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Agrobacterium mediated transformation of ABA biosynthetic pathway coding genes for enhanced drought tolerance in *Nicotiana tabacum*

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Abstract
Abscisic acid (ABA) is an important hormone that regulates stomata closure, developmental stages and drought tolerance in plants. NCED (EC 1.13.11.51) is a key enzyme involved in the biosynthesis of abscisic acid. Transgenic tobacco plants with the integration of *nced* and *rpk* genes under the control of stress inducible promoters *viz., sal T* and *lea P* were developed through *Agrobacterium* mediated transformation. Over expression of integrated genes were confirmed by leaf disc assay method by imposing artificial stress using methyl Viologen (5 µMmethyl Viologen for 36 hrs).On exposure of stress, transgenic plants showed high content of chlorophyll pigments and low level of electrolyte leakage and MDA were observed than the wild type plants. Over expression of *nced* and *rpk* enhanced the abscisic acid content and increased the tolerance to drought stress.

Keywords: Abiotic stress, *Nicotiana tabacum*, *Agrobacterium tumefaciens*, Abscisic acid, Methyl viologen

Introduction
Post genomic era offers unrivalled innovative opportunities in modern agriculture to meet a numerous complex challenge, which include feeding and clothing to a rapidly growing global population. There is a need to improve existing plant characteristics for better crop performance, particularly with respect to improving yield and stress tolerance in crops to adapt into the changing environment. Genetic manipulation of crop plants by using advanced techniques holds great promise for sustainable agriculture and meets the need and expectations in plant sciences and agriculture (Altman *et al.,* 1999) [1]. Genetic engineering directly manipulates the genome of an organism either by introduction of one or several new genes and regulatory elements, or by decreasing the expression of endogenous genes, for either of these cases, a DNA construct is inserted into one or more chromosomes in a random manner and into one or more loci (Naqvi *et al.,* 2009) [18]. Single gene transfers into the plants have been effective in cases of simple traits, such as herbicide tolerance and insect resistance, majority of these transgenic plants are produced through genetic transformation of only one transgenic protein. However, physiological functions and traits rely on coordination of multigene expression. Therefore, a strategy for simultaneous transfer of multiple genes into plants will provide the potential for manipulating sophisticated metabolic pathways, expressing recombinant protein complexes, and studying complex genetic control circuits for crop improvement (Naqvi *et al.,* 2009) [18]. Agricultural production is constrained by a variety of biotic and abiotic stresses that significantly reduce the quality and quantity of crop production. Among abiotic stresses, drought is the most devastating stress factor; Plants have developed several defense mechanisms involving various molecular, physiological and biochemical alterations in response to stress. Signaling pathway of any abiotic stress involves certain key steps such as signal perception, transduction, and responsiveness, combined with activation of physiological and metabolic reactions (Pérez-Clemente *et al.,* 2013; Liu J.H. *et al.,* 2014) [21, 15]. In this way, plant cells first perceive stress stimulus through sensors or receptors localized mostly at the cell membrane. The phytohormone, abscisic acid (ABA) is also reported to be more abundant under water-deficit conditions and this in turn causes stomata closure and induces expression of various stress related genes (Yang *et al.,* 2011) [27]. The ABA dependent pathway regulates stress inducible gene expression through several positive and negative regulators. Developing transgenics tolerance against drought stress involves coordination and controlling of multiple genes related to particular metabolic pathway.

We aimed to develop a transgenics through *Agrobacterium* transformation by inserting abscisic acid pathway genes-*nced* and *rpk* responsible for abiotic stress in a model plant of tobacco, we assessed the transgenic plants with regard to the expression levels of introduced genes in different ways of characterization *viz.*, molecular, biochemical and physiological methods in comparison with wildtype (WT) plants.

Materials and methods

Construct

Hyper virulent strain of *Agrobacterium tumefaciens*, EHA105, carrying a gateway vector pMDC99 has been used to construct plant expression vector with *rpk*and *nced* genes regulated by *leaP* and *salT* promoters respectively. *Nopaline synthase (nos*) has been used as terminator for both genes and*hpt II* was used as plant selectable marker.

Agrobacterium transformation

Sterile fresh leaves of tobacco (*Nicotiana tabacum* cv. Samsun) were chosen as explant for transformation, leaf discs were infected with *Agrobacterium* liquid culture, showing 0.6 OD at 580nm, for 15 min. Then the leaf discs were blotted on sterile tissue paper and co-cultivated on MS medium supplemented with 2.0 mg/L BAP and 0.05 mg/L NAA along with 200 µM AS in dark for 2 days. After two days, leaf discs were transferred onto the selection medium, which contains hygromycin as a selection agent. Cultures were maintained at 25 ^{0}C under 16/8 hrs light and dark photoperiod. After two weeks, the regenerated plantlets were transferred onto the rooting medium (MS-based medium). The rooted plantlets were hardened in pots containing soil mixture and maintained in poly house for further screening.

Molecular analyses of transgenic tobacco

Integration of transgenes was confirmed with PCR and southern blot analyses. Genomic DNA was isolated from the leaves of control and transgenic plants by following C-TAB method (Doyle and Doyle, 1990) [7]. Initial screening of putative plants was done by PCR with a respective plant selectable marker *hpt II;* positively amplified samples were further screened by using promoter and gene specific primers for positive integration of transgenes.

For southern blot analysis, genomic DNA from wildtype and PCR positive plants were digested with *Bam HI* restriction enzyme. Digested DNA was separated on 0.8% agarose gel and transferred onto Hybond-N+ nylon membrane by using upward capillary method. The membrane was hybridized overnight at 60 ^{0}C with *nced* probe and finallyX-ray film was developed.

Stress treatment - methyl viologen (MV) leaf disc assay

Stress tolerance of transgenic plants was assessed by applying methyl viologen to the leaf discs of tobacco plants. Leaf discs of 1.0 cm diameter were excised from healthy and fully expanded leaves of transgenic and wildtype tobacco plants and separately vacuum infiltrated in a 6 ml of different concentrations of MV (2.5, 5.0 and 7.5 µM) solution in a 24-well plate, the leaf discs in water were used ascontrol. Samples were placed in dark for two hrs and then illuminated to light (intensity of 100 µmol /m^2/s) for different time periods *viz.*, 24, 36, 48 and 60 hrs at a temperature of 28 ^{0}C.

Photosynthetic pigments analyses

Photosynthetic pigments *viz.*, chlorophyll a, b, total chlorophyll and total carotenoids analyses were done by using standard procedure of Arnon, (1949) [2]. After MV treatment, leaf discs were placed in one ml of 80% acetone at RT for overnight in dark and absorption was measured at 663 nm, 645 nm and 480 nm using a spectrophotometer. Photosynthetic pigments were estimated based on following equations.

Chlorophyll-A = 12.7 (A663) - 2.69 (A645) V/W × 1000.
Chlorophyll-B = 22.9 (A645) - 4.68 (A663) V/W × 1000.
Total chlorophyll = 20.8 (A645) + 8.02 (A663) V/W×1000.
Total carotenoids = A480+0.114×A663-0.638×A645.

Electrolyte leakage and Lipid peroxidation

Leaf discs, treated with different concentrations of methyl viologen with different time periods, were used for measuring electrolyte leakage and MDA content. Ion leakage studies were done by the protocol described by Kumari *et al.,* (2015) [14] and calculated by using the following formula. Ion leakage (%) = (E1-Ei)/ (E2-Ei) × 100.

Where, Ei = initial reading after MV stress, E1= after 12 hours reading, E2= after incubation at 100°C reading.

MDA content was measured by protocol described by Heath and Packer (1968) [9], the absorbance was recorded at 440, 532 and 600nm. Nonspecific absorbance of 600 nm was subtracted from the absorbance at 532 nm, and the difference was used to calculate the amount of MDA by using an extinction coefficient of 155/mM/cm and the concentration of MDA was expressed in nM/gFW.

ABA extraction and HPLC analysis

Drought stress experiment was conducted in pots using wild type and transgenic lines, during experiment plants were exposed to water deficit condition, ABA was extracted from leaves of transgenic and control plants under stress condition, leaves were homogenized with mortar and pestle in liquid nitrogen to a fine powder and then extracted overnight with 80% cold aqueous methanol in dark at 4°C. The extract was filtered through a whatman filter paper no-1 for twice and dried using nitrogen gas and stored at -20°C. One ml of 80% methanol was added to the dried extract, vortex and filtered through 13 mm diameter nylon membrane (Millex filters Ø 0.22 µm) and placed in vials. ABA was measured by C18 column of HPLC (Waters 2695) by using a mobile phase of methanol and glacial acetic acid (0.1% v/v) in an isocratic method at a wavelength of 254nm.

Statistical analysis

All the experiments were performed at least three times under the same conditions and the effect of various parameters on biochemical data obtained from transgenic plants with reference to their control wild type plants were subjected to statistical analyses by following Snedecor and Cochran (1968) [23].

Results

Simple traits like insect resistance genes were transformed easily into plant cells using simple constructs, whereas, in case of metabolic pathways and physiological functions, where more than one gene is involved and interconnected, gateway technology has made an advantage for the construction of more than a single gene in a single construct. In this context, we developed transgenic tobacco plants with two-genes construct *viz.*, *rpk* and *nced*, through *Agrobacterium* mediated transformation. Callus initiation was observed after a week from cut edge of leaf disc explants of

tobacco (Figure 1 A, B), which were transferred onto hygromycin selection medium (Figure 1C&D) and selected shoots were transferred onto rooting medium and well rooted plantlets were hardened in polyhouse (Figure 1E).

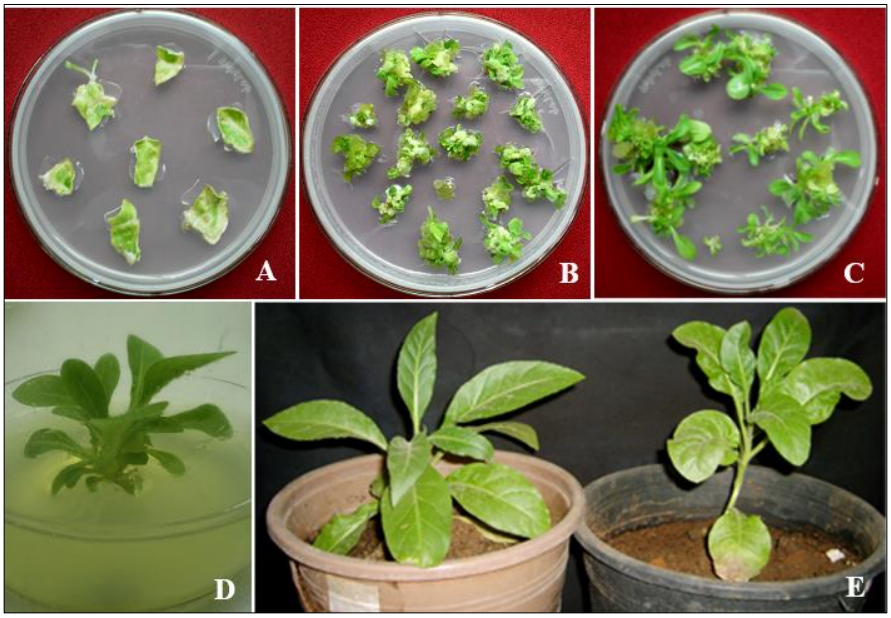

Fig 1: Different stages of *in vitro* culture of tobacco (*Nicotiana tabacum* L.), in *Agrobacterium* mediated genetic transformation: (A) Leaf explants inoculated on MS medium after co-cultivating with *Agrobacterium*; Multiple shoot induction from explants at initial (B&C) and fully grown stage (D) on selection medium; Well grown *in vitro* raised tobacco plants after transferring into pots (E).

Molecular analysis of transgenic plants for the confirmation of transgene integration

DNA was isolated from transgenic plants along with control tobacco plants, which were analyzed for transgene integration. Initially transgenic plants were screened through PCR analysis by using selection marker-*hpt II*(Figure 2), amplification was observed in some plants, later, *nced* and *rpk* primers were used and confirmed the presence of integrated gene in selected amplified samples, whereas in wildtype non-transformed plantsno fragment amplification was observed. Samples which give positive amplification in PCR analysis were selected for southern hybridization and hybridized with *necd*probe, from which single copy lines were selected for further analyses.

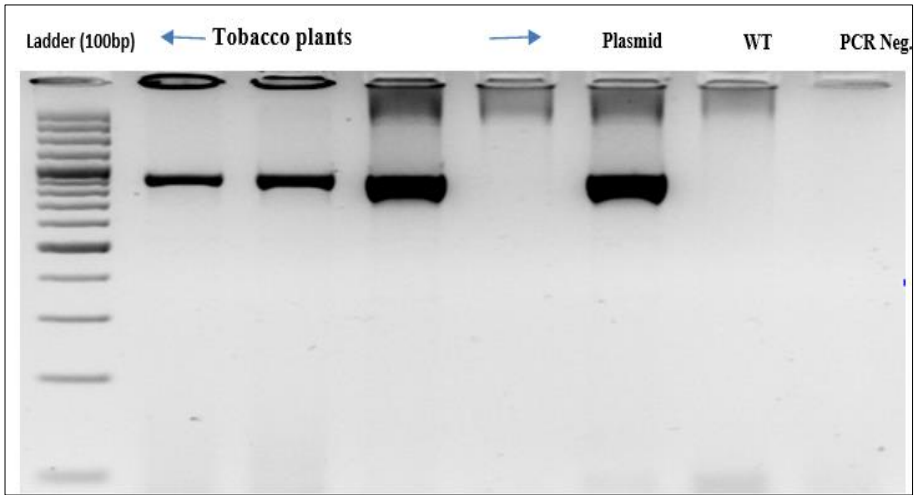

Fig 2: PCR screening of putative transgenic showing the amplification at 980bp and control tobacco plants with no amplification band using *hpt II* selectable marker

Effect of methyl viologen stress

Drought stress tolerant transgenic tobacco plants developed by using two genes construct were analyzed by leaf disc assay method. Leaf discs of transgenic and wild type plants were treated with different concentrations of MV (2.5, 5.0 and 7.5 µM) and exposed to different time periods (*viz.*, 24, 36, 48 and 60 hrs). In all the concentrations significantly higher levels of leaf damage were observed in wild type leaf discs while compared with the transgenic plants in all the time periods. The leaf discs in water (0 µM of MV) were treated as control throughout the experiment. WT plant leaves were severely affected, while the transgenic plant leaves displayed tolerance to MV and this is further proved by the chlorophyll contents.

The effect of different concentrations of MV on chlorophyll pigments *viz.*, total chlorophyll, chlorophyll a, chlorophyll b, and total carotenoids were measured in WT and transgenics. The damage caused by MV stress was visualized by the degree of bleaching of leaf tissues. Most of the WT samples were bleached after exposing leaf discs to MV stress while transgenic samples remained green, the stress effect tended to be more significant with the increased concentration of MV.

Even in all the concentrations of MV solution, the total chlorophyll and total carotenoids content of transgenics was higher than in WT plants. Normal and partial bleaching of leaf discs were observed at 2.5 and 5 µM MV concentrations, whereas leaf discs were strongly damaged and pigment concentration was drastically decreasedat higher end of 7 µM concentration(Figure 3A&B).

Electrolyte leakage (EL) and MDA content was found to be stable in transgenic lines, but the leakage was increased in WT plants as concentration increases (Figure 3C&D). Eventually, 5 µM concentration of MV and 36 hrs photo period exposure was found to be optimized for tobacco leaf disc assay.

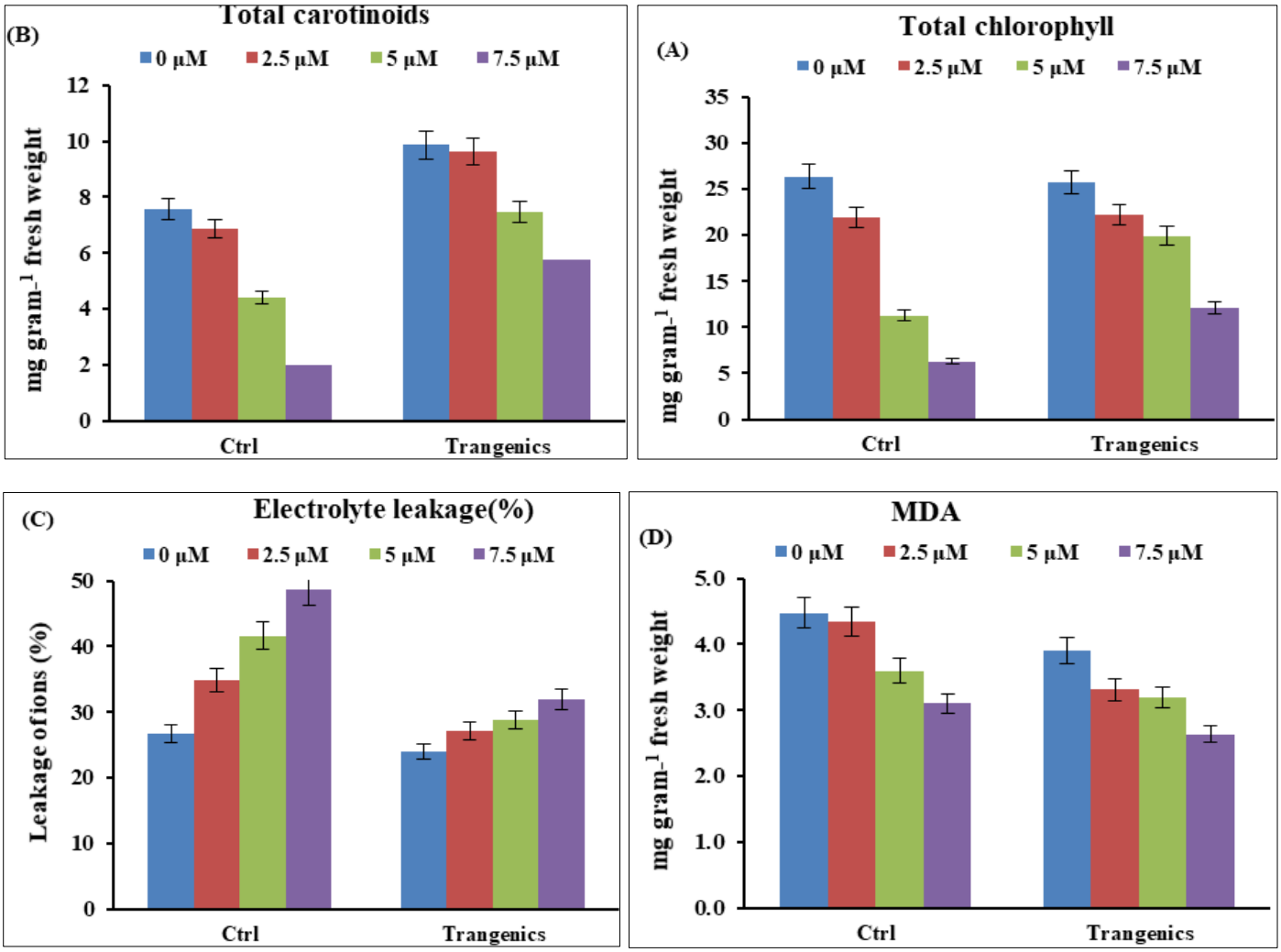

Fig 3: Optimization of Methyl Viologen concentrations (0,2.5,5.0 and 7.5µM)on tobacco leaf disc assay by estimating different parameters *viz.*, (A) Total chlorophyll, (B) Total carotenoids, (C) Electrolyte leakage, and (D) Malonaldehyde concentration of transgenics and control plants leaf under MV stress at 36hrs exposure

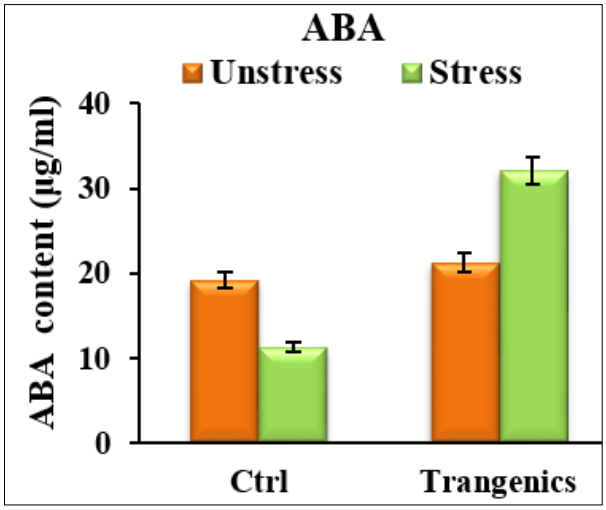

Fig 4: Estimation of Abscisic acid (ABA) in transgenic and control plants under drought stress and un stress. ABA was extracted from leaves of tobacco plants through HPLC method

ABA analysis

Over expression of *nced* gene contributed to the higher ABA accumulation in transgenic plants. Leaf samples were collected under stress and normal condition and measured ABA levels in transgenic and WT plants by HPLC method, under normal condition no difference has been observed in both the lines, whereas under stress condition, transgenic plants showed increased ABA level compared to WT plants (Figure 4) and increased the survival rate under drought condition.

Discussion

Drought stress induces a series of biochemical and physiological changes in plants, which include repression of cell growth, photosynthesis and stomata closure. Previous studies on molecular and biochemical pathways have identified many of stress responsive genes, transcription factors and phytohormones which are responsible for morphophysiological changes occurred in plants due to the

stress environment. ABA is a stress signaling hormone in plants and plays an important role in drought stress tolerance through regulating expression of various stress responsive genes in model plants as well as crop plants (Oztur *et al.*, 2002; Yu and Setter, 2003; Buchanan *et al.*, 2005;Muppala*et al.*, 2021) [20, 28, 5, 17]. Stress responsive genes can be expressed either through ABA dependent or ABA independent pathway or both of the pathways (Chinnusamy *et al.*, 2004; Kobayashi *et al.*, 2006) [6, 13]. Many genes regulate the biosynthesis of ABA, initially the gene *ZEP* was cloned, and expressed in many plant species, but it varies among species and tissues, in this case drought induction observed only in roots not in leaves of *Arabidopsis*, tomato (Audran *et al.*, 1998; Thompson *et al.*, 2000a) [3, 25] and in cowpea they are not drought responsive (Iuchi *et al.*, 2000) [11]. In the pathway of ABA biosynthesis, expression of *nced* gene has received more importance and is considered as a rate limiting enzymes (Bang *et al.*, 2013) [4]. Previous studies have shown that increased NCEDtranscript levelsin plants haveincreased the biosynthesis of ABA (Qin and Zeevaart, 2002; Martinez-Andujar *et al.*, 2011) [22, 16], In present study, transgenic tobacco plants were developed, expressing *nced* and *rpk* genes under the control of stress inducible promoters *leaP* and *salT*respectively. In the process of transgenic development and expression, promoters will also play an important role. Thompson *et al.*, 2000 [26], in his studies revealed using a constitutive promoter such as *CaMV-35S*leads to the over expression of *nced*in tomato, that leads to negative pleiotropic effects, such as over guttation, leafmargin chlorosis, and seed dormancy. Estrada-Melo *et al.*, 2015 [30], in his studies on petunia used stress inducible promoter rd29a; their results demonstrated that over expression of *nced* gene induces ABA biosynthesis and improves drought tolerance by avoiding the negative effects resulting from use of constitutive promoter. In our previous studies (Sreenu *et al.*, 2016) [24], rd29 promoter was used to drive genes related to glutathione pathway in maize;in present study, using stress inducible promoter *salT*and *leaP*, similar results were observed in chlorophyll pigments content, MDA and electrolyte leakage under MV stress in transgenic tobacco plants. Chlorophyll pigments content were increased and EC and MDA levels were decreased when compared with control plantssimilar results were reported by Zhao *et al.*, 2010 [29], were the over expression of ARAG1 in rice promotes the synthesis of higher level of ABA and leads to early stomata closer in transgenic plants which prevent major water loss, similarly over expression of *nced* gene increased ABA level in transgenic tobacco plants. Iuchi *et al.*, 2001[12] reported that over expression of *nced* gene increases endogenous ABA level and improved drought tolerance in various plants. likewise, in present study, increased ABA levels were observed under drought stress in transgenics. RPK1 is a membranelocalized RLK, its expression was induced by drought stress and ABA level (Hong *et al.*, 1997) [10]. Osakabe *et al.*, 2010 [19], reportedin *Arabidopsis*, *rpk1*, induces various stress responsive genes and enhances the physiological responses to drought stress, our results demonstrated that over expression of *nced* and *rpk* genes positively regulates drought stress tolerance by reducing water loss through stomata closure.

Conclusion

Traits controlled by many genes and or regulators in a physiological pathway can be manipulated using the specific promoters of related physiological function and can be introduced into plant system using multigene gateway technology which has been developed using model plant tobacco in the present study.

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References

1. Altman A. Plant biotechnology in the 21st century: the challenges ahead. Electronic Journal of Biotechnology. 1999;2(2):1-2.
2. Arnon DI. Copper enzymes in isolated chloroplasts Poly phenol oxidase in *Beta vulgaris*. Plant Physiology. 1949;24(1):1-15.
3. Audran C, Borel C, Frey A, Sotta B, Meyer C, Simonneau T, *et al*. Expression studies of the *zeaxanthin epoxidase* gene in *Nicotiana plumbaginifolia*. Plant Physiology. 1998;118(3):1021-1028.
4. Bang SW, Park SH, Jeong JS, Kim YS, Jung H, Ha SH, *et al*. Characterization of the stress-inducible *OsNCED3* promoter in different transgenic rice organs and over three homozygous generations. Planta. 2014;237(1):211-224.
5. Buchanan CD, Lim S, Salzman RA, Kagiampakis I, Morishige DT, Weers BD, *et al*. Sorghum bicolor's transcriptome response to dehydration, high salinity and ABA. Plant Molecular Biology. 2005;58(5):699-720.
6. Chinnusamy V, Schumaker K, Zhu JK. Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. Journal of Experimental Botany. 2004;55(395):225-236.
7. Doyle JJ, Doyle JL. Isolation of plant DNA from fresh tissue. Focus. 1990;12(1):13-15.
8. Temesgen Begna. Impact of drought stress on crop production and its management options. Int. J Res. Agron. 2021;4(2):66-74.
9. Heath RL, Packer L. Photo peroxidation in isolated chloroplasts, I. kinetics and stoichiometry of fatty acid peroxidation. Archives of Biochemistry and Biophysics. 1968;125(1):189-198.
10. Hong SW, Jon JH, Kwak JM, Nam HG. Identification of a receptor-like protein kinase gene rapidly induced by abscisic acid, dehydration, high salt and cold treatments in *Arabidopsis thaliana*. Plant Physiology. 1997;113(4):1203-1212.
11. Iuchi S, Kobayashi M, Yamaguchi-Shinozaki K, Shinozaki K. A stress-inducible gene for *9-cis-epoxycarotenoid dioxygenase* involved in abscisic acid biosynthesis under water stress in drought-tolerant cowpea. Plant Physiology. 2000;123(2):553-562.
12. Iuchi S, Kobayashi M, Taji T, Naramoto M, Seki M, Kato T, *et al*. Regulation of drought tolerance by gene manipulation of *9-cis-epoxycarotenoid dioxygenase*, a key enzyme in abscisic acid biosynthesis in *Arabidopsis*. The Plant Journal: for Cell and Molecular Biology. 2001;27(4):325-333.
13. Kobayashi Y, Murata M, Minami H, Yamamoto S, Kagaya Y, Hobo T, *et al*. Abscisic acid-activated SNRK2 protein kinases function in the gene-regulation pathway of ABA signal transduction by phosphorylating ABA

response element-binding factors. The Plant Journal: for Cell and Molecular Biology. 2005;44(6):939-949.
14. Kumari S, Joshi R, Singh K, Roy S, Tripathi AK, Singh P, *et al*. Expression of a cyclophilin OsCyp2-P isolated from a salt tolerant landrace of rice in tobacco alleviates stress *via* ion homeostasis and limiting ROS accumulation. Functional & Integrative Genomics. 2015;15(4):395-412.
15. Liu JH, Peng T, Dai W. Critical cis-acting elements and interacting transcription factors: key players associated with abiotic stress responses in plants. Plant Molecular Biology Reporter. 2014;32(2):303-317.
16. Martínez-Andújar C, Ordiz MI, Huang Z, Nonogaki M, Beachy RN, Nonogaki H. Induction of *9-cis-epoxycarotenoid dioxygenase* in *Arabidopsis thaliana* seeds enhances seed dormancy. Proceedings of the National Academy of Sciences of the United States of America. 2011;108 (41):17225-17229.
17. Muppala S, Gudlavalleti PK, Malireddy KR, Puligundla SK, Dasari P. Development of stable transgenic maize plants tolerant for drought by manipulating ABA signaling through *Agrobacterium*-mediated transformation. Journal of Genetic Engineering and Biotechnology. 2021;19(1):1-14.
18. Naqvi S, Farre G, Sanahuja G, Capell T, Zhu C, Christou P. When more is better: Multigene engineering in plants. Trends in Plant Science. 2010;15(1):48-56.
19. Osakabe Y, Mizuno S, Tanaka H, Maruyama K, Osakabe K, Todaka D, *et al*. Overproduction of the membrane-bound receptor-like protein kinase 1, *RPK1*, enhances abiotic stress tolerance in *Arabidopsis*. The Journal of Biological Chemistry. 2010;285(12):9190-9201.
20. Oztur ZN, Talame V, Deyholos M, Michalowski CB, Galbraith DW, Gozukirmizi N, *et al*. Monitoring large-scale changes in transcript abundance in drought- and salt-stressed barley. Plant Molecular Biology. 2002;48(5-6):551-573.
21. Pérez-Clemente RM, Vives V, Zandalinas SI, López-Climent MF, Muñoz V, Gómez-Cadenas A. Biotechnological approaches to study plant responses to stress. BioMed Research International. 2013;2013: 654120.
22. Qin X, Zeevaart JA. Over expression of a *9-cis-epoxycarotenoid dioxygenase* gene in *Nicotiana plumbaginifolia* increases abscisic acid and phaseic acid levels and enhances drought tolerance. Plant Physiology. 2002;128(2):544-551.
23. Snedecor GW, Cochran WG. Statistical methods, The Iowa State University Press, Ames and Oxford and IBH publishing Co Pvt Ltd, New Delhi, 1968.
24. Sreenu P, Sateesh Kumar P, Reddy MK, Sailaja D, Pavan Kumar G. Resourceful and high efficiency *Agrobacterium* mediated transformation of maize (*Zea maysL.*) using coleoptilar nodal explants. International Journal of Current Research in Biosciences and Plant Biology. 2016;3(12):1-9.
25. Thompson AJ, Jackson AC, Parker RA, Morpeth DR, Burbidge A, Taylor IB. Abscisic acid biosynthesis in tomato: regulation of zeaxanthin epoxidase and 9-cis-epoxycarotenoid dioxygenase mRNAs by light/dark cycles, water stress and abscisic acid. Plant Molecular Biology2000a; 42(6): 833–845.
26. Thompson AJ, Jackson AC, Symonds RC, Mulholland BJ, Dadswell AR, Blake PS *et al*. Ectopic expression of a tomato *9-cis-epoxycarotenoid dioxygenase* gene causes over-production of abscisic acid. The Plant Journal for Cell and Molecular Biology. 2000;23(3):363-374.
27. Yang SD, Seo PJ, Yoon HK, Park CM. The *Arabidopsis* NAC Transcription factor VNI2 integrates abscisic acid signals into leaf senescence via the *COR/RD* Genes. The Plant Cell. 2011;23(6):2155-2168.
28. Yu LX, Setter TL. Comparative transcriptional profiling of placenta and endosperm in developing maize kernels in response to water deficit. Plant Physiology. 2003;131(2):568-582.
29. Zhao L, Hu Y, Chong K, Wang T. *ARAG1*, an ABA-responsive *DREB* gene, plays a role in seed germination and drought tolerance of rice. Annals of Botany. 2010;105(3):401-409.
30. Estrada-Melo AC, Chao, Reid MS, Jiang CZ. Over expression of an ABA biosynthesis gene using a stress-inducible promoter enhances drought resistance in petunia. Horticulture Research 2015;2:15013.