过表达苜蓿*MsDREB1*基因大豆耐旱性分析

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摘要: 本研究目的是利用转基因技术改良大豆(*Glycine max*)的耐旱性, 并研究rd29A和CaMV-35S两类启动子的驱动效果。利用构建的转基因载体pCAMBIA-rd29A-MsDREB1和pCAMBIA-35S-MsDREB1, 通过农杆菌(*Agrobacterium tumefaciens*)介导法, 将苜蓿(*Medicago sativa*)基因*DREB1*导入大豆品种'中黄13号', 获得rd29A和CaMV-35S两类启动子驱动的*MsDREB1*转基因大豆。对T_1至T_2代植株进行PCR、Southern blot分析, 分别筛选到9和12个转基因大豆株系, 各随机选择两个转基因株系作为研究对象。正常水分状态下初花期统计大豆株高及叶面积。苗龄30 d的植株在不同干旱胁迫条件下, 用逆转录定量PCR (RT-qPCR)分析基因表达差异, 测定叶绿素含量、丙二醛含量、相对含水量及植株干重, 并分析各株系干旱后复水的成活率。结果表明, 两种启动子对*MsDREB1*表达的调控存在明显差异, 在非胁迫下35S启动子调控的*MsDREB1*为超量表达, 而rd29A启动子调控的*MsDREB1*表达量较低; 在严重干旱胁迫下, rd29A:MsDREB1表达量高于35S:MsDREB1表达量; *MsDREB1*超量表达抑制植株正常生长。两种启动子各转基因株系均有一定耐旱能力, 但存在差异。*MsDREB1*诱导表达耐旱性效果更明显, 在中度干旱胁迫下, 其植株相对含水量、叶绿素含量、单株干重均显著高于*MsDREB1*超量表达, 而丙二醛含量显著低于*MsDREB1*超量表达。结果说明*MsDREB1*作为转录调节因子参与了植物的干旱调节。该研究为*MsDREB1*基因在大豆耐旱基因工程中的应用提供方法。

关键词: 大豆; MsDREB1; rd29A启动子; 耐旱性

在各种非生物胁迫中, 干旱是限制作物生产的最普遍因素。植物对干旱的响应是由其形态、生理、细胞和生化等变化来综合反应的。干旱相关的环境信号首先被特异性受体感知, 激活后启动级联传递并激活转录因子(transcription factors, TFs), 该转录因子再激活特定基因表达, 随后诱导植物体代谢变化导致植物耐旱性增加(Shinozaki和Yamaguchi-Shinozaki 2007; Tran等2010; Krasensky和Jonak 2012; Jogaiah等2013; Zandkarimi等2015; Chen等2016)。非生物胁迫条件下的基因表达涉及依赖脱落酸(abscisic acid, ABA)和不依赖ABA两种类型(Mizoi等2012; Osakabe等2013a, b; Yoshida等2014; Li等2015; Chen等2016)。DREB属于AP2/EREBP类转录因子, DREB转录因子可以通过与DRE/CRT顺式作用元件特异结合激活大量下游基因表达, 从而使植物较好应对逆境胁迫的危害。Liu等(1998)通过实验证实在35S:DREB转基因拟南芥中有12个基因的表达量较对照增加至少一倍。这些基因产物都可能在植物抗逆中发挥作用。因此可将*DREB*基因作为植物抗逆基因工程的一个工具, 综合改善植物抗逆性。大量转基因实验证明了这一设想。例如, 在拟南芥(*Arabidopsis thaliana*)中过表达酵母*DREB*基因或*OsDREB1*A基因, 提高了转基因植株的抗冻和抗旱能力(Dubouzet等2003); 在烟草(*Nicotiana tabacum*)中过表达*AhDREB1*基因提高了转基因植株的抗旱及耐盐能力(Shen等2003); 在番茄(*Solanum lycopersicum*)中过表达*AtCBF1*基因提高了转基因植株的抗冻能力(Hsieh等2002)。大量实验表明转化一个*DREB*基因因子与转化其他单一基因如脯氨酸合成酶基因、甜菜碱合成酶基因相比能收到更好的抗逆效果, 且往往可以改善植株对几种逆境胁迫的耐受力。同时, 人们发现组成型启动子35S启动子驱动*DREB*基因过表达往往会导致转基因植株矮化现象的发生(Liu等1998; Ito等2006), 为避免这一不利因素, Kasuga等(1999)利用rd29A启动子替代35S启动子驱动*DREB*基因在拟南芥和烟草中表达, 有效解决了植株矮化的问题(Pellegrineschi等2004)。也有人认为这样做没有必要, 因为最近在水稻(*Oryza sativa*)中组成型过表达*CBF3*和*ABF3*基因, 既提高了转基因水稻的抗旱、耐盐能力, 又没有发现转基因植株任何矮化或表型异常现象(Oh等2005), 其中的原因还需进一步研究。在花椰菜花叶病毒(*Cauliflower mosaic virus*) CaMV-35S启动子控制下, 转基因拟南芥过表达*AtDREB1D*具有较强的耐旱、耐冻性(Haake等2002)。基因植株的northern

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blot分析表明, 在正常和冷胁迫下, *AtDREB1D* mRNA水平均比野生型(wildtype, WT)植株高很多, 这表明*AtDREB1D*对拟南芥适应干旱胁迫的信号转导中有重要作用(Haake等2002)。

然而, 在正常生长条件下, *AtDREB1*D结构性过表达造成植物严重生长迟缓。近年来, 过表达葡萄(*Vitis vinifera*) *VrCBF1*和*VrCBF4*基因表现出降低株高和不利于生长的现象, 虽然转入的目的基因表达量很高, 过表达的转基因株系耐冷性增加(Siddiqua等2011)。为了克服这些对植物生长的负面影响, 由诱导表达的转基因植物显得特别重要, 其只有在胁迫条件下才高度表达。有研究报道, 使用不同的启动子如ABA或非生物胁迫诱导的启动子(Kasuga等2004; Fu等2007; 吕兆勇等2016)去诱导水稻、烟草的*DREB*基因使花生(*Arachis hypogaea*)、大豆和小麦(*Triticum aestivum*)的耐旱性提高(Oh等2005; Chen等2008; Bhatnagar-Mathur等2009; Kong等2016)。因此, 这些基因是可能用于提高作物抗旱性的研究与应用的。

大豆是全球主要油料作物, 最大的植物油和蛋白质饲料来源。在开花阶段这一大豆生长最关键的时期水分亏缺将增加花和荚的败育率, 从而导致最终产量的减少。在大豆生产实践中, 干旱导致产量减少可高达40% (Westgate和Peterson 1993)。因此, 寻找在水分匮缺条件下缓解或改善大豆生长和发育的方法是至关重要的。与传统育种相比, 通过基因工程直接引入基因是一个有效和快速的提高大豆非生物胁迫的耐受性的方法。本试验将*MsDREB1*转录因子转入大豆基因组, 通过rd29A诱导型启动子和35S组成型启动子驱动*MsDREB1*基因表达, 用不同干旱胁迫处理来研究转基因植物耐旱性。对其进行生理和基因表达测定, 试图通过分析由不同启动子rd29A、CaMV-35S调控的*MsDREB1*转基因大豆耐旱性, 获得应用价值较高的耐旱转基因大豆, 为*MsDREB1*在大豆耐旱基因工程中的应用提供参考。

材料与方法

1 材料

本实验所用大豆[*Glycine max* (L.) Merr.]材料‘中黄13号’由驻马店市农科院提供, 使用Cl_2消毒16 h, 并在B5培养基中无菌培养。

2 质粒构建与转化

根据GenBank中拟南芥rd29A启动子序列(D13044)设计引物P1和P2 (表1)。以拟南芥基因组DNA为模板, 采用特异引物P1和P2, 进行PCR扩增反应, 扩增所得片段连接到T载体上。测序后, 与GenBank序列进行同源性比对。将载有目的基因rd29A启动子序列的T载体和表达载体pCAMBIA1301分别用限制性核酸内切酶*Bam*HI、*Kpn*I双酶切, 0.8%琼脂糖凝胶电泳检测酶切结果。使用DNA回收试剂盒回收目的片段和载体片段, 1.0%琼脂糖凝胶电泳检测回收效果, 把目的片段与载体以3:1的比例在缓冲液中用连接酶4°C过夜。转化后用PCR与酶切检测质粒。同理, 以苜蓿(*Medicago sativa* L.)基因组为模板, 用P3和P4作为引物, 克隆*MsDREB1*基因(EU233782)。把该目的基因插入到35S启动子和新合成的rd29A启动子之后(图1), 用冻融法把两种载体分别转入农杆菌(*Agrobacterium tumefaciens* Smith et Towns.)中。

表1 引物序列

Table 1 Primer sequences

名称	序列(5′→3′)
rd29A(F)-P1	CGGATCCGCCATAGATGCAATTCAATCAAACT
rd29A(R)-P2	GGGTACCCCAAAGATTTTTTTCTTTCCAATAGAAG
MsDREB1(F)-P3	CGGTACCACACCATTTTCCACTCTATCC
MsDREB1(R)-P4	GCTGCAGTTTCCTATTCTACGATCCAAA
RT-qPCR MsDREB1(F)-P5	CCCTTTGACGCATCATCACC
RT-qPCR MsDREB1(R)-P6	TTCCTCCCTGCTCGCTTCTT
RT-qPCR ACTIN2(F)-P7	TGATGGTGTGAGTCACACTGTACC
RT-qPCR ACTIN2(R)-P8	GGACAATGGATGGGCCAGACTC

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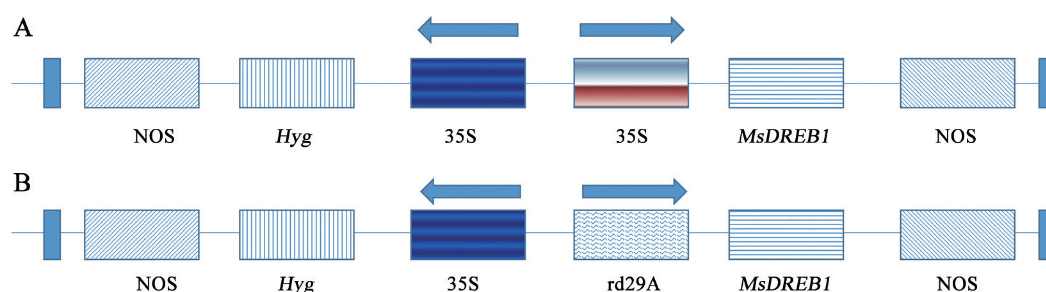

图1 重组表达载体结构简图

Fig.1 Construction of transgenic plant expression vectors

A: CaMV-35S启动子驱动的载体; B: rd29A启动子驱动的载体。NOS: 终止子; *Hyg*: 潮霉素抗性基因; 35S: 花椰菜花叶病毒启动子(组成型); rd29A: 拟南芥rd29A基因启动子(诱导型); *MsDREB1*: 苜蓿*DREB1*基因序列。

3 转基因大豆扩繁

采用农杆菌介导的子叶节转化法转化大豆(Ko等2003; 张银霞等2006)。利用潮霉素进行转基因植株筛选。抗性植株经炼苗后转土移栽。大豆种植在本校实验基地, 按常规水肥病虫害管理。

4 转基因大豆Southern blot和定量表达分析

4.1 转基因大豆Southern blot检测

参照Murray和Thompson (1980)的溴化十六烷基三甲铵(cetrimonium bromide, CTAB)法提取大豆基因组DNA。*MsDREB1*基因分析以P3、P4为引物(表1), 大豆基因组DNA为模板, 复性温度为55°C。大豆基因组DNA用*Eco*RI内切酶37°C酶解。采取以地高辛(digoxin)随机标记*MsDREB1*基因全长作为探针、化学发光信号的Southern blot检测技术参考萨姆布鲁克和拉塞尔(2002)的方法及相关手册进行。

4.2 转基因*MsDREB1* mRNA RT-qPCR分析检测

对扩繁后PCR与Southern blot显示阳性的植株(处理后)作外源基因表达量分析。以T_2代纯合体4个转基因株系(RD-1/RD-2、35S-1/35S-2)及对照苗(WT)叶片为检测材料, 每个株系检测3株苗。选择转基因大豆的叶样品用TRIzol试剂(Invitrogen公司)提取RNA。用紫外分光光度计在260 nm/280 nm测定RNA的浓度。参照SMART cDNA Synthesis Kit说明书合成cDNA第一链。逆转录定量PCR (reverse transcription quantitative PCR, RT-qPCR)在ABI7300实时检测系统进行检测。使用试剂为Power Sybr Green PCR试剂(ABI)。用表1中给出的P5、P6、P7、P8作为RT-qPCR引物。反应体系参考Li等(2009)进行。所有的PCR进行3个重复, 并用同一个条件进行测试。基因拷贝数的定量方法采用双标准曲线法, 具体参照Li等(2009)进行。

5 转基因大豆耐旱性分析

选取长势均匀的大豆幼苗移栽到装有细砂的营养钵(口径为20 cm、高为18 cm)中定植, 定植后每3 d用Hoagland营养液透灌一次。30 d后对幼苗进行干旱胁迫处理及生理指标测定。干旱处理分别为正常水分(绝对含水量80%~85%)、低度胁迫(绝对含水量60%~65%)、中度胁迫(绝对含水量40%~45%)和严重胁迫(绝对含水量20%~25%)。胁迫6 d后分别测定转基因大豆叶绿素含量、丙二醛含量、相对含水量、植株干重等生理指标(高俊凤2006), 复水后2 d测定存活率。在正常状态下, 测定大豆初花时株高及叶面积, 测量植株叶面积使用LI-COR-3000叶面积仪(LI-COR, Inc.)。每个群体测定15株苗, 重复3次。数据使用Excel 2013及SPSS 17.0统计分析。

实验结果

1 转基因株系*MsDREB1*表达量分析

为比较启动子调控目的基因效果, 在不同程度的干旱胁迫下, 对4个转基因株系(RD-1/RD-2、35S-1/35S-2) PCR(数据未显示)及Southern blot阳性(图2)苗实时PCR检测(图3)。转*MsDREB1*株系外源基因均能表达, 但由于启动子不同, 基因表达量存在较大差异。未经胁迫诱导时, 35S启动子调控的*MsDREB1*为超表达, 相对表达量显著高于rd29A启动子调控的*MsDREB*1表达量。经干旱胁

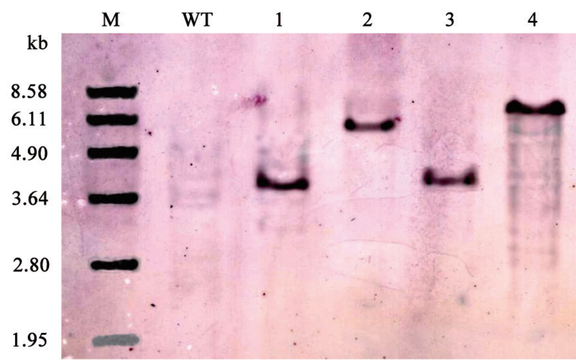

图2 大豆转*MsDREB1*基因Southern blot分析

Fig.2 Southern blot analysis from transgenic lines with expression *MsDREB1* genes in soybean

M: 单分子量标准; WT: 野生型植株, 下同; 1和2: 35S启动子转基因植株35S-1和35S-2; 3和4: rd29A启动子转基因植株RD-1和RD-2。

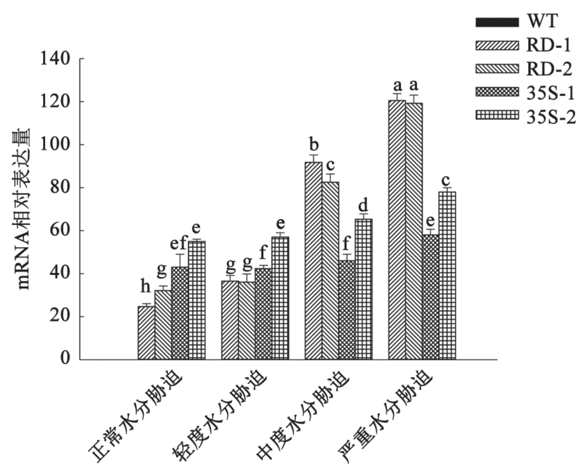

图3 野生型和转基因大豆各株系不同干旱胁迫处理下*MsDREB1*的表达

Fig.3 *MsDREB1* genes expression in wildtype and 35S and rd29A lines under drought stress treatments

迫(分别为低度胁迫60%~65%水分、中度胁迫40%~45%水分、严重胁迫20%~25%水分)处理后, 35S启动子调控的*MsDREB1*表达量有所提高, 而rd29A启动子调控的*MsDREB1*表达量明显增加, 但rd29A启动子调控的*MsDREB1*表达量增加明显高于35S启动子调控表达量。

2 转基因植株的形态差异分析

为了比较启动子对植株生长的影响, 在4个转基因株系正常水分条件下初花时, 测定其株高和叶总面积, 结果表明, 35S启动子的转基因植株高度明显低于WT与rd29A启动子的转基因植株(图4)。

3 不同启动子转基因大豆耐旱性差异分析

选取PCR阳性植株, 对其进行干旱处理。rd29A启动子调控的转基因大豆长势明显好于35S, 主要表现在生根较快, 地上部分生长迅速, 成活率较高。推测CaMV-35S调控的外源基因持续超量表达抑制植株生长, 与Chen等(2009)在大豆中的研究结果相似。转录因子在组成型强启动子驱动下异源超表达, 即使在正常生长条件下, 也会启动基因表达。这种错误基因表达常造成转基因植株在正常生长条件下株型变异和植株矮小(Ito等2006)。

为了比较大豆转基因植株和野生型植株的抗旱性, 将苗龄30 d的转基因植株盆栽进行断水胁迫处理, 所有的植株干旱胁迫6 d后复水。观察复水2 d后的存活率。在复水前测定转基因大豆各株系叶绿素含量、丙二醛含量、相对含水量及植株干重(图5)。结果表明, 各株系处理前后的变化量差异显著。干旱处理条件下, *MsDREB1*诱导表达, 使植株膜脂过氧化程度低于*MsDREB1*超量表达, 而叶绿素含量、相对含水量及植株干重高于*MsDREB1*超量表达。严重干旱胁迫下, 野生型植株存活率只有23.3%, 所有的转基因植株的存活率比野生型高很多, 35S:MsDREB1转基因株系35S-1和35S-2存活率为72.1%和71.9%, RD-1和RD-2成活率为82.5%和80.3% (图6)。这些结果表明*MsDREB1*过表达导致转基因植株耐旱性提高; 相比于野生型对照, *MsDREB1*的诱导表达更有利于转基因大豆耐旱性提高, 且不影响植株正常生长和繁殖。

讨　论

DREB转录因子是参与植物对干旱、高盐和低温等环境胁迫的应答, 调节植物体内产生各种生理代谢反应的关键因子。本研究将苜蓿*MsDREB*1基因转入大豆, 获得由两种启动子驱动的具有一定抗旱性的株系。但有研究表明, 在组成型启动子CaMV-35S调控下, *DREB1A*转基因拟南芥出现了生长阻碍、结实少等不利性状。有研究者提出采用逆境诱导型启动子如rd29A启动子去驱动*DREB*基因表达, 可有效降低上述由*DREB*基因持续过表达导致的对植物的不良影响(Kasuga等

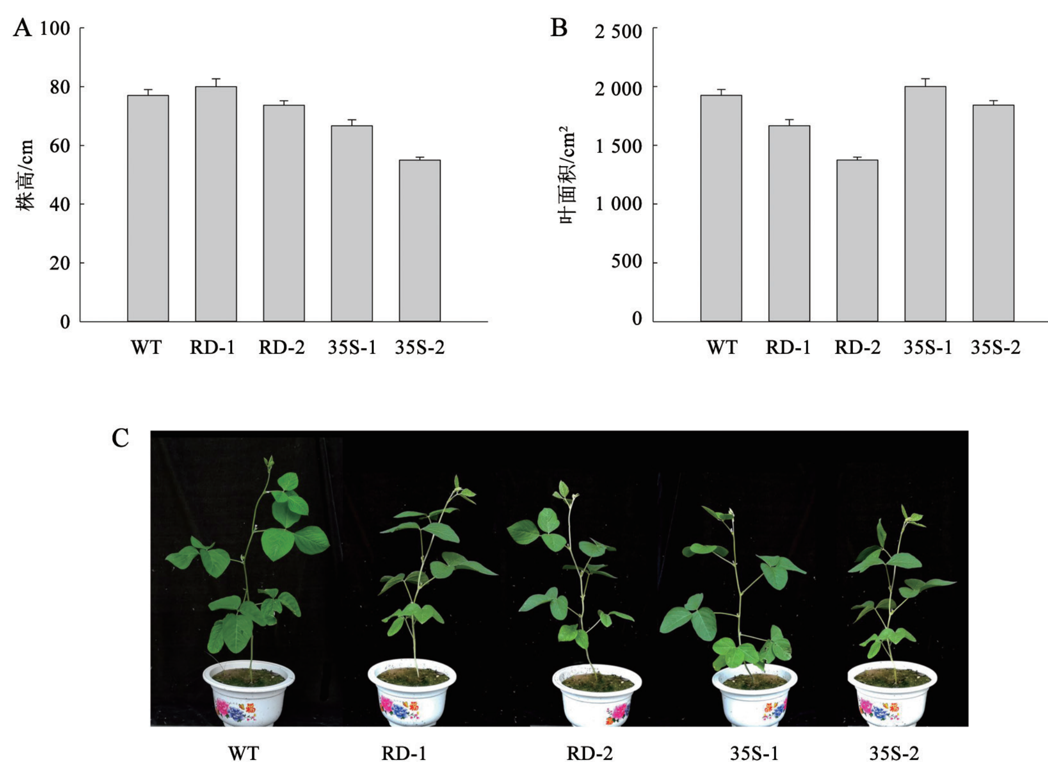

图4 野生型和转基因植株在无水分胁迫条件下的高度和叶面积

Fig.4 Height and leaf area of wildtype and transgenic plants under well-watered conditions

A: 各株系株高; B: 各株系叶面积; C: 各株系外观。

1999, 2004; Lee等2016; Wei等2016)。由于rd29A启动子中存在一个与ABA应答相关的ABRE顺式作用元件和两个与环境胁迫相关的DRE顺式作用元件, 植物细胞只有在受到低温、高盐和干旱时rd29A启动子才会驱动目的基因表达, 而在正常条件下不会或只在低水平上驱动目的基因表达(陶怡等2016)。本研究中, 两个独立的T_2纯系被选择进行进一步分子和生理分析。苗龄30 d的35S:MsDREB1和rd29A:MsDREB1转基因植株被进行6 d断水的水分胁迫试验。35S启动子的转基因植株*MsDREB1*的表达在正常状态和低度水分胁迫时类似。rd29A:MsDREB1显示在正常供水条件下表达较低(含水量80%~85%), 随着胁迫增加, 严重缺水条件下, *MsDREB1*表达量增加4.2倍。而rd29A:MsDREB1转基因植株非胁迫条件下表达量较低(图3)。rd29A启动子GUS在烟草中在干旱或盐胁迫下超量表达而在非胁迫下表达较弱(李新玲等2007)。同样的研究还有rd29A-IPT引入烟草中在胁迫下叶片叶绿素的保留时间明显增加(Qiu等2012)。rd29A启动子驱动*AtCDPK1*基因表达的转基因马铃薯(*Solanum tuberosum*)各株系中*AtCDPK1*基因表达量明显增强, 而在无胁迫条件下, 植株中*AtCDPK1*基因基本不表达; 同时发现35S控制*AtCDPK1*转基因植株在聚乙二醇胁迫前后, 基因转录未见明显差异(聂利珍等2015)。这些结果与本研究是一致的。在这项研究中还发现rd29A:MsDREB1转基因植株在非胁迫条件下仍有部分的表达, 这主要是由于叶片中内源ABA的存在。许多研究表明在干旱胁迫下, 植物地上部和根系ABA积累比正常条件下多(Chow和McCourt 2004; Bhatnagar-Mathur等2009; 赵璞等2016)。

35S启动子和rd29启动子的转基因植株除了基因表达明显不同外, 在正常或干旱胁迫下, 35S启动子转基因株系生长发育受到一定抑制, 而rd29A启动子转基因株系生长与WT相似, 没有明显差异(图4)。特别是在连续干旱胁迫下, 35S-1、35S-2植株存活率显著低于RD-1、RD-2 (图6), 其他生理指标上也有显著性差异(图5), 这表明*Ms-*

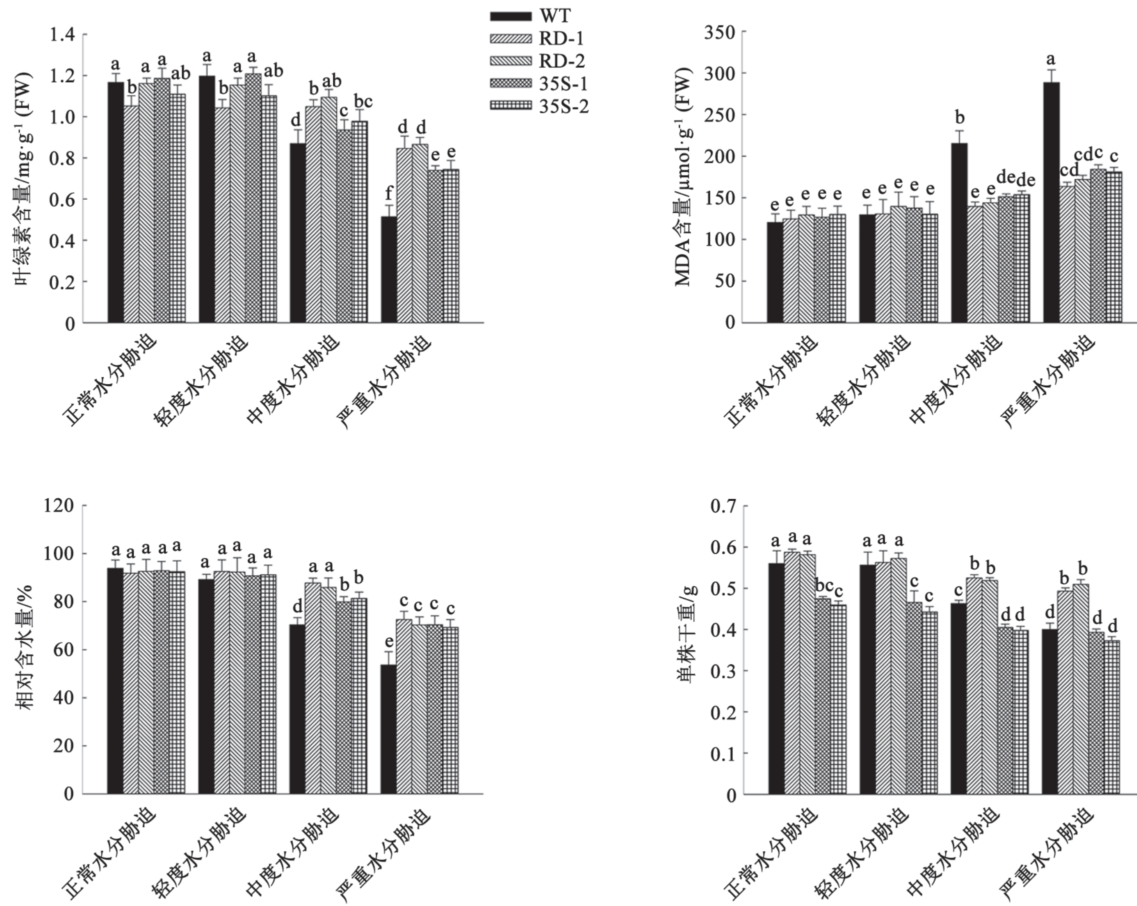

图5 不同干旱胁迫处理6 d后野生型和转基因大豆苗叶绿素含量、丙二醛含量、相对含水量及干重变化

Fig.5 Chlorophyll content, malondialdehyde content, relative water content and dry weight in seedlings of wildtype and transgenic soybeans in drought stress treatment for 6 d

*DREB1*转录因子基因的过表达可以影响植物的生长, 而*MsDREB1*诱导表达不影响植物的正常生长。Ito等(2006)研究结果与此类似, 并提出了转录因子的调控基因, 认为持续过度表达会影响植物的正常生长。

*MsDREB1*基因过表达对植物的生长发育有负面影响, 可能是由以下两个原因造成的。Qin等(2008)报道称, DREB2A转录因子在蛋白泛素化过程中降解, 可能与多种代谢途径相关联。因此, 该基因持续过量表达会影响细胞内基因、蛋白质和生理代谢的变化, 最终影响植物生长和抗逆性。此外, 该研究报告称, DREB类转录因子之间可以相互调控(Novillo等2004), 使DREB蛋白质含量稳定。35S启动子调节基因转录因子在正常条件下也高表达, 使转基因植株的生长受到阻碍(图3和4)。由此可以看出, *DREB1*基因的组成型表达虽然有利于提高植物的抗逆性, 但在正常条件下高表达会导致转基因植株的发育表型滞后。如何尽可能减少这些变化, 使应激反应基因在非胁迫条件下不表达, 而在胁迫下及时响应, 提高植物抗逆性, 这是植物基因工程研究需要考虑的问题。根据这一要求, 选择诱导型启动子是一种有效的方法。在低温、干旱、高盐等胁迫条件下, rd29A启动子可以诱导基因表达, 且只有在受到外部胁迫时才诱导植物外源基因表达, 在正常生长条件下不诱导(Pellegrineschi等2004; Wei等2016), 从而减少植物外源基因过表达对植物带来的不良影响。本研究中, 转基因株系rd29A在生长和抗旱的耐受性上均优于35S, 且转基因植株的抗旱性并没有随着干旱程度的增加而降低(图3~5)。此外, rd29A启动子

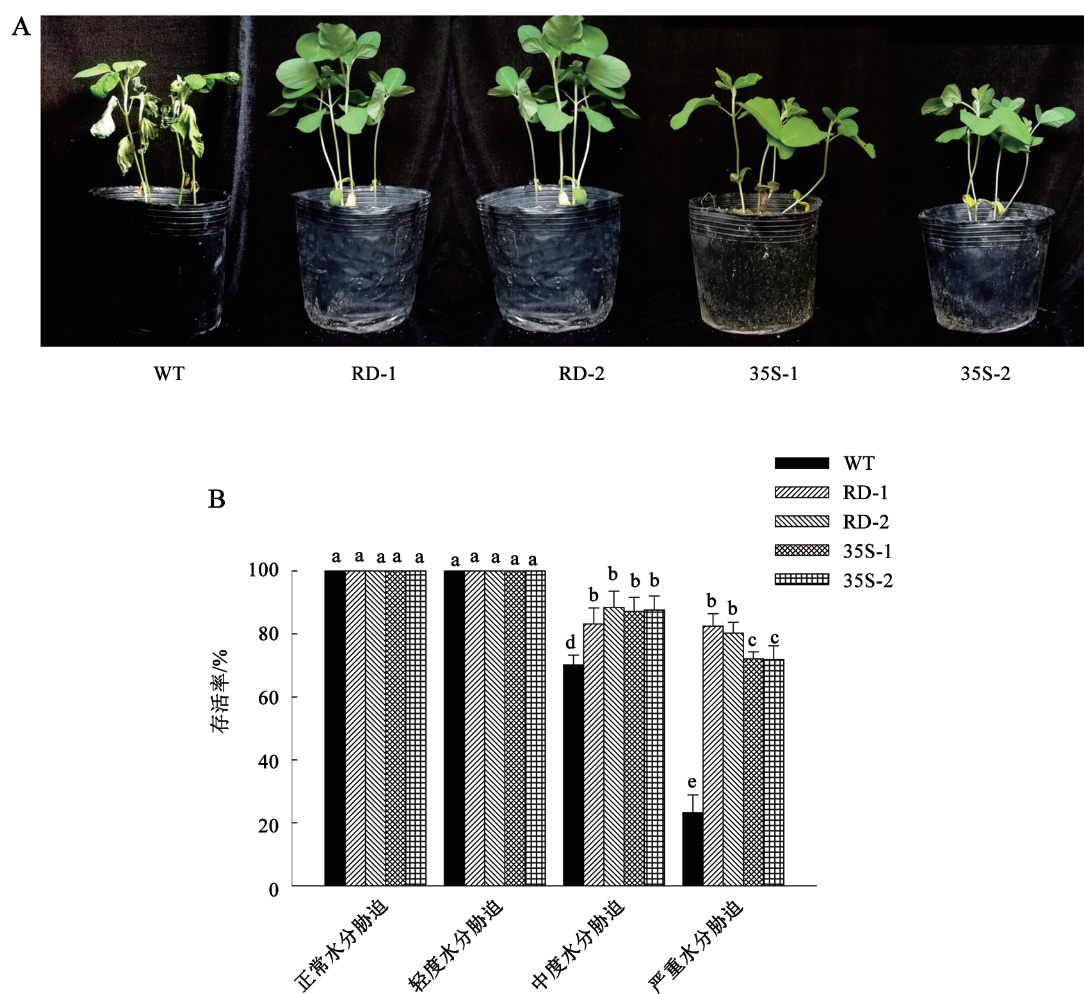

图6 干旱胁迫后复水的转基因植株

Fig.6 Transgenic plants' recovery after drought stress

A: 各植株复水后2 d的外观; B: 各植株复水后2 d的存活率。

的作用不仅限于转录因子的基因工程领域, 对蛋白激酶的表达调控、离子通道蛋白和其他基因也有效(张俊莲等2011; 冯娟等2013)。因此, rd29A启动子和调控基因的使用, 可以有效提高大豆的耐旱性, 为大豆遗传工程的抗旱研究开辟新途径。总之, 转*MsDREB1*基因大豆明显比野生型提高了抗旱能力, rd29A启动子调控的转基因大豆生长及对干旱抗逆性均优于CaMV-35S启动子, rd29A启动子与*MsDREB1*可共同应用于植物耐旱性研究。

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Drought tolerance analysis of transgenic soybean with overexpressed *MsDREB1* gene from alfalfa

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Abstract: The purpose of this study was using transgenic technique to improve drought resistance of soybean (*Glycine max*) and also the driving effects of two kinds of promoters, rd29A and CaMV-35S. By using the transgenic vectors pCAMBIA-rd29A-MsDREB1 and pCAMBIA-35S-MsDREB1 into soybean through *Agrobacterium tumefaciens*-mediated transformation, the transgenic soybeans drived by rd29A and CaMV-35S were produced. By analyzing T_1 to T_2 generations plants by PCR and Southern blot, 9 and 12 transgenic soybean lines were selected, respectively, and then 2 transgenic soybean lines from each generation were randomly chosen as the research object. The height and area of soybean leaves statistics was couducted under well-watered situation at the beginning of flowering period. By analyzing the differences of 30 d old seedlings in gene expression under different drought stress conditions by reverse transcription PCR (RT-PCR), the contents of chlorophyll, malondialdehyde and relative water and dry weight of the plants were measured and the survival rate of every line was calculated after rewatering. The results show that there were significant differences in the regulation of *MsDREB1* expression between the two promoters. Under non-stress condition, *MsDREB1* gene was overexpressed under the regulation of CaMV-35S promoter, but the expression was lower under the regulation of rd29A promoter. Under severe drought-stress condition, the expression level of rd29A:MsDREB1 was higher than that of 35S:MsDREB1. The overexpression of *MsDREB1* inhibited the normal growth of plants. Both promoter-drived transgenic lines had some abilities of drought tolerance, but there were also some differences between themselves. The effect of drought resistance of rd29A:MsDREB1 was more obvious. Its relative water and chlorophyll contents and dry weight were significantly higher than those of 35S:MsDREB1, while the content of malondialdehyde was significantly lower than the overexpression of 35S:MsDREB1. The aim of this study is to obtain transgenic soybean lines with drought tolerance by comparing the drought tolerance of transgenic soybean with *MsDREB1* gene regulated by different promoters, and to provide an effective method of application of drought-stress genetic engineering with *MsDREB1* genes in soybean.

Key words: soybean; MsDREB1; rd29A promoter; drought tolerance

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