncRNA. NaCl treatment inhibited *AtR8* lncRNA expression in the germinated seeds. High concentration of NaCl significantly inhibited seed germination of *atr8*, which had partial deletion of *AtR8* lncRNA, compared to that of the wild type, indicating that *AtR8* lncRNA plays an important role in regulating seed germination in response to salt stress.

**Key words** long non-coding RNA, *Arabidopsis thaliana*, seed germination, salt stress

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