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Optimization of Genetic Transformation on Phytochrome A by Directly Differentiated Buds in Alfalfa

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ABSTRACT

Background: The study showed that phytochrome A (phyA) is involved in alfalfa fall dormancy regulation. To provide the scientific basis for using fall dormancy to predict new varieties' dormancy class from the molecular level, cultivate alfalfa varieties with cold solid resistance and weak fall dormancy and improve the fall dormancy level of existing excellent types, we could study its function through gene silencing. But the genetic transformation rate is not high, which affects the research progress.

Methods: To optimize the genetic transformation system in alfalfa (*Medicago sativa* L.), a binary plasmid vector pART27-phyA was transferred into alfalfa in *Agrobacterium* tumefaciens-mediated way. To improve transformation efficiency, we optimized the mediums by screening different Kan (Kanamycin) concentrations, NAA (a-Naphthylacetic acid) concentrations and adding YE (yeast extracts).

Result: The results showed that using cotyledon nodes as explants, the proper concentration of Kan antibiotics resistance screening was 100 mg/L, the appropriate concentration of NAA for inducing cotyledon node differentiation was 0.1 mg/L and 0.2 g/L YE was suitable to add on the root induction medium; The expression of phyA in terminal buds and leaves of transformed plants of T1 and T2 generations of phyA in Reindeer was significantly reduced ($P<0.05$), but the expression trend in roots and stems had decreasing trend with no significant changes.

Key words: Alfalfa, Cotyledon node, Genetic transformation, phyA.

INTRODUCTION

Alfalfa is a kind of high-quality legume grass with high crude protein content, good palatability, high grass yield and good nitrogen fixation capacity, which is widely known as the king of forage (Elgharably *et al*., 2021; Samac *et al*., 2006). Therefore, cultivating alfalfa with good traits has excellent significance for the sustainable development of animal husbandry. Transgenic technology breaks the breeding boundary, accelerates the breeding process and provides a broader space for breeding work. The prerequisite for breeding improvement using transgenic technology is to establish a rapid and efficient genetic transformation system. The existing transformation methods on alfalfa mainly include: The *Agrobacterium*-mediated method, gene gun transformation method, electric shock method and so on (Block, *et al*., 1984; Narusaka *et al*., 2012; Deak *et al*., 1986). *Agrobacterium*-mediated method, which has the advantages of a single integration site of foreign genes, slight structural variation and few copy numbers, is the most critical in genetic breeding. In recent years, most of the existing alfalfa genetic transformation systems take cotyledons, hypocotyls and true leaves as explants and obtain transgenic plants through dedifferentiation and redifferentiation callus regeneration (Wang *et al*., 2006; Wang *et al*., 2013; Du *et al*., 1994; Zhang *et al*., 2014; Zhang *et al*., 2011), however, it takes a long time to complete the whole system, along with callus induction difficulty, late differentiation ability, low regeneration rate and poor repeatability. In the process of *Agrobacterium*-mediated plant genetic transformation, the concentration of transformed receptors, antibiotics and the proportion of culture medium would all affect the transformation efficiency.

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Direct differentiation bud regeneration is a regeneration method without the stage of dedifferentiation to form callus to get the regenerated plants with advantages: short plant regeneration cycle, small genetic variation and genetic stability of the transferred foreign genes.

Studies have shown that many tissues or organs of alfalfa could be used as receptor materials for genetic transformation (Gordon-Kamm *et al*., 2002; Zhang *et al*., 2021). And making leaves as receptor materials is more common for the genetic transformation of alfalfa (Zhou *et al*., 2013; Amini *et al*., 2016). The direct differentiation bud regeneration pathway usually takes the stem tip or cotyledon node as the explants used on soybeans. Olhoft *et al.* (2003) infected the cotyledon node with *Agrobacterium* tumefaciens and obtained transgenic soybeans through the screening of hygromycin B and detected the expression of the GUS

gene in the transformed plants. Liang *et al*. (2020) got transgenic plants by directly differentiating adventitious buds from hypocotyls and radicles of *Atractylodes macrocephala*. Kumar *et al*. (2013) used stem tips as receptor materials to get transformed plants of alfalfa; Sheng *et al*. (2007) found that cotyledons and hypocotyls had no differentiation, while the cotyledon nodes had a higher differentiation rate under the same conditions. The previous research focused on the different types of fall-dormancy alfalfa metabolism mostly, which was associated with soluble sugar and the regulation mechanism of fall dormancy is not clear. PhyA directly or indirectly affects the synthesis of endogenous hormones GA_3, ZR, IAA and ABA and regulates the fall dormancy of alfalfa (Fan *et al*., 2014; Du *et al*., 2014).

In this experiment, *Agrobacterium*-mediated direct differentiation buds selected cotyledonary nodes as explants and screened culture medium with different Kan concentrations, NAA concentrations and adding YE for further optimization of genetic transformation on phyA in alfalfa. It could provide a foundation for reviling the mechanism of phyA in regulating fall dormancy in alfalfa.

MATERIALS AND METHODS

Plant materials and bacterial fluid

The alfalfa variety was Reindeer (Fall Dormancy Class 1, fall dormancy type, FD1). Selected high-quality and complete seeds and put them on the MS medium: MS+30 g/L sugar +2.5 g/L Phytagel (pH 5.8), after sterilization and absorbing the excess water. When the sterile seedlings were eight days old, excised the true leaves at the cotyledon nodes and then cut them longitudinally along the hypocotyl. Cotyledons with wounds at the leaf nodes would be the experimental explants.

Agrobacterium tumefaciens transferred with a foreign gene antibiotic (phyA RNAi, phyA regulates fall dormancy of alfalfa) was provided by the research group of the Grassland Science Laboratory of Henan Agricultural University. It was cultured on a shaking table at 25! and 280r/min, suspended in MS liquid medium for standby and Kan was used as an antibiotics resistance screening agent (from the vector pART27).

Wild-type seeds of Reindeer and phyA RNAi T1, T2 (the first- and second-generation seeds of Reindeer with transformed phytochrome A-RNAi in alfalfa). Incubate in the light incubator under 16 h/8 h of daylight photoperiod, 24/20°C of daylight temperature (60 days). At the end of the experiment, take samples at 10 a.m. on the day (root, stem, leaf, terminal bud), put them into the high-pressure sterilized cryopreservation tubes, directly put them into liquid nitrogen quick freezing and then transferred them to the -80°C refrigerator for preservation.

Culture medium

MS was the primary medium in the process of alfalfa genetic transformation and the pH value was adjusted to 5.8 (Table 1).

Determination of Kan screening concentration

The uninfected *Agrobacterium* sterile cotyledon node explants were inoculated on the bud induction medium supplemented with 1.0 mg/L 6-BA. The concentration of Kan was set at seven gradients: 0 mg/L, 25 mg/L, 50 mg/L, 75 mg/L, 100 mg/L, 125 mg/L and 150 mg/L. Each treatment has four repetitions and each repetition has 30 cotyledon nodes. After 30 days, observe the growth status of cotyledon node explants, counted the survival rate of cotyledon nodes and analyze the sensitivity of explants to Kan.

Determination of NAA concentrations on bud induction

After co-culture with *Agrobacterium* tumefaciens containing the target gene, the explants were dark cultured on the co-culture medium for three or four days, then transferred to the bud induction medium containing antibiotics (100 mg/L Kan+400 mg/L Carb) with different concentrations of NAA. The concentration of NAA was set with seven gradients: 0 mg/L, 0.05 mg/L, 0.1 mg/L, 0.2 mg/L, 0.3 mg/L, 0.4 mg/L and 0.5 mg/L, with five repetitions for each treatment. After two weeks, the differentiation of adventitious buds was observed and the differentiation rate and vitrification rate of adventitious buds were calculated.

Effects of different concentrations of YE on root induction

After two weeks of bud induction, the cotyledons were inoculated on the root induction medium (containing Kan and Carb) of different concentrations of YE for root induction in a culture flask. The concentration of YE was set with six gradients: 0 g/L, 0.1 g/L, 0.2 g/L, 0.5 g/L, 0.7 g/L and 1.0 g/L, with six repetitions for each treatment. Then observed the growth of the explants and made statistics after two weeks.

RNA isolation and PCR detection

The total RNA in every sample was extracted according to the TRIzol method (Invitrogen). Reverse transcription was performed following the manufacturer's instructions (Takara Bio, Inc.). RNA content and quality were analyzed using Nano2000 (Ultramicro spectrophotometer, ThermoFisher Scientific, USA) and each RNA sample was adjusted to the same concentration. The primers were designed using Primer 5.0 software in Table 2.

When transformed seedlings generate thick and strong roots, we should carry out acclimatization, transplanting and identification. The transgenic plant primers were designed according to the fragment of the npt-II (Neomycin Phosphotransferase II) gene on the vector pART27 used in the experiment (Li *et al*., 2012). Primers were synth esized by Sangong Bioengineering (Shanghai) Co., Ltd.

Statistical analysis

All data were subjected to a one-way ANOVA analysis of variance using SPSS 19.0 and the mean differences were compared with Duncan's multiple comparisons.

Adventitious bud differentiation rate (%) =

$$\frac{\text{Number of explants differentiated}}{\text{Number of explants inoculated}} \times 100$$

Vitrification rate (%) =

$$\frac{\text{Number of explants vitrified from adventitious buds}}{\text{Number of explants from regenerated buds}} \times 100$$

Root differentiation rate (%) =

$$\frac{\text{Number of explants rooted}}{\text{Number of explants inoculated}} \times 100$$

Gene expression: The data were normalized against the expression of the housekeeping gene GAPDH. The expression was calculated relative to a calibrator sample using the formula $2^{-\Delta\Delta Ct}$; the values were presented as mean±standard deviation.

RESULTS AND DISCUSSION

Effects of Kan concentration on cotyledon node differentiation

After one month of screening, the cotyledon nodes on the medium containing low concentrations (0 mg/L and 25 mg/L) of Kan grew normally and well with the differentiation of most adventitious buds. Kan had an inhibitory severe effect on the growth of untransformed explants of alfalfa. 100 mg/L Kan could produce a solid inhibiting effect on the differentiation of adventitious buds from cotyledon nodes. With the increasing Kan concentration, the explants not only had no differentiation of adventitious buds, but also gradually yellowed and dying soon. Under 150 mg/L Kan, explants had no cotyledon nodes to survive. Therefore, 100 mg/L Kan was selected as the appropriate antibiotic concentration for the transformants (Table 3).

The proper concentration of antibiotics was the critical factor in obtaining transformed seedlings. If the concentration of antibiotics were too high, it would cause severe damage to the explants. Not only could the explants not differentiate into adventitious buds, but also most of the explants quickly yellowed and died on the antibiotics screening medium; However, if the concentration of antibiotics were too low, the screening effect would be poor and a large number of false-positive adventitious buds would be produced.

Effects of NAA concentration on bud induction

After co-culture for 3-4 days, cotyledon nodes were inoculated on a bud induction medium with different concentrations of NAA and adventitious bud induction and resistance of antibiotics screening are carried out at the same time. The results were listed in Table 4. NAA concentration had a significant effect on the differentiation rate and vitrification rate of adventitious buds in cotyledon

Table 1: Components of media for alfalfa transformation.

Media	Components
Agrobacterium dilution medium	MS + 30 g/L sugar, pH=5.8
Seed germination medium	MS + 30 g/L sugar + 2.5 g/L PhytagelÿpH 5.8
Co-culture medium	MS + 1.0 mg/L 6-BA + 100 mg/L AS + 30 g/Lsugar + 4 g/L Phytagel, pH 5.8
Bud induction medium	MS + 1.0 mg/L 6-BA + 400 mg/L Carb + 30 g/L sugar + 4 g/L Phytagel, pH 5.8
Root induction medium	1/2MS + 400 mg/L Carb + 20 g/L sugar + 2.5 g/L Phytagel, pH 5.80

Note: 6-BA, 6-Benzylaminopurine; AS, acetosyringone; Carb, carbenicillin.

Table 2: The primers of genes.

Primer name	Base sequence	Amplify length	Amplify condition
phyA-F	GAGAGATAGCTTTATGGATGTC	168 bp	55°C
phyA-R	GCGACCTAAACCAGAAAAC		
GAPDH-F	TGTGCCAATCTATGAGGGTT	131 bp	55°C
GAPDH-R	TCTTTTGGATTGGGCTTCGT		
npt-II F1	CTGAAGCGGGAAGGGACT	484 bp	55°C
npt-II R1	ATACCGTAAAGCACGAGGAAG		

Table 3: Sensitivity test of Kan on Untransformed cotyledonary node.

Kan concentration (mg/L)	Exophyte (sum)	Cotyledon node survival (%)	Cotyledon node growth
0	120	76.67[a]	Grow normally with the differentiation of adventitious buds
25	120	75.00[a]	Grow slowly with more differentiation of adventitious buds
50	120	45.83[b]	Grow slowly with less differentiation of adventitious buds
75	120	44.17[b]	Grow very slowly with occasional differentiation of adventitious buds
100	120	18.33[c]	Grow very slowly with no differentiation of adventitious buds
125	120	8.33[c]	Growth stop with cotyledon nodes gradually etiolated and died
150	120	0	Growth stop with cotyledon nodes etiolated and died

nodes. With NAA concentration ranging from 0 mg/L to 0.5 mg/L, the differentiation rate of adventitious buds increased first and then decreased, while the vitrification rate decreased first and then increased slightly. The differentiation rate of adventitious buds was the highest when NAA concentration was 0.1 mg/L, significantly higher than 0 mg/L, 0.2 mg/L, 0.4 mg/L and 0.5 mg/L treatment groups (P<0.05). Although there was no significant difference between the 0.05 mg/L and 0.3 mg/L treatment groups (P>0.05), 0.1 mg/L treatment was 11% higher than them at least. Adding NAA, the vitrification rates of all treatment groups were all lower than the 0 mg/L treatment group and the lowest vitrification rate was 19.05%. NAA concentration was 0.4 mg/L, the vitrification rate was significantly lower than that of 0 mg/L, 0.05 mg/L, 0.1 mg/L, 0.2 mg/L, 0.3 mg/L treatment groups (P<0.05), but there was no significant difference with 0.5 mg/L treatment group (P>0.05). The data showed that when the concentration of NAA was 0.1 mg/L, the differentiation rate of adventitious buds reached the maximum, more than 90% and the vitrification rate was low. Therefore, the suitable bud induction medium was: MS+1.0 mg/L 6-BA + 0.1 mg/L NAA + 100 mg/L Kan + 400 mg/L Carb + 30 g/L sugar + 4 g/L Phytagel, pH 5.8.

NAA is a kind of auxin plant growth factor, which can promote rapid cell division and expansion. It is often used to stimulate rooting, callus induction and adventitious bud differentiation. Auxin promotes growth at low concentrations and inhibits growth at high concentrations (Pan *et al*., 2008). The proper ratio between auxin and cytokinin can induce adventitious buds to a maximum extent. Kumar *et al*. (2008) studied two kinds of cytokinins, 6-BA and KT and induced buds from shoot tip explants. In addition, Zheng *et al*. (2008) added different concentrations of 6-BA to the bud induction medium. The results showed that the highest budding rate was only 75.0%. In this experiment, the maximum differentiation rate of adventitious buds reached 90.08% by adding NAA. The results showed that 0.1 mg/l NAA was the most suitable in the process of inducing adventitious buds of alfalfa.

Table 4: Effect of different concentration of NAA for shoot induction from cotyledonary node explants.

NAA concentration (mg/L)	Inoculated explants (sum)	Adventitious bud differentiation rate (%)	Vitrification rate (%)
0	60	71.62^{b}	44.55^{a}
0.05	60	78.97^{ab}	35.95^{a}
0.1	60	90.08^{a}	33.49^{ab}
0.2	60	74.19^{b}	34.83^{ab}
0.3	60	75.09^{ab}	32.85^{ab}
0.4	60	52.95^{c}	19.05^{c}
0.5	60	34.98^{d}	21.67^{bc}

Effects of YE concentration on regeneration roots

The cotyledon node explants infected by *Agrobacterium* tumefaciens are inoculated on the root induction medium containing different concentrations of YE. The results were listed in Table 5. The rooting differentiation rates with different YE concentrations were different. When the YE concentration was 0.2 g/L, the root differentiation rate of the explants reached the maximum (33.33%), which was not significantly different from the 0.1 g/L treatment group (P>0.05), but it was higher than all of the other treatment groups (P<0.05); With the concentration increasing of YE, the root differentiation rate decreased rapidly. Therefore, it was appropriate to add 0.2 g/L YE to the root induction medium. The suitable root induction medium for cotyledon nodes was: 1/2MS + 0.2 g/LYE + 100 mg/L Kan + 400 mg/L Carb + 20 g/L sugar + 2.5 g/L Phytagel, pH 5.8.

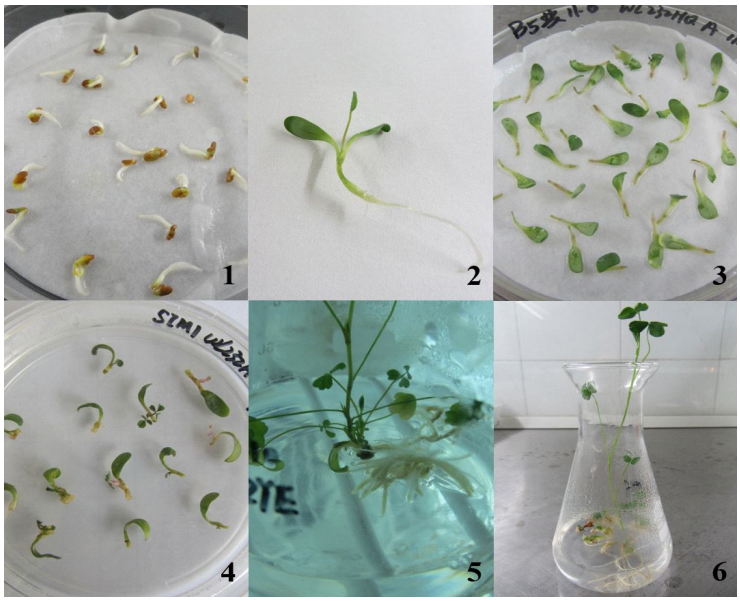
Ma *et al*. (2013) transferred the cotyledon node explants with induced buds on 1/2MS medium containing auxin for rooting. Sheng *et al*. (2007) added auxin to the MS medium to induce rooting. These two primary media are often used (Crane *et al*., 2006; Ding *et al*., 2003; Matt *et al*., 2005). However, in this experiment, explants would form a callus and lose rooting ability on the MS medium; there was no such phenomenon on 1/2MS. In addition, a certain amount of YE would promote the rooting rate, according to the research results of Guan *et al*. (2012). This experiment compared the effects of different concentrations of YE on cotyledon node rooting. The results showed that 0.2 g/L YE concentration on the root differentiation rate was significantly higher than other concentrations. However, the mechanism of YE stimulating root development is not precise. YE is a deeply processed product with edible yeast as raw material. It is rich in nutrition, including peptides, amino acids, B vitamins and other components.

PCR identification of transformed plants

The process of *Agrobacterium*-mediated direct differentiation buds selected cotyledonary nodes with high activity as explants and *Agrobacterium tumefaciens* strain GV3101 was used as a vector to transform the seedings (Fig 1). When

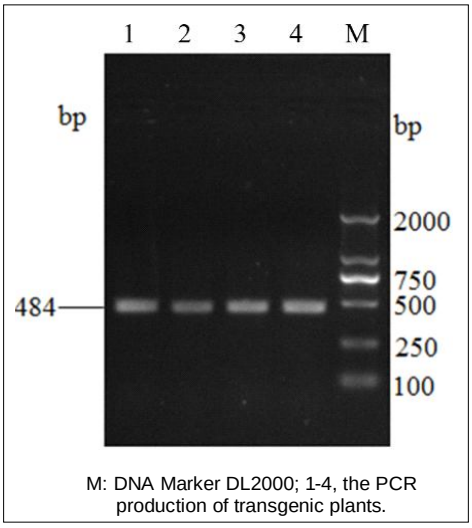
Table 5: Effects of different concentrations of yeast extract on regeneration roots.

YE concentration (mg/L)	Inoculated explants (sum)	Explant root differentiation rate (%)	Rooting condition
1/2MS+0	36	13.89^{b}	Late rooting, 1 or more roots
1/2MS+0.1	36	19.44^{ab}	Rooting earlier, 1~3 roots
1/2MS+0.2	36	33.33^{a}	Early rooting, multiple roots
1/2MS+0.5	36	13.89^{b}	Late rooting, 1 or more roots
1/2MS+0.7	36	11.11^{b}	Late rooting, 1~3 roots
1/2MS+1.0	36	5.56^{b}	Latest rooting, one root mostly



Note: 1, Seeds germination; 2, 8-days-old sterile seedling; 3, Cotyledonary node explants; 4, Bud induction; 5, Root induction; 6, Seedling acclimatization.

Fig 1: The process of genetic transformation mediated by *Agrobacterium* tumefaciens.



M: DNA Marker DL2000; 1-4, the PCR production of transgenic plants.

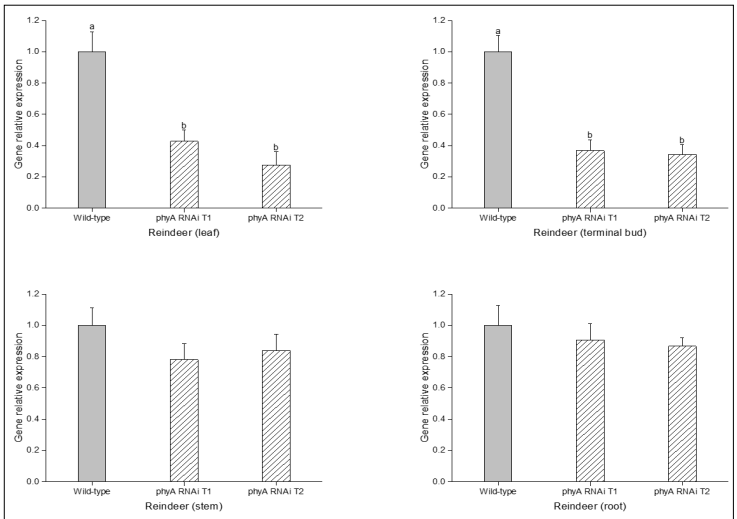
Fig 2: PCR detection of cloning vector in transformed plants.

the transformed plant roots produced multiple thick and strong roots, we could start seedling acclimatization, transplanting and identification.

DNA was extracted from the leaves of pART27-phyA transformed plants and the target fragments were amplified by PCR using the primers of npt-II. The PCR products were detected by 2% agarose gel electrophoresis, shown in Fig 2. It showed 484 bp fragments of the target gene were amplified. The *E. coli* solution with the recombinant cloning vector was used as a template for the PCR reaction and the amplification results were consistent with the expected fragment length too. The *E. coli* solution containing the recombinant vector identified by PCR was sent to Sangong Bioengineering (Shanghai) Co., Ltd. for sequencing. The sequencing results were compared with the fragment sequence of an npt-II gene on NCBI through the blast. It showed 99% homology and indicated the cloned sequence was the npt-II fragment. Then gene quantification of identified transformed plants

The results showed that using cotyledon nodes as explants, the proper concentration of Kan resistance screening was 100 mg/L, the appropriate concentration of NAA for inducing cotyledon node differentiation was 0.1 mg/L and 0.2 g/L YE was suitable to add on root induction medium. Genetic transformation system of alfalfa cotyledon node direct differentiation and bud regeneration: the bud induction medium was MS + 1.0 mg/L 6-BA + 0.1 mg/L NAA + 100 mg/L Kan + 400 mg/L Carb + 30 g/L sugar + 4 g/L Phytagel, pH 5.8; the root induction medium was 1/2MS + 0.2 g/LYE + 100 mg/L Kan + 400 mg/L Carb + 20 g/L sugar + 2.5 g/L Phytagel, pH 5.8.

The expression of phyA in terminal buds and leaves of transgenic plants of T1 and T2 generations of phyA in Reindeer was significantly reduced($P<0.05$), but the expression trend in roots and stems had decreasing trend with no significant changes (Fig 3). Phytochrome A is a central light receptor and terminal buds and leaves could receive light signals; when the phyA gene is silencing, another light receptor may be a compensation action due to decreasing phyA reduction. However, the roots and stems have weak sensitivity to light with no significant changes.



phyA RNAi T1: The first -generation seeds of Reindeer with transformed phytochrome A-RNAi in alfalfa.
phyA RNAi T2: The second-generation seeds of Reindeer with transformed phytochrome A-RNAi in alfalfa.

Fig 3: Gene relative expression of phyA in different tissues of transformed plants.

CONCLUSION

This experiment optimized the genetic transformation system of alfalfa with cotyledon nodes as explants and studied the most suitable concentration of Kan, NAA and YE on the primary medium in the *Agrobacterium*-mediated direct differentiation bud regeneration on PhyA in alfalfa. It would provide technical support for applying transgenic technology in alfalfa breeding and reviling the regulating mechanism of phyA at the molecular level.

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Conflict of interest: None.

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