Genetic engineering of tobacco with *EfIRT2* Gene

Nikam Shweta, S. Varanavasiappan, A. John Joel, T. K. Manape, L. Arul, E. Kokiladevi, K. K. Kumar and D. Sudhakar

ISSN: 0975-928X

Volume: 10

Number:2

EJPB (2019) 10(2):881-888

DOI:10.5958/0975-928X.2019.00115.7

Electronic Journal of Plant Breeding, 10 (2): 881-888 (Jun 2019) DOI: 10.5958/0975-928X.2019.00115.7
ISSN 0975-928X

Research Article

Genetic engineering of tobacco with *EfIRT2* Gene

Nikam Shweta[1], S. Varanavasiappan[1], A. John Joel[2], T. K. Manape[3], L. Arul, E. Kokiladevi[1], K. K. Kumar[1]and D. Sudhakar[1]
[1]Department of Plant Biotechnology,
[2]Centre of Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore
[3]ICAR-DOGR, Rajgurunagar, Pune
* **E-Mail:** dsudhakar@hotmail.com

(Received: 05 Jun 2019; Revised: 11 Jun 2019; Accepted: 11 Jun 2019)

Abstract
IRT2 is involved in regulating uptake of iron (Fe) and is considered as one of the potential targets for genetic manipulation for Fe accumulation in crops. Genetic transformation of tobacco with an *IRT2* gene of *Echinochloa frumentacea* was carried out in tobacco model system to study the expression of the gene. Twenty four putative transgenic tobacco plants were generated through *Agrobacterium* mediated transformation. Thirteen tobacco events were screened for presence of *EfIRT2* and *hptII* genes and all thirteen were found positive for gene of interest as well as for the selectable marker gene, *hptII*. In addition, qRT-PCR analysis confirmed the *EfIRT2* gene expression in three tested transgenic tobacco plants.

Keywords
Iron-Regulated Transporter, *EfIRT2*, Genetic Transformation, *Agrobacterium*, Fe deficiency

Introduction

Iron (Fe), a key micronutrient for plant growth, acts as cofactor in vital cellular processes. In early 1843, the essentiality of Fe was confirmed by E. Gris and this discovery was some 50 years earlier than the discovery of other essential micronutrients such as Mn and Zn (Ma *et al*., 2009). Fe-containing proteins play an important role in oxygen transport, cellular respiration, DNA stability and repair, intermediary metabolism, as well as photosynthesis process in plants. Fe deficiency in human beings results in severe health problems such as anemia, which affects about 1.6 billion worldwide. Anemia is a one of the major global public health problem which is more prevalent in women and preschool-aged children. (McLean *et al*., 2009). Among all the risk factors of death and disability, Fe deficiency is ranked at sixth position with high mortality rates in developing countries (WHO, 2002).

Two distinct strategies have been adopted by plants for the uptake of Fe from low solubility sources (Romheld *et al*., 1987). Dicotyledonous and non-graminaceous monocotyledonous species are found to have Strategy I, whereas Strategy II is found only in graminaceous species. These two strategies have differences in Fe solubilization and transportation within plant system. In the strategy I, there is release of proton (H^+) and reductants/chelators, such as electron (e^-), organic acids, and phenolics, into the rhizosphere from plants, which improves Fe solubility, by enhanced reduction and uptake for ferrous Fe while in strategy II, the Fe-acquisition is characterized by hexadentate Fe^{3+} chelating substances (phytosiderophores, mugineic acids) secretion and by their specific uptake system (Ma and Feng, 2005). Some more adaptive measures include root hair, root morphology changes, and transfer cell development, and increases of citrate concentrations in the phloem (Schmidt *et al*., 1999). Genes encoding Fe^{3+}-chelate reductase have been cloned from *A. thaliana* (*FRO2*, allelic to the *frd1* mutation affecting Fe^{3+}-chelate reductase activity; Robinson *et al*., 1999) and pea (FRO1; Waters *et al*., 2002). The Fe regulated transporter gene *IRT1* has been cloned from *Arabidopsis* (Eide *et al*., 1996) as well as its orthologs from pea and from tomato (Cohen *et al*., 1998; Eckhardt *et al*., 2001).

Fe transporter gene namely *IRT1* has been established as the one of the major Fe uptake systems from the soil, and a candidate for Fe bio fortification (Tan *et al*., 2015; Boonyaves *et al*., 2016). IRTs are the members of the Zinc-Regulated Transporter/Iron-Regulated Transporters (ZRT/IRTs)-Related Protein (ZIP) transporter family. Among the *IRT* gene family members, *IRT2* is expressed in the outer layer of the root epidermal cells of *Arabidopsis thaliana* and up-regulated under Fe deficient conditions, but to a lesser extent (Vert *et al*., 2001). Role of IRT2 in uptake of Fe and avoiding transport of manganese and cadmium has been reported by Vert *et al*., (2002) and Shanmugam *et al*., (2011). This has made IRT2 a potential target for genetic manipulation for Fe accumulation in staple food crops. An *IRT2* gene of barnyard millet was isolated in Genetic Transformation laboratory of Department of Plant

Biotechnology, CPMB&B, TNAU (Manape *et al*., 2017). The present study was aimed at functional validation of *EfIRT2* in tobacco model system.

Materials and Methods

A binary vector, pUH harbouring *EfIRT2* (*Echinochloa frumentacea* Iron regulated Transporter 2) gene under the control of *maize ubiquitin 1* promoter and *nos* terminator (Fig.1) was used in this tobacco transformation study. Besides the gene of interest, the binary vector possessed *hygromycin phosphotransferase II* gene for plant selection. The *Agrobacterium* strain, LBA4404 harbouring pUH-*EfIRT2* was used for infection of leaf explants in tobacco transformation studies. Leaf explants were collected from tobacco plants which were grown under aseptic conditions on half strength MS medium with 1.5 per cent sucrose (w/v) and 0.8 per cent agar at pH 5.8. Leaf bits were pre-cultured on plant regeneration medium (MS + 1.0 mg/L BAP + 0.1 mg/L NAA + 3 % sucrose (w/v) and 0.8 % agar, pH 5.8) for two days and the pre-cultured explants were infected with *Agrobacterium*, LBA4404 (pUH-*EfIRT2*). The infected leaf bits were co-cultivated for two days at 25^{o}C on plant regeneration medium with 100 mM acetosyringone to allow efficient T-DNA transfer. The leaf bits were washed with cefotaxime (250 mg/l) and placed on plant regeneration medium containing 20 mg/L hygromycin. Subsequent sub-culturing was done at every two week's intervals and individual shoots which were actively growing on selection plates were transferred to half strength MS medium containing 1.0 mg/L IBA and 20 mg/L hygromycin. The plants which produced profuse roots were transferred to small paper cups with pot mixture containing soil + sand + vermicompost at the ratio of 1:1:1. Gradually, plants were acclimatized to greenhouse condition and then transferred to pots containing above mentioned pot mixture in transgenic greenhouse (Fig.2.)

PCR analyses were performed to confirm presence of *EfIRT2* and *hptII* gene in putative transgenic tobacco lines by using the primers given in (Table.1). These primers were expected to amplify 1254 bp, 407 bp and 686 bp respectively. The amplified products were resolved on 0.8 percent agarose gel, visualized on UV transilluminator upon ethidium bromide staining. Total RNA was extracted from transgenic tobacco leaves using Spectrum^{TM} Plant Total RNA kit (Sigma Aldrich, USA) as per manufacturer's instructions. Total RNA was resolved on 1% agarose gel and visualized on UV transilluminator to check the integrity. RNA was quantified by using Nanodrop spectrophotometer (Genova Nano, Cole-Parmer Ltd, UK). DNase treated (DNase I, Thermoscientific, USA, Cat. No. EN025) RNA was used as template for synthesis of first strand synthesis of cDNA by using Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit (Thermoscientific, USA). The cDNA was diluted by 4 times and used for real time PCR analysis. First strand of cDNA was used as a template for real time PCR analysis. The qRT-PCR was carried out in BioRad CFX Connect^{TM} Real time PCR (BioRad Inc. USA) using SYBR$^{®}$ Supermix (BioRad Inc. USA) with q*EfIRT2* and q*EF-1α* primers as detailed given in (Table.1). Relative fold expression of target gene was calculated as per manufacturer's instructions.

Results and Disussion

Biofortification is a potential and sustainable strategy in combating micronutrient deficiency. Genetic engineering is one of the strategies to achieve desirable level of micronutrients in target tissues by introducing specific genes of interest into staple food crops. In order to achieve these levels, extensive knowledge and understanding of Fe uptake, trafficking, and homeostasis mechanisms in plants are required. Fe is an important micronutrient for almost all organisms but availability is less due to its property of low solubility (Vert *et al*., 2009). Plants have their own sophisticated mechanisms to maintain cellular Fe homeostasis to cope with Fe deficiency. In the last few decades, a wealth of information on Fe on homeostasis in plants has been obtained and high affinity root Fe uptake systems in grass and non-grass plants have been characterized. Under Fe starvation condition these system are very efficient and they could also play role in uptake of Fe in Fe sufficient conditions. In the present study, an attempt was made to express *IRT2* gene of *E. frumentacea*, in a model plant system. Tobacco is commonly used as model dicot plant for genetic transformation studies due to several reasons: its molecular genetics is well understood, its genomic mapping is almost complete, genetic transformation can be easily achieved, tobacco plants survive well *in vitro* and under greenhouse conditions.*Agrobacterium*-mediated transformation is an effective approach to introduced foreign gene in dicot plants. Tobacco plants are routinely engineered with a view to validating the function of novel genes such as genes conferring resistance to herbicides, insects or viruses; tolerance to drought, salt or cold; and increasing yield. *Agrobacterium*-mediated transformation of tobacco leaf disks procedure was reported by Horsch *et al*. (1985). *Agrobacterium*-mediated transformation of tobacco using disease resistance, herbicide resistance and agronomical trait responsible genes has been reported (Haughn *et al.,* 1988, Svab and Maliga 1993 and Pugalendhi *et al.,* 2009). Tobacco plants expressing *Arabidopsis CAX2* gene which is

involved in metal transport were shown to improve metal uptake (Hirschi *et al.*, 2000). *AhHMA4*expressing tobacco lines were generated to study Zn and Cd accumulation (Barabasz *et al.*, 2010). In this study, tobacco was used as a model dicot plant to study the expression of an *IRT2* gene cloned from *E. frumentacea.* An *Agrobacterium* strain, LBA4404 (*pUH-EfIRT2*), was used for tobacco transformation. Leaf discs with transformed cells, on medium supplemented with hygromycin grew and produced shoots while some of the leaf discs which were not transformed turned yellow and dried. Shoots started emerging from the explants3-4 weeks after co-cultivation. A total of twenty four individual putative transgenic tobacco shoot were generated on selection medium and transferred to rooting medium supplemented with hygromycin. The rooted plants were transferred to transgenic greenhouse for hardening.

PCR analyses with three sets of primers, two for the gene of interest and one for selectable marker gene showed that the all the thirteen events tested were positive for the presence of *EfIRT2* and *hpt* genes (Fig 3a & 3b; Fig.4). They showed amplification of 1254 bp, 407 bp and 686 bp respectively while control plants did not show any amplification indicating the presence of the transgenes in the putative transformants.

In addition, quantitative Real-time Polymerase chain reaction (qRT-PCR) was carried out to study the transgene expression in tobacco plants. Three putative tobacco transgenic events containing *EfIRT2* were tested. Among the tested events, event NS-Tobacco-3 showed higher transgene expression followed by NS-Tobacco-7 and NS-Tobacco-4. (Fig. 5). T_1 seeds of these transgenic events were collected for a detail physiological and molecular characterization. Previous studies showed that the IRT2 codes a protein which is closely related to IRT1 (multi-metal transporter) and transport only Fe and Zn (Vert *et al*., 2001). Under Fe deficient condition, significant induction of *IRT2* was observed in root epidermal cells of Arabidopsis (Vert *et al*., 2001), green gram and tomato (Eckhardt *et al*., 2001). Fe responsive ionomics and gene expression profiling of Fe homeostasis genes in tobacco *EfIRT2* transgenic events could improve our understanding of the role of *EfIRT2* in dicot model system. Further, the information could potentially be exploited in Fe bio-fortification program.

References

Barabasz, A., Kramer, U., Hanikenne, M., Rudzka, J. and Antosiewicz, D. M. 2010. Metal accumulation in tobacco expressing *Arabidopsis halleri* metal hyperaccumulation gene depends on external supply. *J. Exp. Bot*., **61(11):**3057-3067.

Boonyaves, K., Gruissem, W. and Bhullar, N. K. 2016. NOD promoter-controlled *AtIRT1* expression functions synergistically with *NAS* and *FERRITIN* genes to increase iron in rice grains. *Plant Mol. Biol.,* **90 (3):**207-215.

Cohen, C. K., Fox, T. C., Garvin, D. F. and Kochian, L. V. 1998. The role of iron-deficiency stress responses in stimulating heavy-metal transport in plants. *Plant Physiol.*, **116 (3):**1063-1072.

Eckhardt, U., Marques, A. M. and Buckhout,T. J. 2001. Two iron-regulated cation transporters from tomato complement metal uptake-deficient yeast mutants. *Plant Mol. Biol.*, **45 (4):**437-448.

Eide, D., Broderius, M., Fett, J. and Guerinot, M. L. 1996. A novel iron-regulated metal transporter from plants identified by functional expression in yeast. *Proc. Natl. Acad. Sci. USA*., **93 (11):**5624-5628.

Haughn, G. W., Smith,J., Mazur, B. and Somerville, C. 1988. Transformation with a mutant *Arabidopsis acetolactate synthase* gene renders tobacco resistant to sulfonylurea herbicides. *Mol. Gen. Genet.,* **211 (2):**266-271.

Hirschi, K. D., Korenkov, V. D., Wilganowski, N. L. and Wagner, G. J. 2000. Expression of Arabidopsis *CAX2* in tobacco. Altered metal accumulation and increased manganese tolerance. *Plant Physiol*., **124 (1):**125-134.

Horsch, R. B., Frey, J. E., Hofman, N. L., Eichholtz, D., Rogers, S. G. and Fraley, R.T . 1985. Simple and general method for transferring genes into plants. *Science*, **227:** 1229-1231.

Ma and Feng, F. 2005. Plant root responses to three abundant soil minerals: silicon, aluminum and iron. *Crit. Rev. Plant. Sci*., **24 (4):**267-281.

Ma, Feng, J. and Ling, H. 2009. Iron for plants and humans. *Plant Soil.,* **325:**1–3.

Manape, T.K. 2017. Transgenic expression of iron homeostasis genes in rice for enhancement of grain iron content. *Ph.D Thesis*. Department of Plant Biotechnology, Tamil Nadu Agricultural University, Coimbatore.

McLean, E., Cogswell, M., Egli,I., Wojdyla, D. and Benoist, B. D. 2009. Worldwide prevalence of anaemia, WHO vitamin and mineral nutrition information system, 1993–2005.*Public Health Nutr*., **12 (4):**444-454.

World Health. 2002. The world health report 2002: reducing risks, promoting healthy life. World Health Organization. **p. 247.**

Pugalendhi, T., Premalatha,M. and Bhat, G. 2009. *Agrobacterium* mediated transformation of annexin gene in tobacco (*Nicotiana tabacum*). *Afr. J. Biotechnol*., **8 (20):** 5149-5154.

Robinson, N. J., Procter, C. M., Connolly, E. L. and Guerinot, M. L. 1999. A ferric-chelate reductase for iron uptake from soils. *Nature*, **397 (6721):**694.

Romheld, V. 1987. Different strategies for iron acquisition in higher plants. *Physio. Plant*.,**70 (2):**231-234.

Schmidt, W. 1999. Mechanisms and regulation of reduction-based iron uptake in plants. *New Phytol*., **141 (1):**1-26.

Shanmugam, V., J. Lo, C. Wu, S. Wang, C. Lai, Connolly, E. L., Huang, J. and Yeh, K. 2011. Differential expression and regulation of iron-regulated metal transporters in *Arabidopsis halleri* and *Arabidopsis thaliana*– the role in zinc tolerance. *New Phytol*., **190 (1):**125-137.

Svab, Z. and Maliga, P. A. L. 1993. High-frequency plastid transformation in tobacco by selection for a chimeric *aadA* gene. *Proc. Natl. Acad. Sci. USA.*, **90 (3):**913-917.

Tan, S., Han, R., Li, P., Yang, G., Li, S., Zhang, P., Wang, W., Zhao, W. and Yin, L. 2015. Over-expression of the *MxIRT1* gene increases iron and zinc content in rice seeds. *Transgenic Res.*, **24 (1):**109-122.

Vert, G., Barberon, M., Zelazny, E., Séguéla, M., Briat, J.F. and Curie, C. 2009. Arabidopsis *IRT2* cooperates with the high-affinity iron uptake system to maintain iron homeostasis in root epidermal cells. *Planta*., **229(6):** 1171-1179.

Vert, G., Briat, J. and Curie, C. 2001. Arabidopsis *IRT2* gene encodes a root-periphery iron transporter. *Plant J.*, **26 (2):**181-189.

Vert, G., Grotz, N., Dedaldechamp, F., Gaymard, F., Guerinot,M. L., Briat, J. and Curie, C. 2002. *IRT1*, an Arabidopsis transporter essential for iron uptake from the soil and for plant growth. *Plant Cell*., **14 (6):**1223-1233.

Waters, B. M., Blevins, D. G. and Eide, D. J. 2002. Characterization of *FRO1*, a pea ferric-chelate reductase involved in root iron acquisition. *Plant Physiol*., **129 (1):**85-94.

Table 1. List of the primers used in this study

Sr. No	Name of the gene	Forward and Reverse Primers (5' ——→ 3')	Amplicon size
1.	*EfIRT2*	*EfIRT2* F: AGGATCCATGTCTTCACAAACAGTGCCAAG *EfIRT2* R: AGGTACCTCACGCCCACTTTGCCATG	1254 bo
2.		*EFIRT2* Int F: CAACCTCTTCGTCGTCGTCAAGG *EFIRT2* Int R: CCTGAACGATGACACGGTTCC	407 bp
3.	*hptII*	*hptII* F: GACGTCTGTCGAGAAGTTTC *hptII* R: GCCTCCAGAAGAAGATGTTG	686 bp
4.	q*EfIRT2*	q*EfIRT2* F: ACTCCTTCAACGACCTCACC q*EfIRT2* R: CTGTGGAAGGTGAGCATGAG	133 bp
5.	q*EF-1α*	q*EF-1α* F : TGAGATGCACCACGAAGCTC q*EF-1α* R : CCAACATTGTCACCAGGAAGTG	51 bp

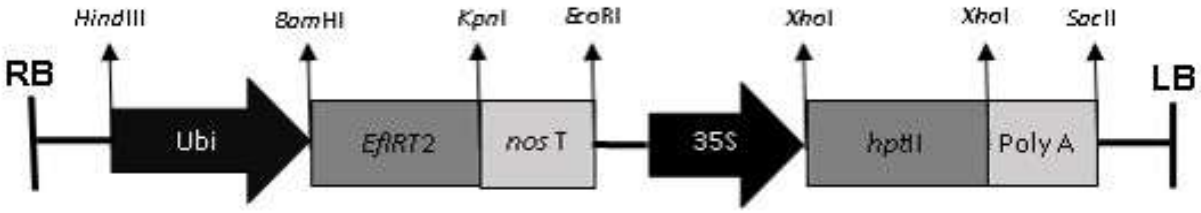

Fig. 1. T-DNA region of binary vector pUH-*EfIRT2*

Fig. 2. *Agrobacterium*-mediated transformation of tobacco

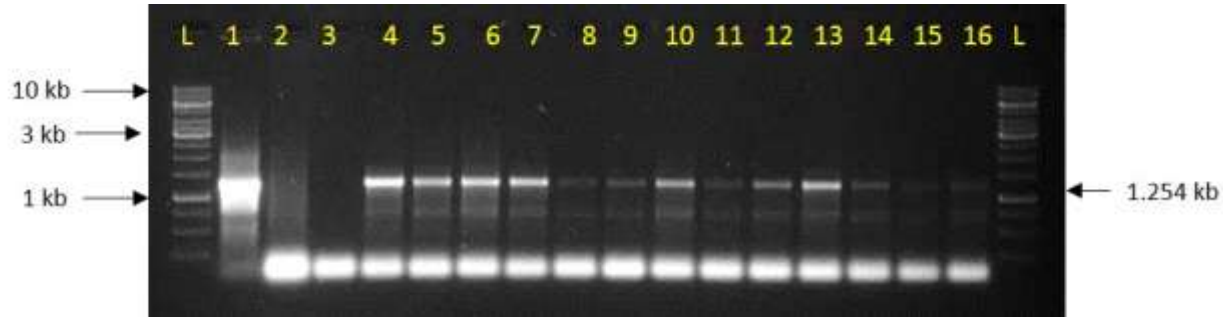

Fig. 3.a. PCR analysis of putative transgenic tobacco for presence of *EfIRT2* gene
Lane L: 1kbp ladder; Lane 1: Positive control; Lane 2: Negative control;
Lane3: Water control; 4 – 16: Transgenic tobacco events

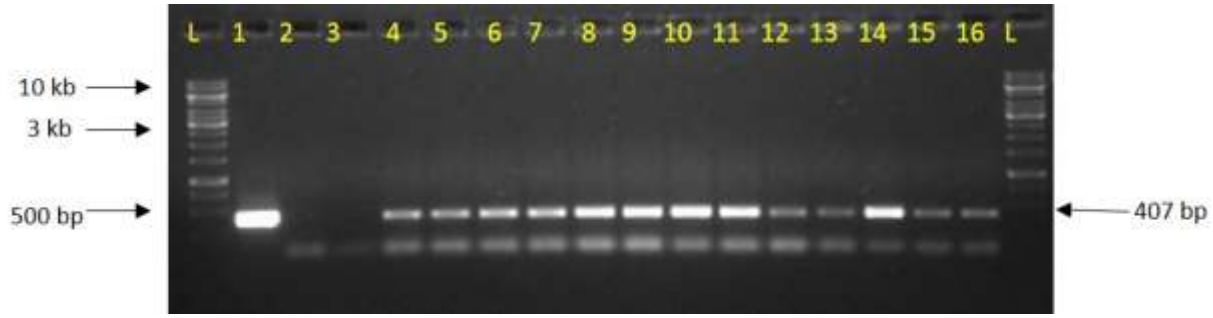

Fig.3.b. PCR analysis of putative transgenic tobacco for presence of *EfIRT2* gene
Lane L: 1kbp ladder; Lane 1: Positive control; Lane 2: Negative control;
Lane 3: Water control; 4 – 16: Transgenic tobacco events

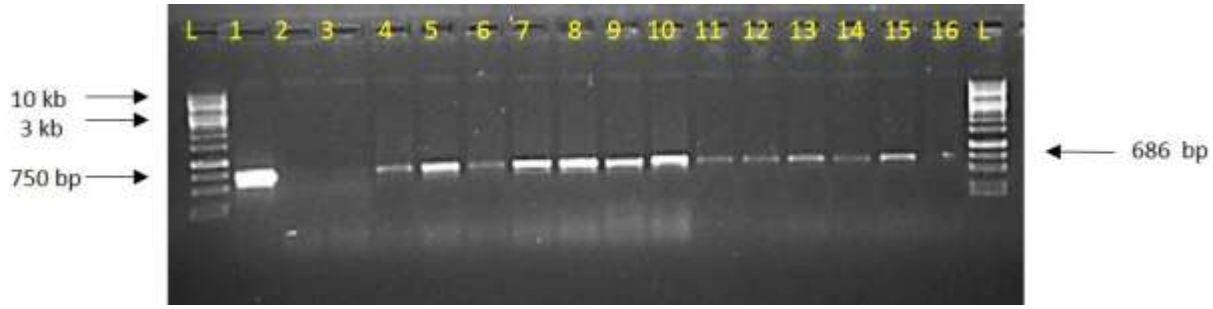

Fig. 4. PCR analysis of putative transgenic tobacco for presence of *hpt II* gene
Lane L: 1kbp ladder; Lane 1: Positive control; Lane 2: Negative control;
Lane 3: Water control; 4 - 16: Transgenic tobacco events

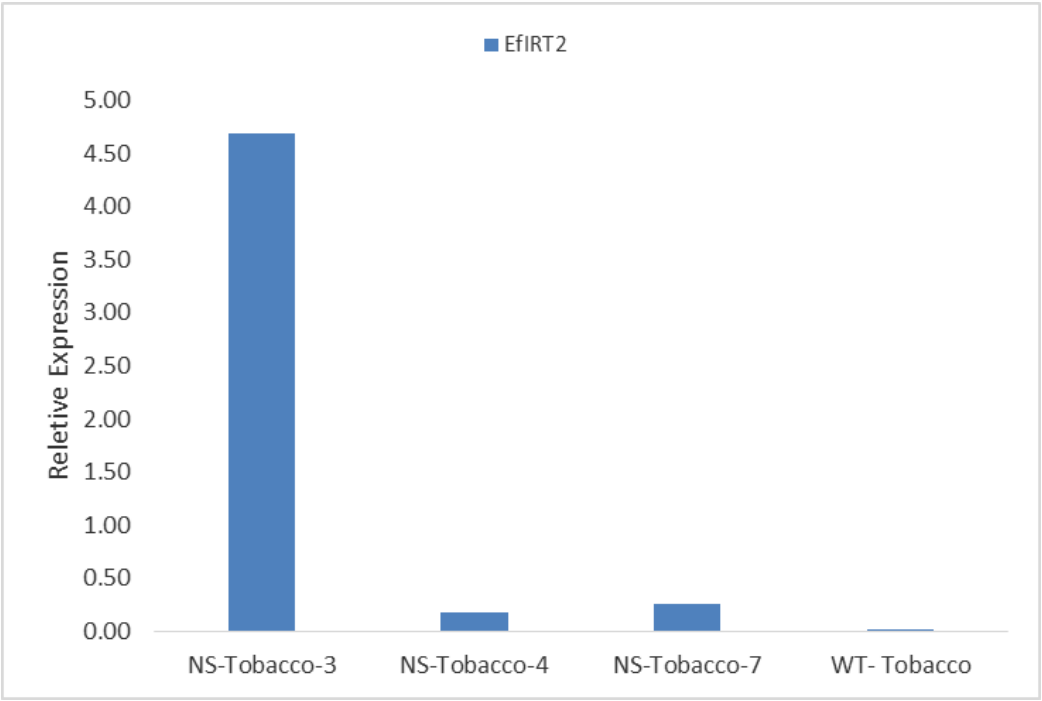

Fig. 5. Relative expression of *EfIRT2* transgene in transgenic tobacco lines. (T_0)

https://ejplantbreeding.org