OVEREXPRESSION OF NICOTIANAMINE SYNTHASE (NAS) GENE RESULTS IN ENHANCED DROUGHT TOLERANCE IN PERENNIAL RYEGRASS

Z.-X. Zhang and Y.-Z. Zheng
Hanshan Normal University, Department of Biology, Chaozhou, China
Correspondence to: Zhenxia Zhang
E-mail: zhangzhenxia@yahoo.com.cn

ABSTRACTS
As important forage crops, gramineous forage plants can also play role in improving natural environment, maintaining water and soil erosion with turfgrass plants together. Using the particle bombardment transformation system nicotianamine synthase gene was introduced into the callus of Lolium perenne. Twenty transgenic ryegrass lines were obtained from 105 pieces of G418-resistant calli, giving a 5.15% transformation frequency. PCR and Southern blotting analysis showed that target gene was integrated into the genome of the transgenic plants. Transgenic plants displayed excellent drought resistant by expression of NASHOR gene in the field aridity experiment. The chlorophyll content of leaves in transgenic plant was higher than that of the controls in normal condition. The further observation in field experiment and identified resistance for these transgenic plants are underway.

Keywords: Lolium perenne, transformation, transgenic plants, biolistic bombardment

Introduction
Drought, or insufficient water, is the single most important environmental factor that limits plant growth and affects crop productivity. It will become even more serious in future as the global climatic warming trend continues, aquifers are drawn down, and competition between agricultural and nonagricultural water use intensifies. Thus improving drought tolerance is an important objective in plant breeding programs. The development of genetically engineered plants with enhanced tolerance to drought is an important challenge in plant gene technology. However, genetic mechanism of drought tolerance in plants could not be completely understood because drought tolerance is a complex trait controlled by many genes. At the present researchers have described a number of genes associated with drought tolerance (1, 9, 30), and try to enhance plant drought tolerance by transgenic approach (16, 22). Kishor et al. (15) obtained transgenic tobacco plants with improved tolerance to water stress by introducing of pScs gene involving proline biosynthesis from the V. aconitifolia. Rohila et al. (24) transformed the hav1 gene encoding group 3 lea protein of the barley into the rice. Third generation (R2) of transgenic plants raise salt stress and water deficit by cell integrity and growth after salt and water stress. The tsp1 gene of trehalose biosynthesis in A. thaliana, affecting sugar metabolism, increase drought tolerance which is determined by better survival of leaves after withholding of water for 15 days in 3-month-old transgenic N. tabacum plants (12, 25). Subsequently Serrano et al. (27) introduce successfully the tsp1 gene from yeast regulatory genes to tobacco. The int1 gene involving D-Ononitol biosynthesis in transgenic N. tabacum plants increase salt stress and water deficit resistance by better photosynthesis rate (28). Pilon-Smits et al. (20) produced transgenic plants with increased dry weight and more efficient photosynthesis under drought by introducing ostA and ostB gene attending trehalose biosynthesis. Capell et al. (2) used the adc gene of putrescine synthesis to minimize chlorophyll loss under drought stress in carrot. The sacB transgenic tobacco and sugar beet plants increase drought by accumulating bacterial fructans and possess an extra sink for carbohydrate(21). Karaka et al. (13) introduced mannitol-1-phosphate dehydrogenase gene into tobacco that enables to enhance the resistance to drought through raising mannitol accumulation and improving osmotic adjustment. DREB1A, a transcription factor, increase tolerance to drought, salt and freezing of Arabidopsis (14) The avp1 gene encoding vacuolar H+-pyrophosphatase of Arabidopsis demonstrate that vacuoles from these transgenic plants enhance cation uptake capability (23). Obviously genetic transformation is a better approach to increase plant drought tolerance. Transgenic approaches offer powerful means to gain valuable information towards a better understanding of the mechanisms that govern stress tolerance, and create new chances to improve tolerance to dehydration stress by introducing a gene involved in stress protection from any source into the objective plants.

Generally graminaceous plants could absorb and utilize iron element by secreting iron-chelating mugineic acid family phytosiderophores (MAs) from their roots to solubilize iron in the rhizosphere (18). Nicotianamine synthase (NAS) is the key enzyme of the MAs biosynthetic pathway (8, 11, 17), and increase the activity of NAS is crucial for the enhanced absorption of iron under Fe-deficient condition (7, 10, 11). Takahashi et al. (29) transformed the barley nicotianamine aminotransferase (NAAT) gene that is another key enzyme of the MAs biosynthetic pathway into rice. Under iron deficiency, transgenic rice had higher levels of NAAT activity than nontransformants. This increased release improved growth of the transgenic plants in alkaline soils. The transgenic rice plants yielded fourfold as much grain as control plants.
We suppose that the engineering of other steps of the MAs biosynthetic pathway could further increase MAs release, and improve plant growth in adverse condition, as increasing nickel tolerance (6).

Graminaceous perennial ryegrass (*Lolium perenne* L.) is a cool-season perennial bunchgrass, which is widely distributed all over the world. It is important forage crop and turfgrass with many favorable agronomic traits, including high yield, abundant nutrition, fast establishment, suitability for reduced-tillage renovation, can be used on heavy/waterlogged soils, and very palatable to grazing animals. However, perennial ryegrass is susceptible to drought, which greatly influence its planting area. Here we have report that enhanced drought tolerance can be achieved by introduction of the barley NASHOR1 gene into graminaceous perennial ryegrass (*Lolium perenne* L.) plant.

### Materials and Methods

#### Materials

Mature seeds of perennial ryegrass (*Lolium perenne*. L. cv. Springwave (Fescue× Lolium)) used in this experiment was kindly provided by Clover Company.

All the chemicals were purchased from Sigma unless other is specified. The pH of all media was adjusted to 6.0 with 0.1M NaOH prior to autoclaving.

The plasmid PBNAS (Fig. 1) bearing the barley nicotianamine synthase NASHOR1 gene driven by the cauliflower mosaic virus (CaMV) 35S gene and the selectable marker nptII gene encoding the enzyme neomycin phosphotransferase II was used for selection.

![Fig. 1. Scheme of the chimeric gene used for transformation of Perennial ryegrass](image)

**Plant transformation and regeneration**

Seeds were sterilized for 20 min in 0.2% NaClO solution, followed by 4-5 times washing with sterilized water. Surface sterilized seeds were immersed in sterilized water at 4°C, the sterilization was repeated after 2 days, and the sterilized seeds were inoculated on MS medium (19), containing 2 or 5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.05 mg/L 6-benzylaminopurine (BAP), 2 mg/L abscisic acid (ABA), and 1g/L casein hydrolysate (CH), 3% sucrose, 3.4 g/L phytagel.

After 25-30 days the derived calli were separated from endosperm, and transferred to above medium every 3 weeks. Approximately 2- to 3-months-old calli were used for gene transfer. Calli were cultured on above medium with 90g/L mannitol 12h prior to particle bombardment, and retransferred to subculture medium for a week after 12h bombardment. After that calli were transferred to the same medium supplemented with 50mg/L G418. G418 concentration was increased to 75mg/L in second and third selections. For the regeneration of plants, calli were transferred to the pre-differentiation media (MS medium contains 2mg/L BAP, 1mg/L NAA, 2mg/L ABA, 75 mg/L G418) for 10 days, and then transferred to the differentiation MS medium (contains 3 mg/L BAP, 1mg/L NAA, 0.5-1mg/L TDZ). After about 3 weeks the regenerated plantlets were rooted in the plant strengthening medium (1/2 MS salts, B5 vitamins, 0.5-1 mg/L NAA, 1mg/L MET, 2% sucrose).

#### Plant growth and rearing conditions

Plants in tissue culture were grown in a 12/12 h light-dark conditions (irradiance 150 μmol m⁻² s⁻¹), at 26°C and 50% relative humidity (3).

Each transformant was propagated *in vitro* to 15~30 plants which were grew in 21-cm (in diameter) pots and transferred to greenhouse. The greenhouse had a 16h light (34°C) and 8h dark (25°C) cycle (irradiance 300 μmol m⁻² s⁻¹).

#### Microprojectile bombardment

The plasmid DNA was coated with gold particles (1μm in diameter), and bombardment to target callus tissue using a Biolistic Particle Delivery System. The 1μg/μl plasmid DNA suspension was spread on the surface of the macrocaera at 1100 psi.

#### PCR examination

Total genomic DNA was isolated from leaves of regenerated putative plants was used as a template in PCR for *nptII* gene. The PCR was performed with the following primer combinations: 5′-TCGGCTATGACTGCGCACAAACAGA-3′, and 5′-AAGAAGCTGATGAAAGCGATGCG-3′. PCR was performed in a volume of 25μl containing 50ng of genomic DNA, 25mM of primers, 1 unit *Taq* DNA polymerase. The cycle parameters was as following: 94°C predenaturation for 4 min, 30 cycles of 94°C denaturation for 30s, 61°C annealing for 40s, 72°C extension for 1min, and as a last step incubation at 72°C for 10 min. PCR-amplified products were separated by electrophoresis on 0.8% agarose gel. Molecular masses were estimated by using HindIII-EcoRI-digested λ DNA as a standard.

#### Genomic DNA preparation and Southern blot analysis

Total genomic DNA was prepared according to Dellaporta et al. (5). About 20μg DNA was completely digested with *EcoRI*. The digestion mixtures were separated on 0.8% agarose gel.

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**TABLE 1**

<table>
<thead>
<tr>
<th>Number of callus</th>
<th>Number of resistant callus</th>
<th>Efficiency of resistant callus (%)</th>
<th>Number of PCR-positive plant</th>
<th>Efficiency of transformation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>388</td>
<td>105</td>
<td>31.06</td>
<td>20</td>
<td>5.15</td>
</tr>
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and blotted onto nylon membranes according to Sambrook et al. (26). For the filters the full length NAS gene fragment was used for generating $^{32}$P-labeled probes. The hybridization was performed in 0.5M sodium phosphate buffer, pH 7.2, containing 7% Na dodecyl sulfate (SDS), 1% bovine serum albumin, and 20 mM EDTA (4). Radioactively labeled bands were visualized using an X-ray film (FUJI).

**Results and Discussion**

**Analysis of transgenic plants**

The barley NASHEOR1 gene was introduced into perennial ryegrass. Two- to three- month-old embryogenic calli were used for biolistic bombardment. Twenty transgenic ryegrass lines were obtained from 105 pieces of G418-resistant calli, giving a 5.15% transformation frequency. Regenerated plants were transferred to soil and further grown in a glasshouse (Fig. 2). The result of transformation was summarized in Table 1.

![Figure 2](image2.png)

**Fig. 2.** Transgenic *Lolium perenne* plants from biolistic bombardment of mature seeds embryogenic calli

A. Embryogenic calli of *L. perenne* cultured in MS medium. B. G418-resistant *L. perenne* calli obtained from biolistic-bombarded embryogenic calli. C. Shoots differentiation from G418-resistant calli. D. Regenerating *L. perenne* plants in vitro 12 weeks after bombardment of calli. E. Transgenic soil-grown *L. perenne* plants 4 months after bombardment of calli

Genomic DNA extracted from the leaf tissue of transgenic perennial ryegrass plants was analyzed by PCR assay using primers designed to amplify an nptII fragment (795 bp). PCR analysis revealed the presence of a DNA fragment of the expected size in the transgenic plants examined and absence in untransformed (control) plant (Fig. 3).

![Figure 3](image3.png)

**Fig. 3.** PCR analysis of nas gene transformation ryegrass plants

1. Marker, 2. plasmid, 3. control, 4-9. transgenic plants

The presence of transgene in these plantlets was also confirmed by Southern hybridization with nas probe, using digested genomic DNA with restriction enzyme BamHI. The expected DNA fragment hybridizing with the nas probe appeared in the plantlets (Fig. 4), indicating that transformants contained intact copies of nas. The result also showed several lines containing two NAS hybridizing bands, indicating the integrated multiple and rearranged nas transgene copies.

![Figure 4](image4.png)

**Fig. 4.** Southern hybridization analysis of putative transformants of *Lolium perenne* digested with BamHI and hybridized with the nas probe

1. plasmid, 2. control. 3-12. transgenic plants

**Aridity stress experiment**

To test whether transformation of nas gene in perennial ryegrass enhanced the tolerance of plant to low iron availability and drought condition, to untransformed and transgenic plants were enforced aridity stress. Three lines of transgenic plants were selected and every line had at least five plants. These plants were planted in pots respectively with control plants together. The stress experiment was repeated three times. The control and transgenic plants transferred to the greenhouse after 4-6 weeks were not watered. After a week water-deficient, the leaves of control plants were slightly wilted. Untransformed plants showed severe wilt symptom after ten days without watering, including wilt, curly and chlorosis leaves, scorched the top of leaf, the shoot could not grew erectly, while transgenic ryegrass plants slightly withered and had not obviously growth inhibition for two weeks without water (Fig. 5). The leaf of transgenic plants appeared significantly wilt and curly until more than three weeks.

![Figure 5](image5.png)

**Fig. 5.** Phenotype of control (left) and transgenic plants (right) in aridity stress

10 days (left) and 14 days (right)

**Measurement of leaf chlorophyll content**

Measurement of the leaf chlorophyll content of transgenic and untransformed perennial ryegrass plants under stress and non-stress conditions indicated that the chlorophyll content of leaves in transgenic ryegrass plants was higher than that of the controls in normal condition (Table 2). By aridity stress
approach for two weeks, the chlorophyll content of leaves from transgenic ryegrass plants was still high, revealing that the structure and function of chloroplasts of plant cells were not destroyed in aridity condition and plants could sustained normal photosynthesis. From the other hand control plants lost their function of photosynthesis after water-deficient for two weeks because of withered and chlorosis.

### TABLE 2

Comparison the leaf chlorophyll content of control and transgenic plants

<table>
<thead>
<tr>
<th>Material</th>
<th>Days of aridity stress</th>
<th>Leaf chlorophyll content (mg/L)</th>
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<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>107.27±11.56</td>
</tr>
<tr>
<td>Control</td>
<td>14</td>
<td>—</td>
</tr>
<tr>
<td>Transgenic plant</td>
<td>0</td>
<td>117.10±8.25</td>
</tr>
<tr>
<td>Transgenic 1-2-1</td>
<td>14</td>
<td>100.49±8.79</td>
</tr>
<tr>
<td>Transgenic 2-3-1</td>
<td>14</td>
<td>129.62±10.11</td>
</tr>
<tr>
<td>Transgenic 4-1</td>
<td>14</td>
<td>136.66±3.41</td>
</tr>
</tbody>
</table>

From these results, we supposed that the tolerance of perennial ryegrass to drought was attributed to transformation of NASHOR1 gene. Overexpression of nicotiamine synthase (NAS) gene enhanced the secretion of mugineic acid (MAs) in transgenic perennial ryegrass plants under adverse condition. Transgenic plants under water deficiency would secrete greater amounts of MAs from the root, corresponded to strengthen the life activity of plant, and maintain the intact structure and function of chloroplasts of plant cells in aridity condition, thus transgenic ryegrass plants could keep normal functions of photosynthesis.

### Conclusions

In this study, we showed that introducing the barley NAS gene in perennial ryegrass enhanced tolerance to aridity condition. Genetically engineered plants tolerance to drought, such as the transgenic perennial ryegrass carrying the barley NAS gene described here, have not been reported before. We considered that increasing the activity of NAS is crucial for improving plant tolerance to adverse condition. Further detailed analyses of NAS transgenic plant will facilitate us to clarify how the NAS gene works in drought conditions, and the mechanism of plant tolerance to adverse conditions.

### REFERENCES