Tobacco plants expressing the defensin NaD1 enhances drought tolerance characteristics in transgenic lines

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Abstract

Biotic and abiotic stresses are main factors limiting crop plants yields and adaptability. Antimicrobial peptides (AMPs) play a pivotal role in plant immune responses to diverse stresses, and hence, becoming novel and essential molecules for studying plant responses to environmental harsh conditions. In this study, for the first time, overexpression of recombinant defensin *NaD1*gene under control of 3x 35S promoter was incorporated into tobacco plants resulting in generating *NaD1* transgenic lines. Stable expression of *NaD1* in transgenic tobacco lines was confirmed by RT-PCR, and next, presence of NaD1 recombinant peptide was verified by ELISA and western blot analysis in transgenic lines. *In Silico* bioinformatic analysis revealed that the most abundant components in Cis-regulatory elements in eleven *NaD1* homologs in *Nicotiana attenuate* (*NaDEF* genes) are MYB, MYC and ABRE elements suggesting that *NaD1* promoter is involved in regulation of abiotic stresses. Overexpression of the *NaD1* in transgenic plants led to a significant ($P \leq 0.01$) increase in the content of chlorophyll *a*, *b*, and total chlorophyll under drought stress. Correspondingly, the index of chlorophyll stability significantly increased in 3 transgenic lines. Moreover, activities of Catalase (CAT), Peroxidase (POD), Ascorbate peroxidase (APX) and S uperoxide dismutase (SOD) were significantly enhanced in response to drought stress in transgenic lines. Among three transgenic lines, line 1 showed the highest chlorophyll level and chlorophyll stability index and a high level of POD, CAT, and SOD enzyme activity under drought stress. The data together suggest that increased antioxidant activity of the enzymes might presumably lead to eliminate ROS levels and maintain the chlorophyll content and stability in response to drought stress. Therefore, the antimicrobial peptide defensin NaD1 can be considered an essential factor in regulation of plant responses to drought stress and could be used in developing transgenic lines resistant to abiotic stresses.

Introduction

Biotic and abiotic stresses are considered as the most critical factors reducing crop yield worldwide. The plant's response to biotic or abiotic stress varies depending on the type of stress. Expression of specific genes or production of common secondary messengers play crucial roles in the regulation of plant responses to stress (Zhang and Sonnewald 2017). Using their extensive defense networks, plants produce high concentrations of some secondary metabolites including tannins, kinins, terpenoids, phenols, and antimicrobial peptides (AMPs) to cope with adverse environmental conditions (Isah 2019; Sharma et al. 2021). The biosynthesis of antimicrobial molecules such as phytoanticipins and phytoalexins (secondary metabolites), proteins associated with pathogenesis, and antimicrobial peptides is stimulated in response to biological stresses resulting in reactive oxygen species (ROS) accumulation and death of plant cells (De Vos et al. 2005; Karpun et al. 2015).

AMPs as an essential part of plant immunity system play a crucial role in maintaining homeostasis and prevention of plant cell infection spread (Karpun et al. 2015). In fact, plants produce these peptides as the first line of defense against plant pathogens (Sher Khan et al. 2019). AMPs are composed of five to more than one hundred amino acids encoded by a single gene (Sabokkhiz et al. 2019). They can destroy microorganisms with minimal energy consumption during primary infection. In addition, AMPs have been successfully employed to improve plant resistant to biotic stresses in agriculture (Lee et al. 2017). Mechanism of action of AMPs on target organisms (pathogens) includes disruption of the cell membrane, RNA synthesis and protein synthesis, inhibition of specific enzymes and induction of ROS biosynthesis (Campo et al. 2008). Many AMPs have been isolated from plants, animals and human and transferred to plants (Campo et al. 2008; Chen et al. 2006; Holaskova et al. 2015). For instance, cecropin was isolated from *Hyalophora cecropia* L. and transferred to rice plants, which increased resistance to the blast disease (Campo et al. 2008). Also, the expression of human LL-37 peptide under the pGD1

promoter in rice significantly inhibited the growth of *Xanthomonas oryzae* (the blight disease) in the leaves, and high resistance against the blast disease caused by *Magnaporthe oryzae* (Lee et al. 2017).

In addition to their main role in response to biotic stress, AMPs are also considered as components of plant response to abiotic stress (Olga et al. 2020; Tam et al. 2015). The role of some AMPs, especially defensins, in response to abiotic stress has also been reported (Olga et al. 2020; Tam et al. 2015). Under drought stress, for instance, the Dhn8 defensin was activated in the *Glycine max* and its transcript levels showed a 10-fold increase in leaves and roots of the drought-resistant transgenic plants (Lay and Anderson 2005). Additionally, transcription of defensin genes *NeThio1*, *NeThio2* in *Nicotiana excelsior*, and *NpThio1* in *Nicotiana paniculate* were induced under salt stress, which was associated with an increased tolerance to 250 mM NaCl. Furthermore, cold-tolerant genotypes of *Triticum aestivum* seedlings showed a higher level of *Tad1* defensin expression (Koike et al. 2002). Defensin NaD1 (defensin1 *Nicotiana alata*) contains a sequence of 47 amino acids (Lay and Anderson 2005). Its transcripts reach to the highest levels in the peripheral cell layers of plant immature flower bud and NaD1 peptides are stored in the cell vacuole (Lay et al. 2003). It was found that NaD1 has an antifungal activity against some pathogenic fungi and yeast *Saccharomyces cerevisiae* (Poon et al. 2014). The fungi cells die when NaD1 defensin contacts the fungal cell wall and moves into the cytoplasm via plasma membrane. Upon entrance, ROS are rapidly produced, and permeability of the fungal plasma membrane increases and finally causes cell death (Bleackley et al. 2016). In addition to its antifungal activity, NaD1 has also insecticidal activity in such a way that when expressed in an artificial diet or transgenic plants, it reduced the growth of Lepidopteran insect pests such as *Helicoverpa armigera* and *Helicoverpa punctigera* via inhibition of the critical digestive enzymes trypsin and chymotrypsin (Lay et al. 2003). Therefore, although, antifungal and insecticidal activity of NaD1 defensin is somehow clear, but its action in response to abiotic stresses is unknown.

In this study, for the first time, we investigated the role of NaD1 antimicrobial peptide in drought stress. The coding sequence of *NaD1* was overexpressed in *Nicotiana tabacum* under control of 35S promoter. The developed *NaD1* transgenic tobacco lines were evaluated for several physiological and biochemical drought tolerance-related characteristics. Moreover, *In Silico* analysis of Cis-regulatory elements was performed in eleven *NaD1* homologs in *Nicotiana attenuate* (*NaDEF* genes). The experimental data together with the bioinformatic results displayed that NaD1 defensin is involved in plant drought regulation and its overexpression in the tobacco transgenic lines leads to a considerable enhancement in drought tolerance characteristics.

Materials And Methods

Design and synthesis of pUC57: NAD1**recombinant construct**

The coding sequence of *NaD1* was obtained from NCBI database under accession number AF509566.1 (www.ncbi.nlm.nih.gov). To make *NaD1* cassette, first, the sequence encoding signal peptide (SP) of *vicilin* gene containing 15 amino acids under accession number Q702P1 from *Pisum sativum* was fused to the 5' terminal part of construct to ensure secretion of recombinant protein in the intercellular (apoplastic) space (Lee et al. 2017; Pang et al. 1992). The Poly histidine tag $(His)_6$ sequence (HT) was engineered to the 5' terminal after SP to recognize the recombinant protein. Three times promoter cauliflower mosaic virus 35S (CaMV 35S) promoter was used to overexpress the *NaD1* in transgenic plants (https://web.expasy.org/protparam/) (Fig. 1). After optimizing the amino acid codons based on *Nicotiana tabacum* codon preferences by Genscript database (www.genscript.com), flanked by *Nco*I and *Bam*HI restriction enzymes, the recombinant construct was chemically synthesized and cloned in the pUC57 cloning vector (Shinegene, China). The resulting plasmid was sequenced (Shinegene, China) to confirm the

construct. The recombinant plasmid pUC57: *NaD1* was transferred to the *E.coli* strain *DH5α*, using the heat shock method (Russell and Sambrook 2001).

Construction of pGSA1285 expression vector

pUC57: *NaD1* plasmids were extracted from *E. coli DH5α* according to Roch instructions on High Pure Plasmid Isolation Kit. Enzymatic digestion of recombinant vectors including pUC57: *NaD1* and expression vector pGSA1285 was performed by *Bam*HI and *Nco*I enzymes according to the instructions of Thermo Fisher Scientific at 37°C. Inactivation temperature of the digestion reaction was done at 80°C (Fig. 2a and b). Then, *NaD1* gene fragment (Cut out from pUC57) and linearized expression vector pGSA1285 were purified from agarose gel according to the instructions of the DNA extraction kit from GeneJET Gel Extraction Kit - Thermo Fisher Scientific. Next, both digested segments including *NaD1* (239 bp) and linearized vector pGSA1285 (9553 bp) was ligated by use of the Sinaclon T4 DNA Ligase enzyme kit according to the company's instructions. The ligation reaction was performed for 2 hours at 22°C, and at the end, the enzyme was deactivated for 15 minutes at 64°C. Subsequently, the ligated products were transferred to competent cells of *E.coli DH5α* by heat shock (Russell and Sambrook 2001). After 24 hours, clones grown at 37°C on a solid LB culture medium containing 34 µg/ml of chloramphenicol antibiotic were checked. The presence of desired gene fragment in pGSA1285 plasmid was confirmed by a colony PCR (Fig. 3c) by *NaD1* specific primers following observation of 106 bp band. The PCR reaction program is presented in Supplementary file, Table S1. Next, enzyme digestion was accomplished by *Bam*HI and *Nco*I cleavage sites (Fig. 4d) To ensure the correct fusion of two fragments and sequence accuracy, Sanger sequencing was performed by Pishgam and Applied biosystem, and the sequences was checked by Snap gene software and Clastal omega program of the EMBL site. Transfer of the recombinant plasmid to *Agrobacterium tumefaciens* strain *GV3101 pmp90* was achieved by thawing and freezing method (Russell and Sambrook 2001). The colony PCR reaction was done by *NaD1* gene-specific primers on *Agrobacterium* colonies grown after 48 hours at 28°C in a selective culture medium containing antibiotics chloramphenicol 50 µg/ml, rifampicin 100 µg/ml and gentamicin 64 µg/ml (Fig. 2d, Supplementary file Table S1).

Table 1
The sequence of specific forward and reverse primers of *NaD1* and *elf*α genes of PCR reactions

Gene name	Sequences	Annealing temperature (C∘)	Size (bp)
NaD1	Forward: 5'- AGGATCTCATCATCACCACCA-3'	57	106
	Rerverse:5'- AGCTTTTCTACATGGTGGCT-3'		
elf α	Forward: 5'- AGGATCTCATCATCACCACCA-3'	57	177
	Rerverse:5'- AGCTTTTCTACATGGTGGCT-3'		

Plant materials

Nicotiana tabacum L. var *Xanthi tobacco* was utilized in this research. First, the seeds were immersed in 70% ethanol for 30 seconds, washed with sterile distilled water, and then disinfected with 5% sodium hypochlorite for 10 minutes and rinsed three times. Later, they were cultured on MS medium containing 30 g/L sucrose and 6.5 g/L

agar with pH 5.8. The seeds were kept under 2000 lux light and temperature 25 ± 1°C with a 16/8-h light/dark photoperiod.

Agrobacterium **-mediated transformation**

Leaf explants (3-week-old plants) were transferred to a suspension of *Agrobacterium tumefaciens* strain *GV3101 pmp90* containing the pGSA1285: *NaD1* gene constructs for 20 minutes. Then, the inoculated leaves were transferred to a co-cultivation medium (MS containing 50 g L^{-1} sucrose and 150 µm acetosyringone, pH 5.8, 0.1 mg L^{-1} NAA and 12 mg L^{-1} BAP) for 72 hours at 24 ± 2°C in dark. Afterward, the leaf explants were transferred to a culture medium containing 100 mg L^{-1} kanamycin and 500 mg L^{-1} cefotaxime to remove *Agrobacterium* and plant growth regulators; 0.1 mg L^{-1} NAA and 2 mg L^{-1} BAP, to regenerate seedlings. Upon the emergence of 4–6 leaf buds, the samples were separated and re-cultured in a medium containing 100 mg L^{-1} kanamycin, 200 mg L^{-1} cefotaxime, 0.1 mg L^{-1} NAA, and 2 mg L^{-1} BAP. Then young seedlings were transferred to the rooting medium containing 0.5 mg L^{-1} NAA and 2 mg L^{-1} IBA (Indole-3-butyric acid). After the emergence of roots, the seedlings were transferred to a hormone-free medium. After sufficient root growth, young plants were transferred to pots containing sterile soil (perlite, peat moss, vermiculite, and vermicompost) for development and kept in the growth chamber at 25 ± 1°C, 8/16-h dark/light cycle, and 50% relative humidity for further analyses (Fig. 3).

Molecular analysis of transgenic plants

Genomic DNA of selected transgenic plants (grown in 100 mg L^{-1} kanamycin) and non-transgenic plants was extracted using the CTAB method (Richards et al. 1994), and its quality was evaluated with 1% agarose gel. The presence of the *NaD1* gene sequence in the transgenic plants (the non-transgenic plant as control plants) were confirmed by using its specific primers (Supplementary file, Table S1, Table 1), and the result of the reaction was checked by 2% agarose gel. To investigate *NaD1* gene expression, RNA extraction from transgenic and non-transgenic plants (control) was performed by the DENAzist RNA isolation kit, the quality and quantity of the extracted RNA using 1% agarose gel electrophoresis and the Thermo Scientific™ nanodrop according to ng μL^{-1} was determined. After treating the total RNA with DNase I to remove possible DNA contamination, cDNA was synthesized using the CinaClon First Strand cDNA Synthesis Kit. To confirm the correctness of cDNA synthesis and also expression of *NaD1* genes in transgenic plants and its non-expression in non-transgenic plants, a semi-quantitative RT-PCR reaction was performed using internal control gene *elf1*α primers with a length of 177 bp and *NaD1* specific primer on cDNA synthesized from transgenic and non-transgenic plants (Table 1), and the result of the reaction was separated on a 2% agarose gel and photographed (Fig. 4).

Investigation of NaD1 recombinant peptide production

Transgenic and non-transgenic leaf tissues were powdered in liquid nitrogen, then 1 ml of phosphate buffer (50 mM, pH 7) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) protease inhibitor was added to each sample (Sigma-Aldrich) and vortexed for 5 minutes. After 30 minutes of centrifugation at 4°C with a speed of 13000 rpm, the upper phase was separated and filtered through a 0.45-micron filter, and the protein concentration was measured by the Bradford method (Bradford 1976). Next, to determine the quality and presence of protein bands of transgenic plants containing NaD1 peptide (KDa9), sodium dodecyl sulfate-polyacrylamide gel electrophoresis [15% SDS-PAGE (15% separating gel and 5% stacking gel)] and Coomassie Brilliant Blue R-250 staining was performed (not shown) (LAEMMLI 1970). The production of NaD1 recombinant peptide containing histidine [Histidine tag, $(His)_6$] was confirmed by the ELISA according to the instructions of the supplier. In order to perform a western blot, samples of proteins (10 µg each) were separated on a 15% SDS-PAGE gel and transferred to a polyvinylidene difluoride

membrane (Millipore) and blocked with 3% BSA in PBS phosphate-buffered saline for 1 h. The membrane was incubated overnight at 4°C with 1:200 dilution of the mouse anti-His sc-8036 antibody (Santa Cruz Biotech). The appropriate secondary antibodies m-IgG_1 BP-HRP Bundle (Santa Cruz Biotech) were used to detect the antibody-antigen complex on membrane. The blots were developed with chemiluminescence detection system (Pierce ECL, Thermo Fisher Scientific). For their development, HyBlot CL (Denville Scientific, Metuchen, NJ) and Amersham Biosciences Hyperfilm were used to detect multiple proteins, membranes were stripped and then reprobed.

Drought stress induction, experimental setup and data analysis

To induce drought stress, all soil-based grown plants were equally irrigated up to generation of 10 leaves, then they were divided into two groups, the first group was not irrigated for 20 days, and the other group irrigated regularly. A Factorial Completely Randomized Design was employed to perform the experiment in which the first factor includes two level of drought stress treatment (no irrigation and regular irrigation), and second factor comprises *NaD1* transgenic lines and non-transgenic plants (Control), with three replications, following sample collections and measurement of photosynthetic pigments and enzyme activity assay. The percentage of chlorophyll stability index was also investigated in an entirely random basic design. The growth conditions were conducted as follows: Temperature 25 ± 1°C, humidity 50%, and light condition LUX 6000 with 16/8-h light/dark photoperiod. Data analyses were performed by use of SAS software ver 9.1. The average comparison for all investigated traits was performed by the LSD test ($P \leq 0.01$).

Total protein extraction

In a laboratory porcelain mortar, 50 mg of transgenic and non-transgenic plant leaves were ground in liquid nitrogen, then 1 ml of extraction buffer (50 mM phosphate buffer) was added to each sample. After vigorous vortexing, the samples were centrifuged for 15 minutes at 14,000 rpm at 4°C, and the supernatant was centrifuged again for 10 minutes at 14,000 rpm at 4°C (Esfandiari et al. 2007). Total protein concentration was determined by the Bradford method and according to the instructions of the DNAbiotech kit (Bradford 1976).

Evaluation of enzyme activity

Catalase (CAT) activity was evaluated by the method provided by Dhindsa and Motowe's method (1981) with including some modifications. For that, the amount of decrease in light absorption at the wavelength of 240 nm was measured using a spectrophotometer. Enzyme activity was measured regarding changes in absorbance over time in one minute (Dhindsa and Matowe 1981). Peroxidase (POD) enzyme activity was measured by Chance and Meahly (1955) with slight modifications. To measure POD activity, light absorption at 470 nm wavelength was measured and recorded for one minute (Chance and Maehly 1955). In addition, the method of Nakano and Asada (1987) was employed to determine the activity of the ascorbate peroxidase (APX) activity. APX activity was determined by spectrophotometer by measuring the amount of ascorbate oxidation during one minute at 290 nm (Nakano and Asada 1987). The activity of the superoxide dismutase (SOD) was determined by measuring the ability of the enzyme to prevent the photochemical reduction of nitroblue-tetrazolium (NBT) by method of Beauchamp and Fridovich (1971). The optical absorbance was measured using a spectrophotometer at 560 nm (Beauchamp and Fridovich 1971). All enzyme activities were measured by the spectrophotometer (Biochrom) model Libra S22.

Investigation of photosynthetic related pigments

Plant leaves were powdered in liquid nitrogen, and 1 ml of extraction solvent (99% methanol and 1% acetic acid) was added to extract chlorophyll *a*, *b*, and carotenoids. Afterward, the samples were stored at 4°C for 24 hours. After

centrifugation for 10 minutes at 1000 rpm at 4°C, the supernatant extract was read for chlorophyll *a*, *b*, and carotenoids by spectrophotometer at wavelengths of 665.2, 652.48, and 470 nm. Finally, chlorophyll *a*, *b*, and carotenoids were determined using the following formulas (Wellburn 1994).

$$Chla = (16.72 \times A665 - 9.16 \times A652)$$

$$Chlb = (34.09 \times A652 - 15.28 \times A665)$$

$$Car = 1000\,(A470) - 1.63(chl.\,a) - 104.96(chl.\,b)/221$$

Total Chl = Chl a + Chl b

$$CSI = Totalchlorophyllunderstress/Totalchlorophyllundernormal * 100$$

Identification of Cis-regulatory elements of NaD1 **genes and** in Silico **gene expression**

Defensin (*NaDEF*) *NaD1* gene sequence of *Nicotiana attenuata* with 96% similarity and coverage with *Nicotiana alata* was obtained from the Ensembl plants database (https://plants.ensembl.org/index.html). Two thousand base pairs upstream of the start codon of *NaD1* defensing (*NaDEF*) gene of *Nicotiana attenuata* as the promotor region were investigated to identify Cis-regulatory elements involved in abiotic stress using PlantCARE database. The elements based on function and frequency were counted and presented according to (Lescot et al. 2002).

Results

Verification of the recombinant expression vector pGSA1285:NAD1

Both vectors pUC57:*NaD1* and pGSA1285 expression vector were digested with *Bam*HI and *Nco*I (Fig. 2a and b). After purification of *NaD1* and pGSA1285 from agarose gel, segments of 239 bp (*NaD1*) and 9553 bp (pGSA1285) were ligated. Recombinant plasmids of *pGSA1285:NaD1* were verified by employing medium containing chloramphenicol, and colony PCR approach using *NaD1* primer (Table 1; Fig. 2c). The presence of *NaD1* in right orientation in the expression vector was confirmed by performing additional enzyme digestion by *Bam*HI and *Nco*I (Fig. 4d). Finally, Sanger sequencing was also accomplished to verify the exact sequence of *NaD1*.

Characterization of transgenic plants

To screen the putative tissue culture-driven tobacco transgenic lines, genomic DNA was isolated from 20 putative transgenic lines. (Table 1 and Fig. 2d). Out of 20, 12 lines amplified a 106 bp PCR fragment corresponding well with *NaD1* gene (Fig. 4b,). We randomly selected three transgenic lines for further analysis and subsequent experiments.

The presence of *NaD1* in genome (Table S1, Table 1) was confirmed by amplifying 106 bp fragments in transgenic plants (Fig. 4b). Subsequently, the expression of *NaD1* was approved in the selected transgenic lines by Semi-quantitative RT-PCR approach (Supplementary file, Table S1, Table 1, Fig. 4e). Investigation of production of NaD1 recombinant peptide in the selected transgenic lines by ELISA shown that amount of NaD1 recombinant peptide in transgenic lines was about 800 µg mL^{-1} g^{-1} fresh weight indicating the production of NaD1 recombinant peptide in the transgenic lines, while this value was zero for non-transgenic plants (Fig. 5a). Western blot analysis was carried out to visualize the presence of NaD1 peptide. A 9 kDa protein was detected in selected transgenic lines

corresponding well with the expected size of NaD1 peptide. No NaD1 peptide was found in control non-transgenic plants.

photosynthetic related pigments

The effect of irrigation conditions (regular irrigation, and 20 days no irrigation) on various plant lines (3 transgenic lines and non-transgenic control plants) was evaluated by measurement of photosynthetic related pigments including chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids. The data specified a significant ($P \leq 0.01$) difference amongst four lines, between two irrigation conditions, and also among their interaction (irrigation conditions * lines) (Table 2). Mean comparison of three *NaD1* transgenic lines and the control plants on photosynthetic related pigments using LSD method showed a significant ($P \leq 0.01$) difference amongst them (Fig. 6a, b, and d). But the most notable finding was the significant increase in chlorophyll *a*, *b*, and total chlorophyll content in all transgenic lines 1, 2, and 3 compared to the control plant under drought stress conditions (Fig. 6- a, b, and d). The carotenoid content also increased in all plants with maximum levels detected in the non-transgenic plants. (Fig. 6c). Correspondingly, mean comparison of CSI chlorophyll stability index showed a significant difference ($P \leq 0.01$) between 3 transgenic and non-transgenic plants (variance analysis not shown) by use of LSD method. So, the highest amount was found in transgenic line 2 (96%) and then lines 1 and 3. The lowest one was belonged to the control plants (Fig. 6e). Therefore, *NaD1* defensin transgenic lines, which could probably be considered as different transgenic events in tobacco, particularly lines 1 and 2 could improve the resistance related characters in response to drought stress.

Table 2
Variance analysis of activities of CAT, POD, APX, SOD, chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids after 20 days of no irrigation in transgenic tobacco plants compared to non-transgenic plants.

S.O.V		Ms							
	DF	Chl *a*	Chl *b*	Carotenoid	Total Chl	CAT	POD	APX	SOD
Plant lines	3	27.73**	263.77**	14.54**	403.44**	1.33**	0.88**	376.41**	21462.40**
Irrigation condition	1	43.47**	603.71**	201.83**	971.18**	0.82**	9.55**	2034.30**	10608.69**
Plant lines * Irrigation	3	37.11**	145.08**	11.32*	240.10**	0.04$^{n.s}$	0.95**	327.72 **	21863.002**
Error	16	0.20	2.22	0.44	2.34	0.002	9.4	2.64	14.29
Total	23								
CV		4.63	5.98	15.43	4.39	15.76	4.79	5.66	5.19
*: significant at 0.05 **: significant at 0.01 n.s: not significant									

Evaluation of enzyme activity

Quantification of the enzyme activity of CAT, POD, APX and SOD was evaluated under irrigation and non-irrigation conditions. The achieved data indicated a significant ($P \leq 0.01$) difference amongst four lines, between two irrigation conditions, and among their interaction (irrigation conditions * lines) (Table 2). Moreover, by applying LSD

method, the mean comparison of activity of four enzymes in four plant lines showed a significant difference among them ($P \leq 0.01$). CAT activity in transgenic lines was shown to be higher that of the non-transgenic with the highest one in transgenic line 1 (Fig. 7a). The POD and SOD activities in lines 1, 2, and 3 was significantly elevated ($P \leq 0.01$) during drought stress. plant (Fig. 7b and d). The activity of APX in all plants significantly ($P \leq 0.01$) increased under non-irrigation condition with transgenic line 3 showing the highest level (27% increase), which is two times higher than control plants (Fig. 7c).

Based on the results, high levels of POD, CAT, and SOD activity were recorded in transgenic line 1 compared to other lines in response to drought stress. Moreover, photosynthetic pigments in transgenic lines 1 and 2 had a higher level. Together, the transgenic line 1 can be considered as the most improved drought-tolerant line due to its ability to activate antioxidant system and maintaining photosynthetic related pigments in *Nicotiana tabacum*. We can conclude that establishment of *NaD1* defensin in the transgenic lines (Specially line 1) caused a significant increase in the photosynthetic pigments and activity of the antioxidant enzymes, which could improve plant tolerance to drought stress conditions.

Cis-elements upstream of NaDEF genes

Frequencies and distributions of Cis-regulatory elements in promoter region can indicate different regulatory and functional characteristics of genes (Koul et al. 2019). There are 502 Cis-regulatory elements (67 types) in eleven *NaD1* homologs in *Nicotiana attenuate* (*NaDEF* genes) (Supplementary file, Table S2). Nine functional groups of Cis-regulatory elements including cell cycle, development, hormone response, light response, binding site, elicitor, stress, transcriptional regulators, and time-related elements were identified. Most of the elements are involved in response to hormones (138 elements), in response to light (132 elements), and in response to biotic and abiotic stress (154 elements) (Fig. 8a). Moreover, 77 Cis-regulatory elements involved in drought stress were identified in eleven *NaDEF* homologous genes indicating important regulatory role of these genes in response to drought stress (Fig. 8b; Supplementary file, Table 3). Investigation of these elements displayed that 31% of stress-related elements are DRE core, MBS, MYB, MYB recognition site, and MYC, which are related to drought stress (Fig. 8b; Supplementary file, Table 3). Comparison of *NaDEF* homologs in response to hormones emphasized that the most abundant Cis elements are related to ABA, which were in turn 9, 7, and 6 in *NaDEF4*, *NaDEF3*, and *NaDEF5*, respectively, and 5 in *NaDEF2* and *NaDEF11*. The most abundant element involved in hormonal response is ABRE (32 elements) which plays a role in the ABA response. Other identified elements in response to ABA include ABR3a, ABRE4, and MYC, which are a total of 18 elements out of 50. The ERE element is also one of the elements involved in the Ethylene-response, which includes 10 elements (Fig. 8b; Supplementary file, Table 3). As a result, by including the results regarding to characterization of *NaD1* transgenic lines and due to the large number of Cis-regulatory elements involved in drought stress and hormonal response especially ABA, we could conclude that NAD1 defensin is involved in regulation of drought stress response in plants, and the transformation of tobacco by *NaD1* could improve plant tolerance to drought stress conditions.

Discussion

Although involvement of most AMPs in response to abiotic stress is not known (Sui et al. 2016), but, the contribution of some groups of AMPs specially different members of the defensin family in various abiotic stresses was reviewed (Olga et al. 2020; Tam et al. 2015); for instance, CADEF1 is associated with salinity, Gmdefensin is involved in drought, AhPDF1.1, CAL1, AtPDF2.6 in heavy metals and TAD1 plays a role in cold tolerance (Olga et al.

2020; Stolf-Moreira et al. 2010). The NaD1 defensin is considered as an important AMP, which displayed a certain function against fungal pathogens and pest insects, however, its involvement in abiotic stresses such as drought stress is unclear. In this study, we explored the role of NaD1 antimicrobial peptide in drought stress.

Overexpression of NAD1 maintain photosynthetic related pigments under drought stress

The chlorophyll content is used as a physiological marker to evaluate plant drought tolerance, and, reduction and degradation of photosynthetic pigments are associated with increased levels of ROS (Sairam et al. 1998). Increased ROS accumulation causes peroxidation and destruction of chlorophyll pigments, and results in significant changes in plant yield (Arazmjo et al. 2010; Tahkokorpi et al. 2007). During drought stress, chlorophyll content decreases due to the activation of chlorophyllase enzyme in response to enhancement in ethylene and abscisic acid levels (Ajithkumar and Panneerselvam 2014). In this study, a significant enhancement in chlorophyll *a*, *b*, and total chlorophyll content was detected in all transgenic lines under drought stress when compared with the non-transgenic plants. Therefore, chlorophyll *a*, *b*, and total chlorophyll in all transgenic lines remained more stable and intact compared to the non-transgenic plant under drought stress (Fig. 6a, b, and d). Accordingly, it is clearly shown that transgenic plants prolonged stay greening by maintaining chlorophyll content (Fig. 6a, b, and d). Moreover, presence of *NaD1* in transgenic plants could postpone chlorophyll degradation (Fig. 6a, b, and d). A higher carotenoid content, which is an essential component in plant antioxidant defense system could induce more tolerance to plants under drought stress (Kiani et al. 2008). Carotenoids are normally present in chloroplasts of all green plants and are exclusively connected to photosystem I and photosystem II complexes protecting photosystem against the damaging effects of ROS caused by drought stress (Allen et al. 2008). Photosynthetic pigments in plants are responsible for absorbing light and producing photosynthesis-reducing powers; both types of chlorophyll *a* and *b* are affected by drought stress, but carotenoids play a supporting role in drought resistance (Tahkokorpi et al. 2007). The current research showed that amount of carotenoid in non-transgenic plants is twice that of transgenic lines showing that non-enzymatic antioxidant system probably tries to eliminate ROS and prevent the destruction of chlorophyll (Fig. 6c), but they are not able to prevent the devastation of chlorophylls (Fig. 6). Consequently, NaD1 could improve the plant response to drought stress through maintaining the content of chloroplast pigments in transgenic lines (Fig. 6a, b, c, and d).

The chlorophyll stability index (CSI) indicates the plants' stress tolerance capacity and is measured based on the comparison of total chlorophyll under drought stress and the control once (Mohan et al. 2000). In this research, the average chlorophyll stability index in transgenic plant lines and the control were significantly different. The highest value was observed in the line 2, followed by lines 1 and 3, and the lowest value was belonged to the non-transgenic plant (Fig. 6e). Normally, plant stress tolerance improves when chlorophyll availability increase or plant CSI enhance, which is reflected in higher photosynthetic rates, higher drought matter production, and higher productivity (Mohan et al. 2000). Since limitation of photosynthesis under drought stress is a complex phenomenon and occurs mainly through removing plant photosynthetic pigments (Farooq et al. 2009), it could be concluded that transformation of the tobacco plants by *NaD1* preserved chlorophyll content, increased chlorophyll stability, and improved the tolerance of transgenic plants to drought stress.

Overexpression of NAD1 activates plant antioxidant system under drought stress

ROS accumulation in plants under abiotic stress causes oxidative damage and cell death. Plants use a complex set of antioxidant strategies to eliminate overproduction of ROS to reduce destructive effects and maintain redox homeostasis (Gill and Tuteja 2010). In the current study, the activity of CAT, POD, APX, and SOD enzymes in transgenic lines under drought stress significantly increased in comparison with non-transgenic plants. But under

regular irrigation, the activities of these enzymes were not significantly different among the transgenic and non-transgenic plants. This indicates that *NaD1* defensin gene in the transgenic lines activate antioxidant enzyme activity system under drought stress resulting in lower oxidative damage and likely reduction of ROS levels. Overexpression of the *NaD1* defensin gene in tobacco behaves like that reported in Arabidopsis in which expression of *Ca-AFP defensin* gene increased drought tolerance by regulation of SOD, APX, CAT, and the proline content (Kumar et al. 2019). Therefore, NaD1 could probably be considered as one of the regulators of antioxidant system to cope with drought stress in the transgenic lines.

Plants regulate ROS levels in cells through ROS-scavenging enzymes such as CAT, POD, APX, and SOD (Kumar et al. 2019). SOD enzyme is one of the most crucial defense enzymes against ROS toxicity because it quickly converts the superoxide anion into oxygen and H_2O_2. Then, its downstream enzymes including APX, CAT, and POD, detoxify and convert H_2O_2 into water and oxygen (Kumar et al. 2019). CAT and APX are two most important intracellular H_2O_2 absorbers. APX regulates ROS levels in the intracellular space, and CAT is the major scavenger of excess ROS under stress conditions (Mittler 2002). JA signaling in wheat enhances the activity of antioxidant enzymes such as CAT, POD, APX, and SOD and induces more resistance to abiotic stress in plants (Qiu et al. 2014). The current research shows that probably *NaD1* transgenic lines employed enzymatic antioxidant system by activation of SOD and its downstream enzymes CAT, POD, and APX to reduce ROS levels. Moreover, during abiotic stresses, up-regulation of plant growth phytohormones such as jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA) induce high levels of ROS resulting in increased production of Ethylene Response Factor 1 (ERF1) in plants (Kumar et al. 2019). ERF1 plays a vital role in pathogen resistance, salinity, drought, and heat stress tolerance through the regulation of specific stress-responsive genes such as AMPs. ERF1 binds to different Cis-elements in response to various stress signals (DRE element or GCC box). By constitutive expression of ERF1, downstream effector genes such as plant defensins are activated to promote ET responses (Kumar et al. 2019).

Therefore, under drought stress, NaD1 as one of antioxidant system regulators in plants activate antioxidant enzyme activity system which might result in lower oxidative damage and likely reduction of ROS levels in the transgenic lines.

Cis-elements in the NaD1 gene play a role in drought stress

Analysis of the promoter regions of eleven *NaD1* homologs in *Nicotiana attenuate* (*NaDEF* genes) revealed the presence of numerous cis-elements involved in response to abiotic stress particularly drought stress. The most common stress related elements among *NaDEF* include DRE core, MBS, MYB, MYC recognition site. In the case of elements involved in hormonal response, ABRE, which plays a role in the ABA response, was found to be the most abundant element. Other identified features in response to ABA include ABR3a, ABRE4, and MYC (Supplementary file, table S3). MYB, MYC, and ABRE were identified as the most prominent elements in response to drought stress and hormonal response (Wang et al. 2021). Following the transduction of stress signal, several transcription factors are activated, some of which are specific to various abiotic stresses. Activated transcription factors induce the transcription of other genes such as AMPs, which are effective at regulating and responding to environmental stress (Karpun et al. 2015; Nongpiur et al. 2012; Ye et al. 2017). MYB transcription factors play an important role in plant drought tolerance by regulating the expression of biosynthesis genes for metabolites (Wang et al. 2021). Further, the MYB transcription factors are also involved in movement of stomatal cells, primarily via ABA signaling and also in response to light. The MYB family contributes to drought resistance through the regulation of flavonoids and cuticle synthesis (Wang et al. 2021). Transgenic plants overexpressing MYB15 and MYB37 were significantly more tolerant to drought and oxidative stress (Wang et al. 2021). Also, tolerance to drought is enhanced by overexpression of

MYB15 and MYB37, which are necessary for ABA-induced stoma closure. Jung et al. (2008) demonstrated that overexpression of MYB44 increased drought tolerance by enhancing ABA sensitivity in both germination and stomatal closure (Jung et al. 2008). It can be concluded that due to the presence of these Cis-elements in the promoter region of *NaDEF* genes, they have a special role in hormonal and stress responses. According to the expression profiles of *MYC* genes, *NaDEF* genes are likely to function in response to both biotic and abiotic stressors. *Arabidopsis MYC2, MYC3, MYC4, and MYC5* are induced by jasmonate; MYC3 and *MYC4* regulate insect herbivory defense by acting additively with MYC2; MYC5 is involved in JA-mediated plant defenses against herbivores and pathogen resistance. Overexpression of MYC2 has resulted in bacterial blight resistance in rice (Chen et al. 2019). During drought stress, two MYC genes, *ZmbHLH103* and *ZmbHLH104*, are significantly upregulated. The expression of 6 MYC genes is induced by different abiotic stresses further indicating that MYC genes participate in response to abiotic stresses, especially drought stress. Taken together, it appears that MYC TFs are primarily involved in growth and development, and in response to environmental stresses (Chen et al. 2019).

AREB is another Cis-regulatory element that is abundant in the promoter region of the *NaDEF* genes, and its frequency was equal to that of MYB and MYC. This element plays an important role in ABA signaling. ABA is a phytohormone and a key regulator of the drought stress response that regulates gene expression and controls stomatal closure in plants to prevent water loss through transpiration during drought stress. ABA-responsive element binding proteins/ABRE binding factors (AREB/ABFs) play an important role in ABA signaling. Overexpression of AREB1 in Arabidopsis, rice, and soybean demonstrated enhanced drought stress tolerance, whereas AREB1 loss of function caused drought stress sensitivity. In response to drought stress, AREB1 regulates numerous genes downstream of the ABA signaling pathway, also ABA biosynthesis and antioxidant signaling. Thus, AREB1 has an important role in drought stress response.

In Silico investigation of the promoter region of the *NaDEF* genes are consistent with the laboratory results on *NaD1* transgenic lines and confirm important role of *NaDEF* genes in enhancing drought tolerance. Therefore, based on bioinformatic studies, *NaD1* expression is likely influenced by MYC, MYB, and ABRE Cis-elements. Probably, certain (unknown) transcription factors bind to these Cis elements in the promoter region of *NaD1* gene during abiotic stress such as drought, therefore, it can be concluded that this group of defensins have an effective role in abiotic stress in addition to biotic stress. Therefore, transformation of some defensins can be very effective in improving crop tolerance to drought stress.

Based on the data together we found that chlorophyll *a*, *b*, and total chlorophyll contents in all transgenic lines were significantly increased. By consideration the fact that the only difference between non-transgenic and transgenic plants is overexpression of *NaD1* defensin gene, it could be concluded that presence of *NaD1* as a regulators of antioxidant system enhanced the activity of antioxidant enzymes of CAT, POD, APX, and SOD, and subsequently, contributed to the protection of chloroplast pigments in the *NaD1* transgenic lines, and probably reduces oxidative damage and ROS levels resulting in improving drought stress response in the transgenic lines. *In Silico* analysis of the promoter region of the *NaDEF* genes are consistent with the laboratory results on *NaD1* transgenic lines and confirm important role of *NaD1* in enhancing drought tolerance in tobacco. Therefore, in addition to its involvement in biotic stress, NaD1 has an effective role in abiotic stress specially drought stress. Transformation of crop plants by *NaD1* can be very effective in improving crop tolerance to drought stress.

Declarations

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Conflict of interest: The authors declare no conflict of interest.

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

SR did the research experiments and data analysis, RSH conducted and supervised the whole research project. AZ, MBN and FNF cooperate as advisors in different parts of the work, respectively. All authors have read and approved the manuscript.

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Figures

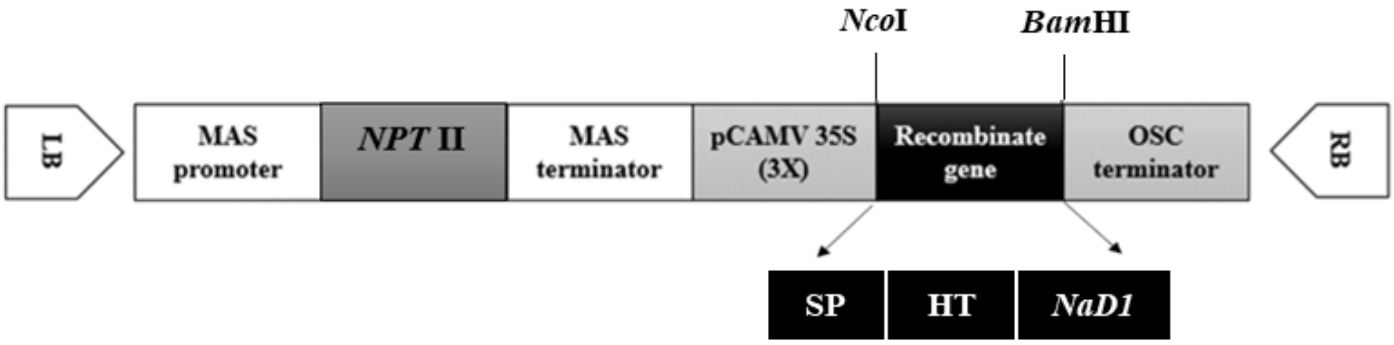

Figure 1

The various sections present in T-DNA cassette in pGSA1285-NaD1**.** MAS: Mannopine synthase, *NPT* II: Neomycin phosphotransferase II, CaMV35S: Cauliflower mosaic virus 35S promoter, OSC: Octopine synthase, SP: *vicilin* signal peptide, HT: Histidine tag $(His)_6$, RB: right border, LB: left border. The size of different parts in the figure does not correspond to their actual size.

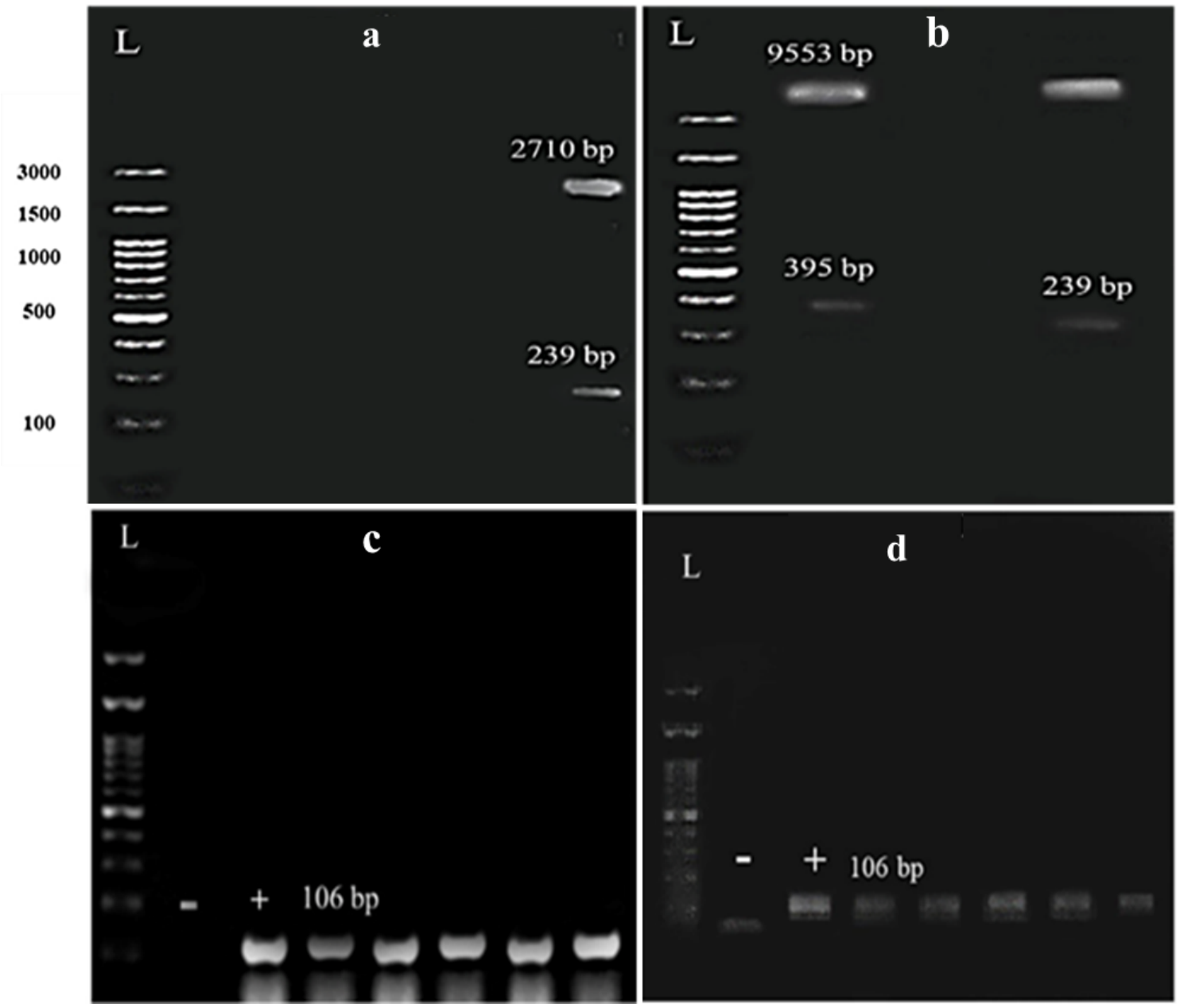

Figure 2

Digestion of recombinant vectors and colony PCR of *Ecoli* and *Agrobacterium tumefaciens*.

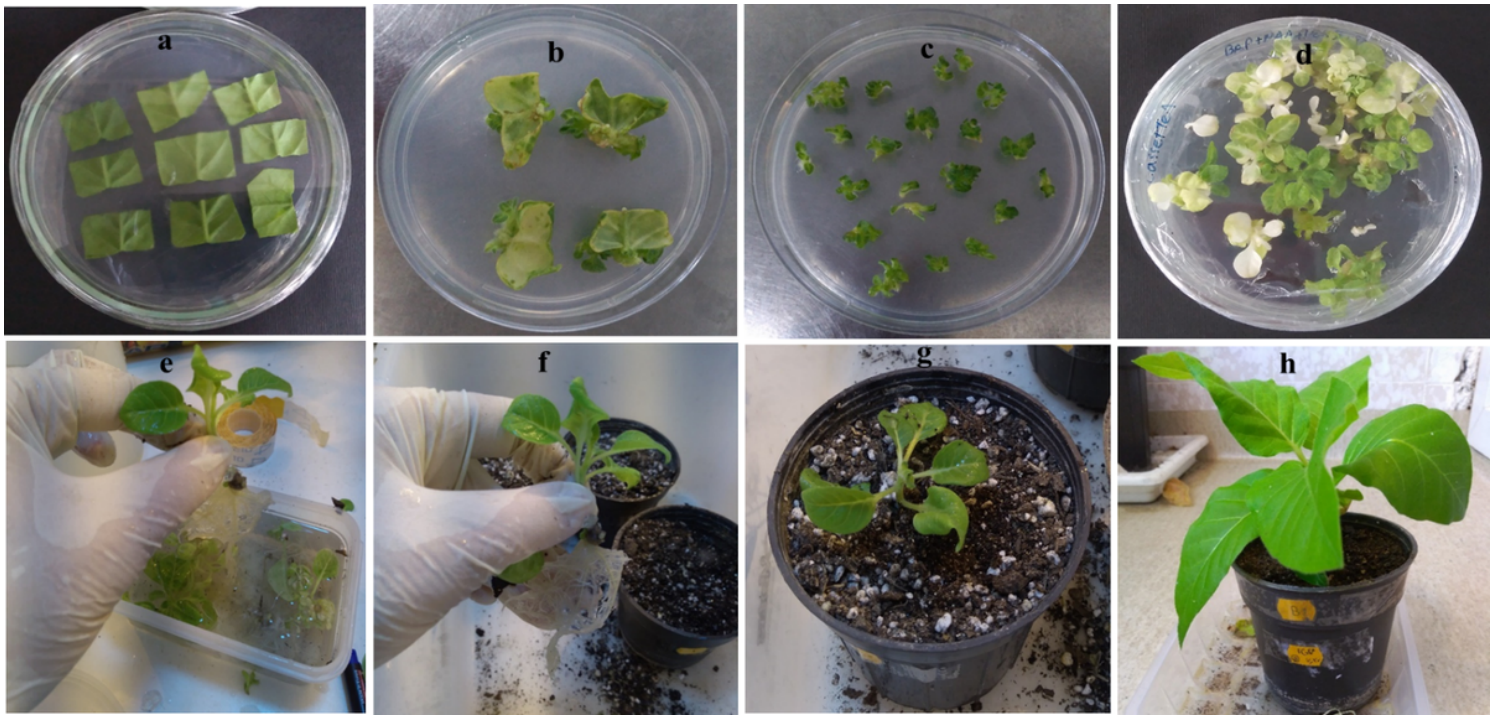

Figure 3

Tissue culture, inoculation, Agrobacterium -mediated transformation and generation of transgenic lines.

a Co-cultivation of leaf explants and *Agrobacterium tumefaciens GV3101 pmp90* containing recombinant plasmid pGSA1285:*NaD1*. **b** Shoot formation around leaf explants on MS medium containing kanamycin, cefotaxime, and plant growth regulators including NAA and BAP. **c**Isolation and cultivation of transgenic seedlings with 4-6 leaves in MS medium containing the same antibiotics and growth regulators as **c**. **d** Transgenic seedlings growing on MS medium containing kanamycin, cefotaxime, NAA, and IBA to develop roots. (**e**-**h**) various transfer stags of transgenic seedlings to pots and growth them under proper.

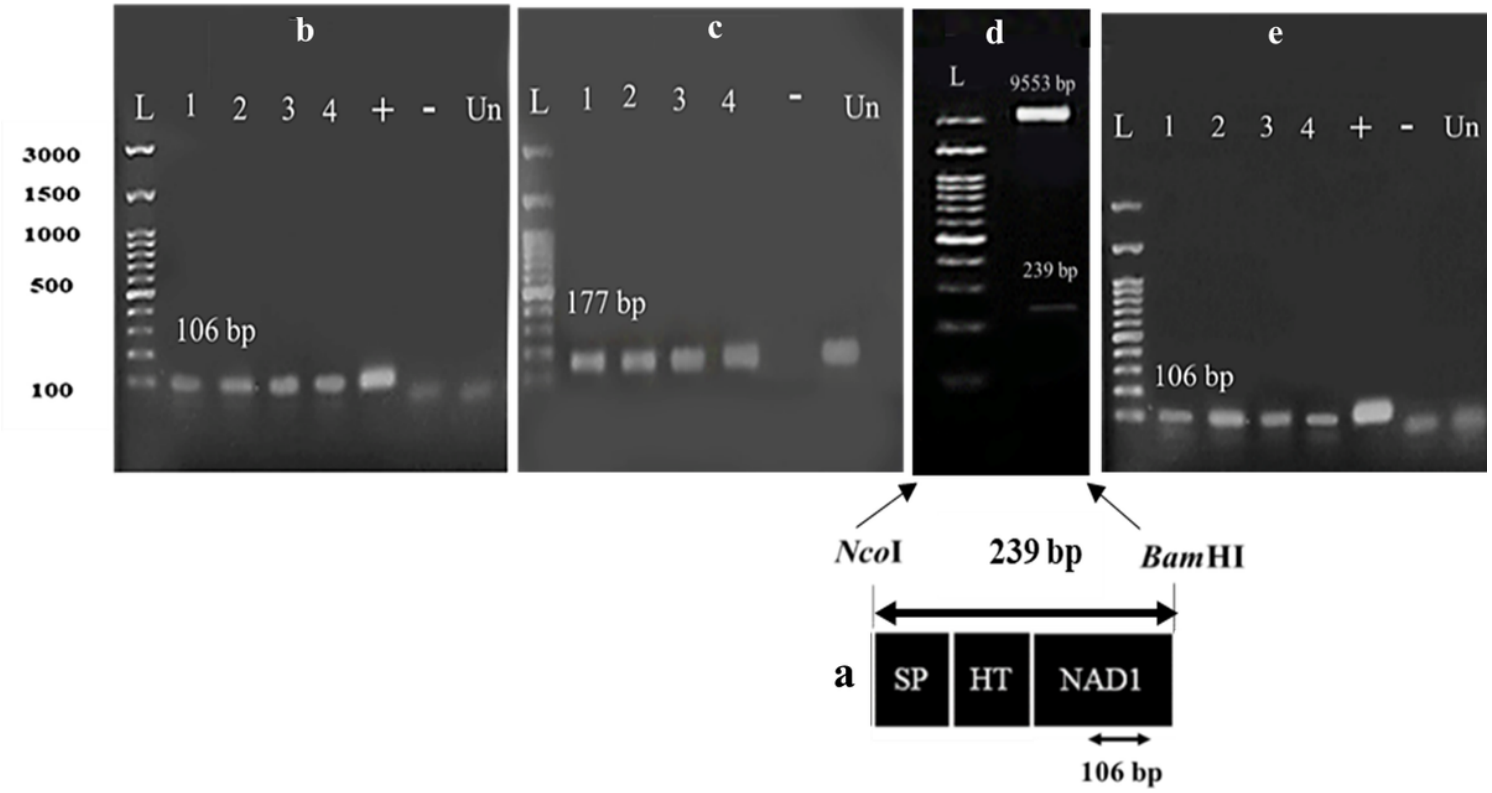

Figure 4

Transformation of Agrobacterium by recombinant pGSA1285: *NAD1*, transfection and gene expression peptide presence analysis in tobacco plants.

a Different part of *NaD1* cassette. **b** the electrophoresis of PCR amplification of *NaD1* (106 bp), 1-4 represent the transgenic lines, Un designates for untransformed control once, negative control (-), and a plasmid containing the mentioned gene fragments as positive control (+). **c**) Semi-quantitative RT-PCR on cDNAs using *elf1a* housekeeping gene as internal control on 2% agarose gel (177 bp corresponds for presence of *elf1a*in 1-4 transgenic samples, and non-transgenic plants (Un)). **d** double digestion of pGSA1285: *NaD1*using *Bam*HI and *Nco*I, the observed fragments include 9553 bp (Corresponding to pGSA1285 expression vector) and 239 bp (Related to *NaD1* gene). **e** Semi-quantitative RT-PCR for amplification of *NaD1* transcripts on 2% agarose gel (1-4 transgenic lines, 106 bp bands), non-transgenic plants as control (Un), negative control (-) and a plasmid containing the mentioned gene fragments as positive control (+). The ladder in the left corner is 100 bp.

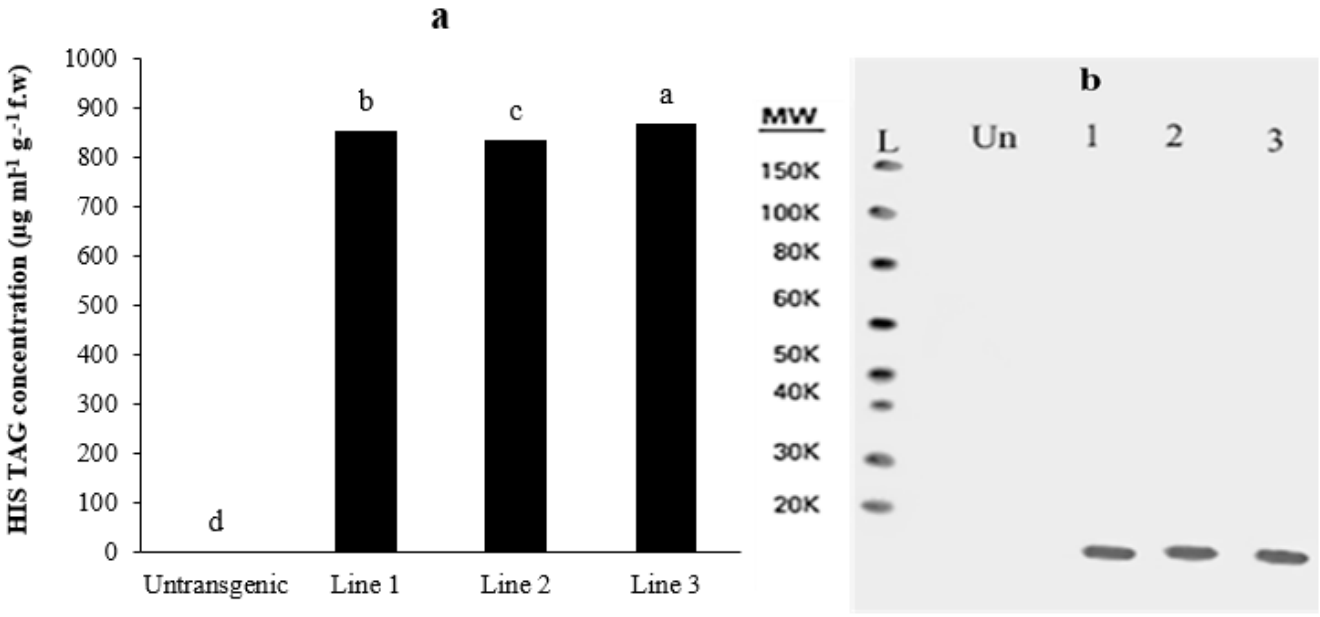

Figure 5

$(His)_6$-NaD1 peptide presence analysis in tobacco plants.

a Production of the *NaD1* recombinant peptide containing Histidine tag sequence, $(His)_6$ by ELISA test at 450 nm wavelength in terms of µg mL^{-1} g^{-1} fresh weight, the concentration of peptides with Histidine tag for the total protein extract of non-transgenic plants was zero. **b**) Anti-His monoclonal antibody sc-8036 (Santa Cruz Biotech) was used to detect recombinant peptides. Transgenic plants (1, 2, and 3) contain $(His)_6$-NaD1 (KDa9) peptide. The non-transgenic line indicated by un.

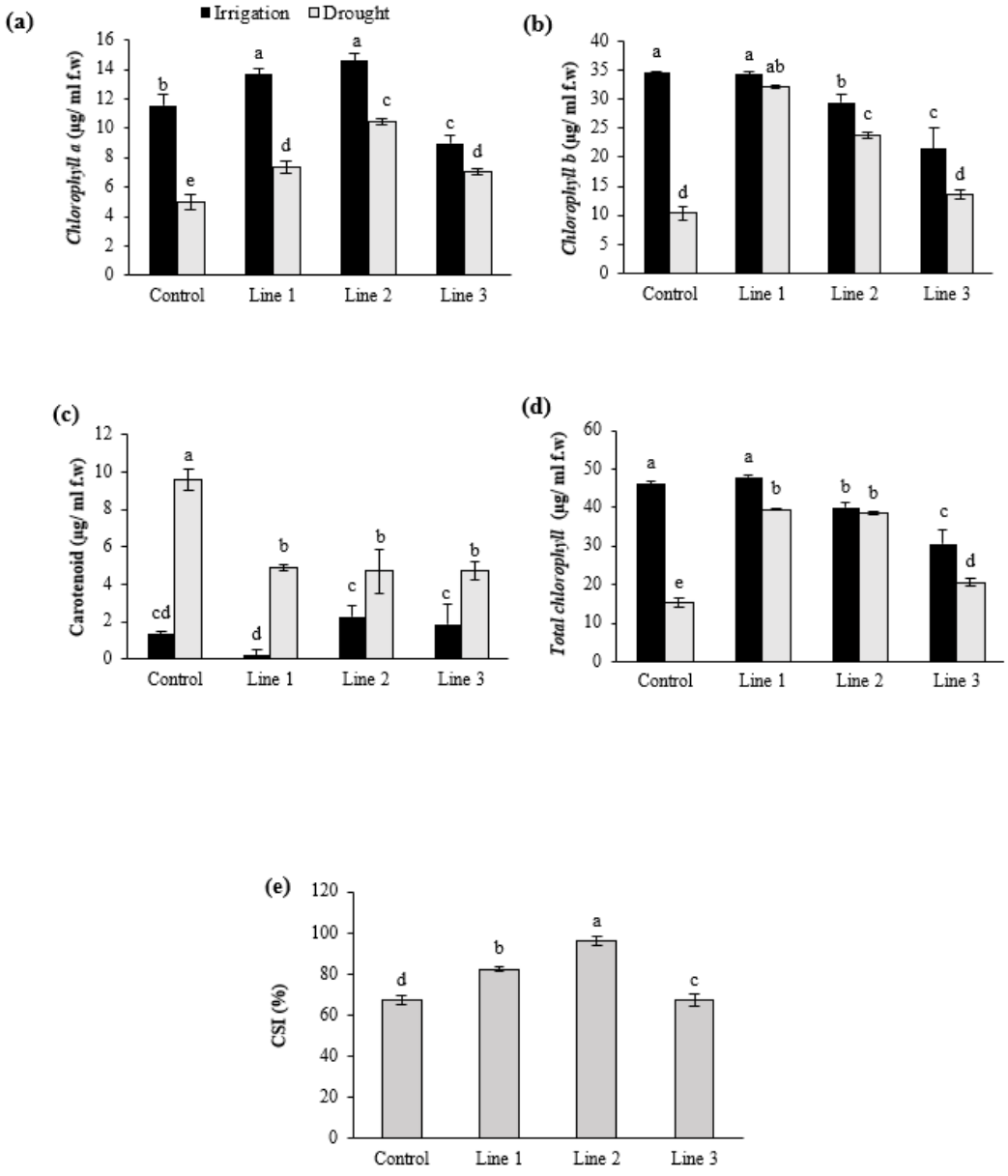

Figure 6

Mean comparison of chloroplast related pigment contents

achlorophyll *a*, **b** chlorophyll *b*, **c** carotenoid, **d**) total chlorophyll, and **e** chlorophyll stability index after 20 days of non-irrigation in transgenic NaD1 tobacco plants compared to non-transgenic plants (Control). Common letters indicate no significant difference between the means based on the LSD test at P<0.01. CSI is an acronym for chlorophyll stability index.

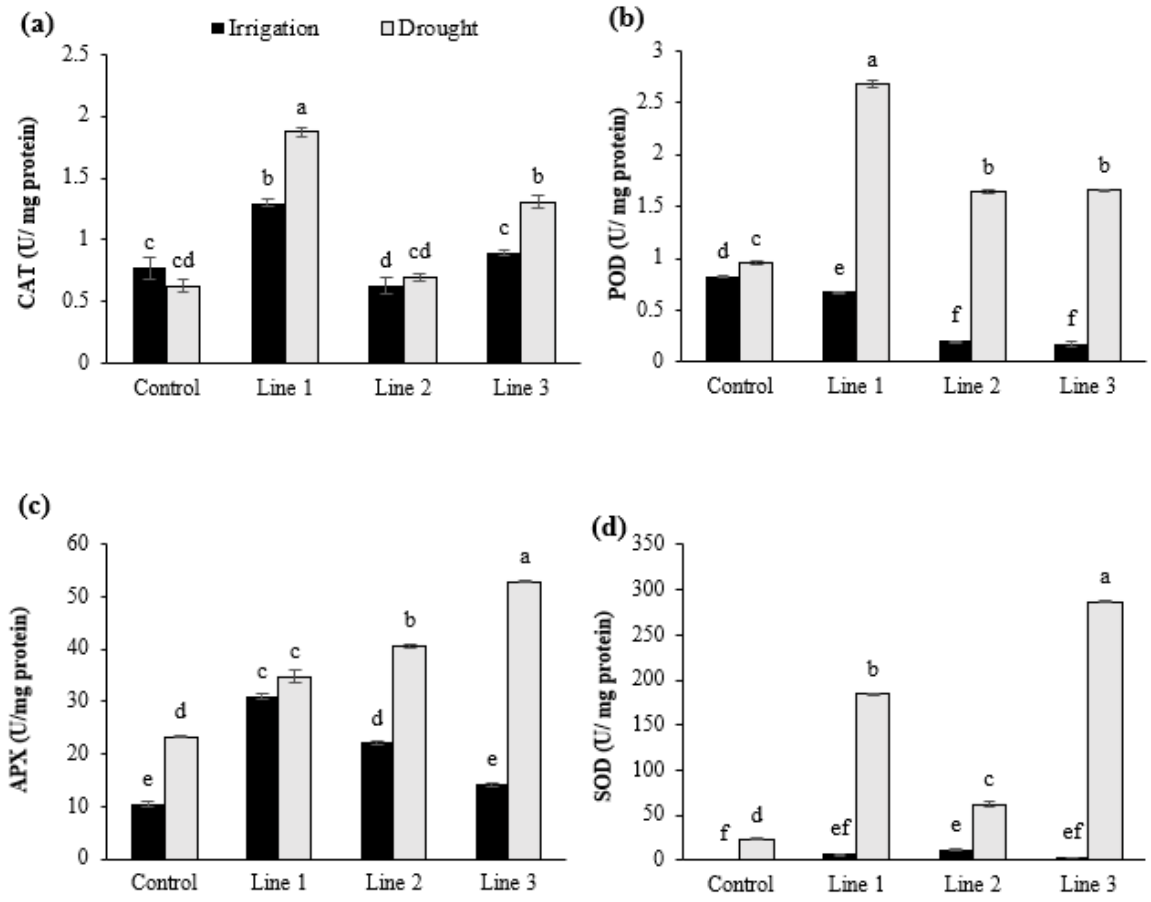

Figure 7

Mean comparison of various enzyme activities. **a** mean activity of CAT, **b** POD, **c** APX, and **d** SOD after 20 days of no irrigation in NaD1 transgenic tobacco plants were compared to the non-transgenic plant (Control) using the LSD test ($P \leq 0.01$). the same letters indicate no significant difference between those means.

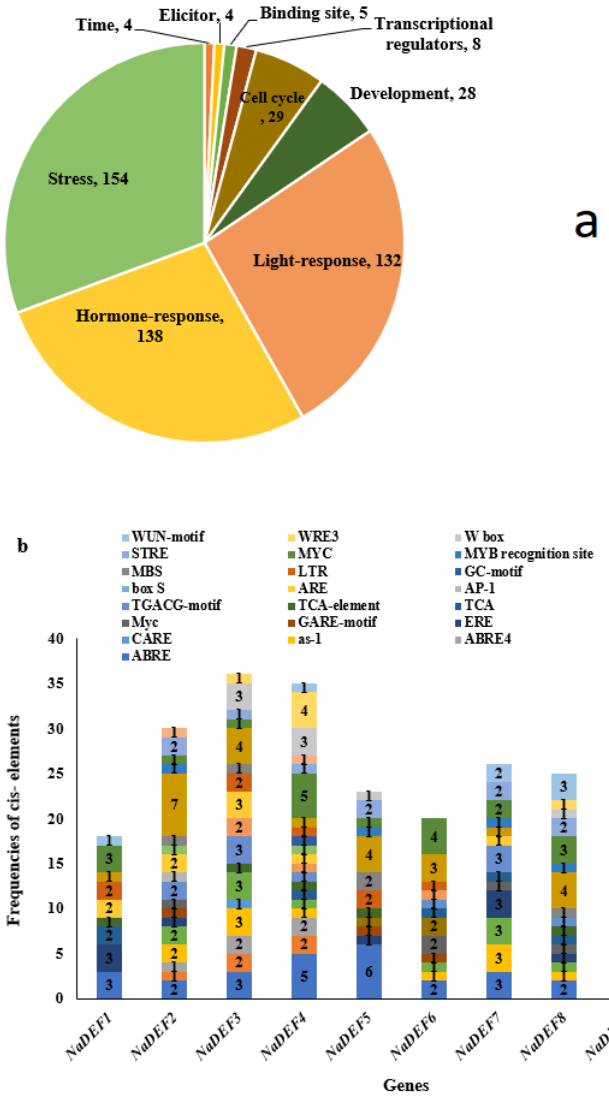

Figure 8

The frequency of regulatory elements in *Nicotiana attenuata*.

a The regulatory elements identified in the promoter region of *NaDEF* genes in *Nicotiana attenuata* based on information from the Plant Care database. The eleven genes *NaDEF* family of *Nicotiana attenuata* contain 67 types of regulatory elements with a frequency of 502 numbers. A total of nine functional groups of cis-elements were identified, including cell cycle, development, hormone response, light response, binding site, elicitor, stress, transcriptional regulators, and time-related elements. **b**Frequency of Hormonal response and stress regulatory elements in *Nicotiana attenuate*based on information from the Plant Care. Most of the elements are involved in the response to hormones (138), the response to light (132), and the response to stress (154) The highest accumulation

of cis-elements was detected in *NaDEF 3* and *NaDEF 4* genes, and the lowest diversity of cis-elements was identified in *NaDEF 1*.

Supplementary Files

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- supplementaryinformation2.pdf