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STUDY ON INHERITANCE PATTERN AND NUTRITIONAL ANALYSIS OF *ATGRF2* TRANSGENIC 'BURGALTAI' (*Medicago varia* Marthz)

НАСЛЕДОВАНИЕ *AtGRF2* ГЕНА И ХИМИЧЕСКИЙ СОСТАВ СУХОГО ВЕЩЕСТВА ТРАНСГЕННОГО СОРТА ЛЮЦЕРНЫ ИЗМЕНЧИВОЙ (*Medicago varia* Marthz) БУРГАЛТАЙ

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Alfalfa (*Medicago varia* Marthz) 'Burgaltai' cultivar is one of the most important forages of its palatable and nutritional values. But still, national scientists had reported that there is lack of characteristic have been appeared in Mongolian cultivated *Medicago* sp., and it is the percentage of leaf in the yield is low (up to 45%) and it had decreased dramatically in development cycle. Over-expression of *AtGRF2* resulted in larger leaves and cotyledons comparing to wild type. In this study, transgenic 'Burgaltai' with *AtGRF2* gene had increased leaf area by 0.52 cm^2 and leaves in percentage per bush up to 48.6%. The transgenic lines showed 3:1 segregation ratio in T_1 progenies ($\chi^2 = 0.04–0.42$, $p \leq 0.52–0.84$). Hygromycin antibiotic concentration is 50 mg/l for selective media. According to the feed evaluation, some chemical composition increased in transgenic 'Burgaltai' except total fat.

Keywords: alfalfa, *AtGRF2* gene, transgenic 'Burgaltai' and wild 'Burgaltai' varieties, hygromycin_antibiotic.

Сорт люцерны изменчивой Бургалтай является ценной кормовой культурой по поедаемости и питательности. Тем не менее, отечественные ученые считают, что у возделываемого в Монголии вида *Medicago* sp. недостатком является низкая облиственность (до 45%), которая в процессе развития растений люцерны резко снижается. Установлено, что высокая экспрессия *AtGRF2* гена приводит к увеличению размеров листьев и семядолей по сравнению с диким типом. В исследовании показано, что у трансгенного сорта Бургалтай с геном *AtGRF2* площадь листьев увеличилась на 0,52 $см^2$, а процентное содержание листьев на растении — до 48,6%. Трансгенные линии показали соотношение расщепления 3:1 в потомстве T1 (χ^2 = 0,04–0,42, p ≤ 0,52–0,84). Концентрация антибиотика гигромицина в селективных средах составляет 50 мг/л. Согласно результатам оценки корма, в трансгенном Бургалтае увеличилось содержание некоторых химических веществ, за исключением общего жира.

Ключевые слова: люцерна, *AtGRF2* ген, трансгенный и дикий сорт Бургалтай, антибиотик гигромицин.

Introduction. There are about 2823 species of plant spreading in Mongolian rangeland and around 600 species can be used as forage. Among them alfalfa (*Medicago* sp.), is the most important forage of its palatable and nutritional values. It has been reported that alfalfa puts about 50–100 kg/ha biologically fixed nitrogen into the soil and increasing the soil fertility [5].

Moreover, it is suitable for cultivated pastures and hay production and improvement of vegetation. But still, national scientists had reported that there are two lacks characteristic have been appeared in Mongolian cultivated *Medicago* sp., the first one is the percentage of leaves in the yield is low (up to 45%) and it had decreased dramatically in development cycle [3]. The second it cannot be used in the first year of cultivation since the height of the plant reaches 20–25 cm while foreign cultivars gave 30–40 c/ha hay production in the first year of introduction [1]. However, foreign cultivars of alfalfa gave higher yield than native it is not able to be wintering in Mongolian harsh and extreme condition. Thus, it is necessary to increase the yield of Mongolian native alfalfa which is more adapted Mongolian specific condition. It would be creating an opportunity for combat to desertification, biological reclamation of the mining production, reclaiming the degraded grassland, to increasing the fodder supply for intensive and semi-intensive farm [1; 4].

On the other hand, Korean scientists had described *GRF* gene family in 2003 from *Arabidopsis thaliana* (*AtGRF*) which comprises nine members (*AtGRF1–AtGRF9*) [6]. Over-expression of *AtGRF1* and *AtGRF2* resulted in larger leaves and cotyledons comparing to wild type. Far from here, we had obtained transgenic alfalfa sample with *AtGRF2* gene through *Agrobacterium* — mediated genetic transformation. In this study, our main goal is to determine *AtGRF2* gene activity comparing the transgenic 'Burgaltai' and wild 'Burgaltai' varieties. To reaching the goal the following specific objectives were implemented.

1. Determining the inheritance of *AtGRF2* gene in T_1 progeny of transgenic 'Burgaltai' by Mendel’s law.
2. Comparing some phenotypic characteristics of transgenic 'Burgaltai' and wild 'Burgaltai' varieties.
3. Comparing the biomass and nutritional value of transgenic 'Burgaltai' and wild 'Burgaltai' varieties.

Materials. The seeds of transgenic 'Burgaltai' with *AtGRF2* gene and wild 'Burgaltai' varieties.

Methods.

– Seed sterilization and selection of hygromycin antibiotic concentration for selection.

Seeds were washed on 0.1% soap water with gentle stirring for 3–4 min and rinsed three times with sterilized distilled water. Seeds were then soaked in 70 % ethanol with gentle shaking for 3–4 min and rinsed with sterilized distilled water. After surface-sterilized in 30% Clorox for 10 min seeds were rinsed more than three times with sterilized distilled water. To evaluate the effect of different selection pressure, seeds were placed on MSB medium supplemented with different hygromycin concentration (0–control, 10, 20, 30, 40 and 50 mg/l) for 2 weeks at 28 ºC in fluorescent light (200 $E/m^2/s$) at a photoperiod of 16h for the germination.

– Determining the inheritance of *AtGRF2* gene in T_1 progeny.

Self-pollinated seeds (T_1) of transgenic plants were placed on segregation medium comprising MSB5 with 50 mg/l hygromycin. The treatment was carried out in triplicate (10 seeds per dish) and was repeated three times in each transgenic line. After 2 weeks, hygromycin-resistant plants developing true leaves were counted, and data were analysed by the χ^2 test at $p \leq 0.05$.

– Comparing some chemical analysis of transgenic 'Burgaltai' and wild 'Burgaltai' varieties.

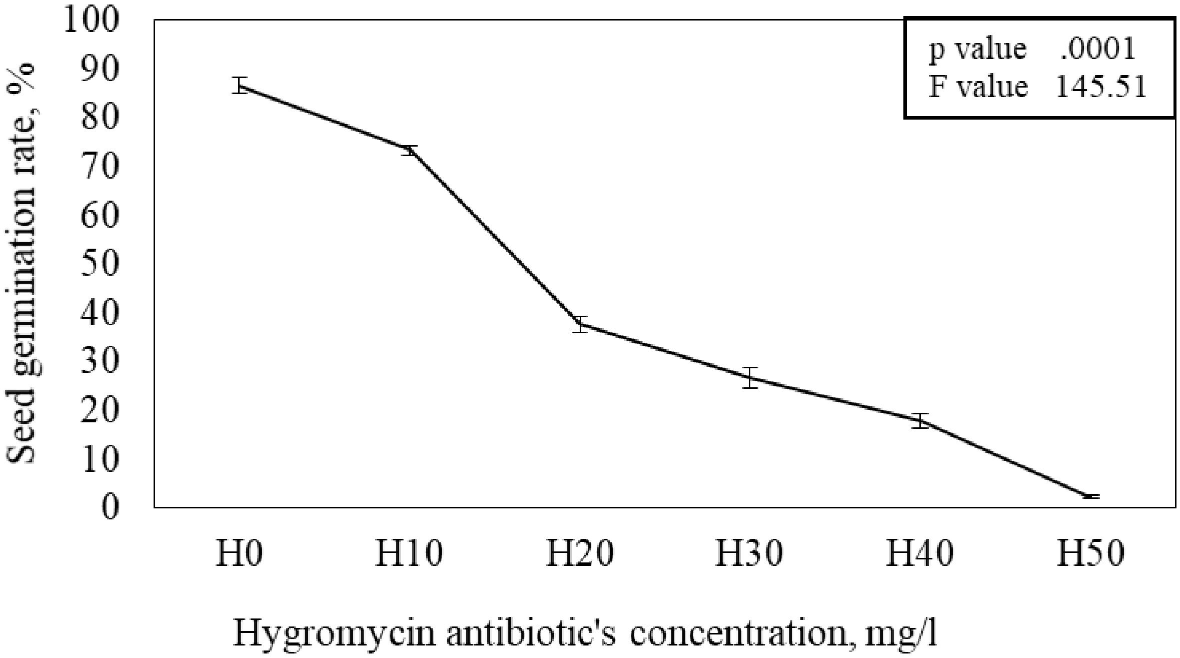
Transgenic and wild 'Burgaltai' variety seeds were planted in the soil and cultivated under laboratory condition for 3 months. Green biomasses were harvested and dried for analysis. Total protein determined by MNS6550:2015, fat by MNS3058:1981, calcium by MNS4265:1995, phosphorus by MNS4266:2015, ash by MNS6548:2015 biomass and dry matter by weigh method. The leaf area was calculated by its width and length measurements at 2–4 apical leaves of wild and transgenic plants.

Results And Discussion.

– Selection of hygromycin antibiotic concentration for transgenic plant selection.

The T-DNA vector region in this study contains hygromycin antibiotic resistance gene (*hptII*) as a selection marker gene, and by determining the antibiotic resistance of the plant seeds, it is possible to select the lines in which the *AtGRF2* gene has been transferred. Concentration of antibiotic in the selection medium is essential in transformation experiments, in which the antibiotic serves as the selective agent that allows only transformed cells or plants to survive [2]. Wild 'Burgaltai' variety seeds placed onto a selective medium containing hygromycin at 0-control, 10, 20, 30, 40 and 50 mg/l concentration in MSB5 medium replicated three times for each concentration. Over a period of 2 weeks the number of germinated seeds were counted. The result is presented below (Graphic 1 and Figure 1).

Seed germination was 86.6% in control group on MSB5 absent of hygromycin and seed germination were dramatically decreased while hygromycin antibiotic concentration increased. Seed germination getting lower by 15.3% on 10 mg/l, by 56.4% on 20 mg/l, by 69.2% on 30 mg/l, by 79.5% on 40 mg/l and by 97.4% on 50 mg/l hygromycin concentration respectively comparing to the control group.



Graphic 1. Survival rate of seeds at different concentration of hygromycin in two weeks

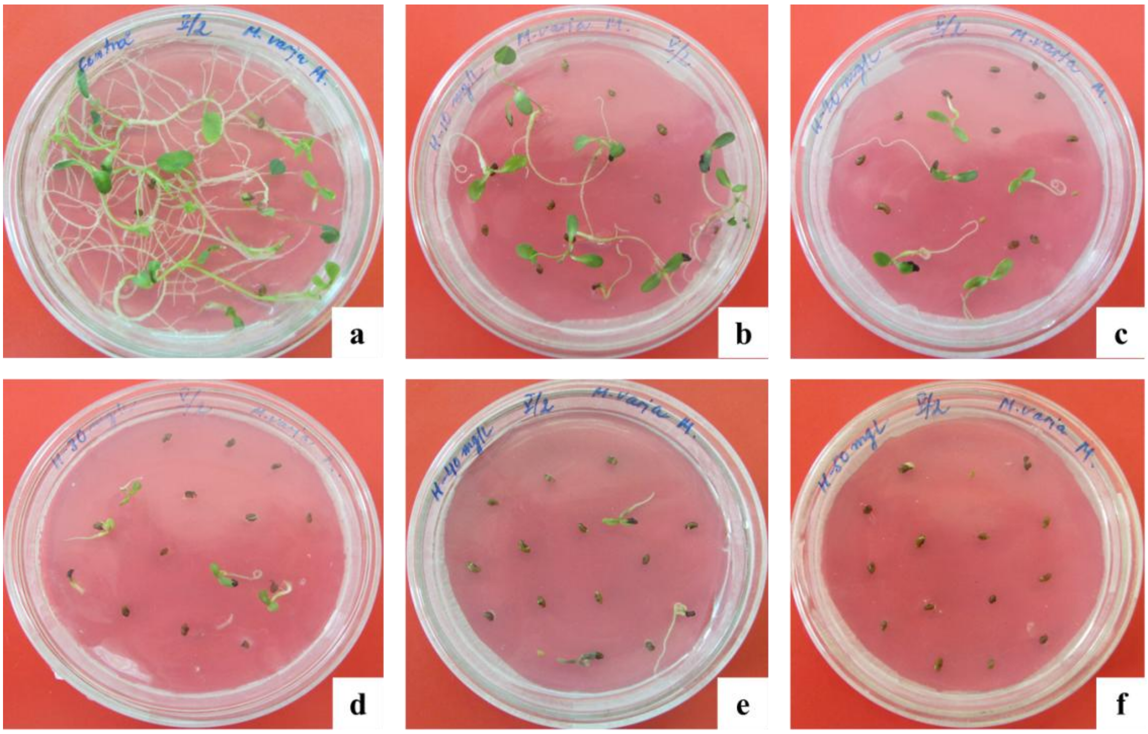


Figure 1. Seed germination rate on hygromycin containing selective media
a – control, no hygromycin, b – 10, c – 20, d – 30, e – 40, f – 50 mg/l hygromycin containing MSB5 media

– Determining the inheritance of *AtGRF2* gene in T_1 progeny.

To determining the inheritance of *AtGRF2* gene of transgenic 'Burgaltai' variety in T_1 progeny, eleven sterilized seeds were placed in MSB5 medium supplemented with 50 mg/l hygromycin antibiotic in three replicates, and seed germination was counted for 2 weeks.

The transgenic T6 and 11 lines showed 3:1 segregation ratio in T_1 progenies ($\chi^2 = 0.04–0.42$, $p \leq 0.52–0.84$). From table 1, the inheritance of the *AtGRF2* gene in these transgenic lines is 3:1 or according to Mendel's II law. *AtGRF2* gene in *Brassica napus* showed the similar segregation to T_1 progenies ($\chi^2 = 0.29$, $p \leq 0.59$) in previous study [8].

Table 1. Inheritance ratio of the *AtGRF2* gene in transgenic 'Burgaltai'

Transgenic line	Hygromycin resistant (R)	Hygromycin sensitive (S)	Ratio (R:S)	Hypothesis	χ^2 value	p value
T6	9.7 ± 0.6	2± 0.8	4.9:1	3:1	0.42	0.52
T11	8.7 ± 1.0	3.3 ± 0.9	2.6:1	3:1	0.04	0.84

– Comparing some chemical analysis of transgenic 'Burgaltai' and wild 'Burgaltai' varieties.

Transgenic and wild 'Burgaltai' variety seeds cultivated under laboratory condition for 3 months. Green biomasses were harvested and dried for analysis.

In transgenic 'Burgaltai' had increased its leaves percentage up to 48.6% and it is 26.2% increase compared to wild 'Burgaltai' variety's leaves percentage per bush. The leaf area in transgenic 'Burgaltai' was 0.52 cm^2 wider than wild plant. Moreover, moisture had increased 8.3%, crude protein 2.1%, phosphorus 71.4%, respectively, compared to wild 'Burgaltai'. There is no difference between ash and calcium between two groups. However, total fat was 20% lower in transgenic 'Burgaltai' compared to wild 'Burgaltai' (Table 2). *AtGRF2* gene transferred in *Brassica napus* showed 0.5% higher in leaf area than wild type plant, and total protein content of the transgenic plants, the total amount of the dietary fiber was the same, and the calcium content was 1.7% lower. About calcium, total ash, organic matter, and total fat contents were very little difference [8]. Gene expression of *BnGRF2* gene in *Brassica napus* showed 20% larger leaves than wild plants [7].

Table 2. The biomass and some nutritional value of transgenic 'Burgaltai' and wild 'Burgaltai' varieties

Sample	Total crude protein, %	Total fat, %	Ash, %	Phosphorus, %	Calcium, %	Moisture, %	Leaf area, cm^2	Percentage of leaves per bush
TB	19.1	1.2	9.9	0.12	1.28	5.2	1.39 ± 0.11	48.6 ± 9.1
WB	18.7	1.5	9.8	0.07	1.26	4.8	0.87 ± 0.23	38.5 ± 0.05

TB – Transgenic 'Burgaltai', WB – Wild 'Burgaltai'

Conclusion. Hygromycin has been extensively used as selective antibiotic in transformation vectors include hygromycin phosphotransferase gene as selectable marker for transgenic plants. The number of seeds that germinated on MSB5 selective medium was significantly reduced under 50 mg/l selection pressure. Therefore, a concentration of 25 mg/l hygromycin was used for further studies. However, explants exposed to lower levels of hygromycin concentration, ranging from 10 mg/l to 40 mg/l restrict the germination rate. In transgenic 'Burgaltai' T_1 genetic inheritance were 3:1 to Mendelian ratio means 1 copy number of transgene was integrated into 'Burgaltai' genome. The leaf area was increased by 0.52 cm^2, leaves per bush increased by 26.2% phosphorus amount by 71.4% in transgenic plant than wild type plants. Moreover, moisture had increased 8.3%, crude protein 2.1%, phosphorus 71.4%, respectively, compared to wild 'Burgaltai'. There is no difference between ash and calcium between two groups. From the results, we concluded that the nutritional value of transgenic and wild type alfalfa plants was not shown significant differences.

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