Overexpression of differentially expressed \textit{AhCytb6} gene during plant-microbe interaction improves tolerance to N$_2$ deficit and salt stress in transgenic tobacco

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\textit{Stenotrophomonas maltophilia} has plant growth-promoting potential, and interaction with \textit{Arachis hypogaea} changes host-plant physiology, biochemistry, and metabolomics, which provides tolerance under the N$_2$ starvation conditions. About 226 suppression subtractive hybridization clones were obtained from plant-microbe interaction, of which, about 62% of gene sequences were uncharacterized, whereas 23% of sequences were involved in photosynthesis. An uncharacterized SSH clone, SM409 (full-length sequence showed resemblance with \textit{Cytb6}), showed about 4-fold upregulation during the interaction was transformed to tobacco for functional validation. Overexpression of the \textit{AhCytb6} gene enhanced the seed germination efficiency and plant growth under N$_2$ deficit and salt stress conditions compared to wild-type and vector control plants. Results confirmed that transgenic lines maintained high photosynthesis and protected plants from reactive oxygen species buildup during stress conditions. Microarray-based whole-transcript expression of host plants showed that out of 272,410 genes, 8704 and 24,409 genes were significantly ($p < 0.05$) differentially expressed (>2 up or down-regulated) under N$_2$ starvation and salt stress conditions, respectively. The differentially expressed genes belonged to different regulatory pathways. Overall, results suggested that overexpression of \textit{AhCytb6} regulates the expression of various genes to enhance plant growth under N$_2$ deficit and abiotic stress conditions by modulating plant physiology.

Plant growth-promoting rhizobacteria (PGPR) improve plant growth and development directly and/or indirectly: directly by nitrogen fixation, phosphate solubilization, siderophore, and phytohormone production and indirectly by acting as a biocontrol agent or by activating induced systemic resistance (ISR) in the host plant$^{1,2}$. Interaction of PGPR or pathogenic bacteria with the host plants causes various signalings, which leads to the activation of the host immune system. However, plants differentiate between PGPR and pathogenic bacteria based on response times, activation of genes, and their expression levels$^{3,4}$. Utilization of the potential of the PGPR and their effect on the host transcriptional machinery is a great avenue for the development of sustainable agriculture for biotic and abiotic stress-affected crop plants.

According to the Intergovernmental Panel on Climate Change$^{5}$, changes in climatic conditions and agricultural habits, and increased use of chemical fertilizers cause various abiotic stresses in legumes, which affect their growth and productivity. Abiotic stresses in soil (salt, cold, drought, waterlogging, metal toxicity, pH, and low availability of nutrients, among others) cause an alteration in the microbial flora of soil, which affects the symbiotic relationship between legumes and rhizobia$^{6,7}$. Nitrogen is a major nutrient element for plant growth and development due to its central role and presence in many biomolecules like protein, chlorophyll, and nucleic acids.

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Nitrogen also acts as a regulator for the carbon cycle, which directly affects the photosynthetic machinery of plants. It is well established that nutrient homeostasis plays a key role in plant growth and development. Nutrient deficiency, including the $N_2$ starvation condition, leads to stress conditions and activates the nutrient-deprivation signal transduction. In nitrogen starvation conditions, plants use their stored nitrogen, and more than half of the leaf nitrogen is used in photosynthetic machinery, thus plants have to compromise with growth (less nitrogen for structural proteins) and yield (early senescence). Differential expression of key genes coordinates with plant physiology to manage the demand for nutrients. The low availability of nitrogen in the soil decreases the yield of the crop, which could be compensated for by the application of $N_2$-fixing bacteria. In this scenario, we need diazotrophic bacteria that are tolerant to abiotic stress and act as PGPR to balance the nutrient cycle between the plant-microbe-soil dynamic in stress conditions. The use of PGPR for the enhancement of crop productivity under various biotic and abiotic stresses is better for sustainable and environmentally friendly agriculture.

There are a plethora of studies that show the improvement in yield and health of plants after application of PGPR, and some studies showed changes at the molecular level (transcript expression) in the host plant after interaction with PGPR. There is a need to understand the changes and events taking place at the molecular level in the host plant after interaction with PGPR under abiotic stress, and utilizing the differentially expressed gene for the potential candidate for the bioengineering of the host genome could be a highly translational strategy.

In nature, the peanut plant is associated with various nitrogen-fixing, nodulating rhizobacteria, which help in nitrogen fixation. However, the lack of specificity of this interaction make it difficult to understand the specific changes that occur at the molecular level during the interaction. To understand the effect of a specific PGPR on plant growth promotion and molecular changes in A. hypogaea, we used the strain Stenotrophomonas maltophilia BJ01, isolated from non-crop and non-leguminous plants from the coastal saline area. We reported the effect of single PGPR S. maltophilia BJ01 on the physio-biochemical and metabolic changes on the host plant under nitrogen starvation and salt stress conditions. Differential expression of genes due to plant-PGPR interaction will provide the molecular mechanism of PGPR-action as well as useful insight about the potential gene candidates to be explored for sustainable agriculture under stress conditions.

In this study, we found that the AhCytb6 gene is differentially expressed in peanut under nitrogen-starved conditions after interaction with S. maltophilia BJ01. To understand the role of this gene in the host plant, we engineered the genome of the model plant (tobacco) and inserted AhCytb6 along with the expression cassette in the genome. Ectopic overexpression of the AhCytb6 gene in transgenic tobacco enhances plant performances under nitrogen starvation and salt stress. The role of AhCytb6 was also explored for the growth and development of plants and their stress responses. Cytb6 is a key regulatory unit of the electron transport chain in plants and affects the photosynthetic efficiency and yield of plants. Recently, Lande et al. reported that abiotic stress drastically decreases the proteins related to Cytb6 in chickpea. Overexpression of this gene increases the photosynthetic ability, biomass and yield of the plants under various abiotic stresses (in vivo). Our results showed that the photosynthesis gene AhCytb6 is differentially expressed in host plants due to interaction with PGPR, and overexpression of this gene provides tolerance to the model plants under $N_2$ starvation and abiotic stress conditions.

**Results**

**Differential expression of genes in the response of S. maltophilia BJ01 under N$_2$ starvation condition.** There were 400 SSH (suppression subtractive hybridization) clones sequenced and subjected to chimera analysis, and 226 resultant clone sequences were obtained, which were subjected to BLAST and categorized into eight-groups (Fig. 1A). Interestingly, about 62% of differentially expressed gene sequences did not show significant similarity with known genes and were categorized as unknown/uncharacterized/hypothetical. Similarly, 23% of EST sequences were involved in photosynthesis. About 3% of sequences belonged to apoptosis, while 1% EST were signaling molecules, transcription factors, stress regulators, and metabolism. About 3% of sequences did not show any resemblance and fell under the miscellaneous category. Transcript profiling of representative genes from selected categories showed differential up-regulation in PGPR-treated peanut plants (Fig. S1). Based on transcript expression profiling, clone SM409 showed 4.1-fold upregulation and resemblance with uncharacterized/hypothetical protein and was selected for further study. The full-length SM409 clone (ORF) sequence showed resemblance (99.69% sequence similarity with 100% query coverage) to the chloroplast with uncharacterized/hypothetical protein, and was selected for further study. The full-length SM409 clone (ORF) sequence showed resemblance (99.69% sequence similarity with 100% query coverage) to the chloroplast genome (CDS: cytochrome b6) of A. hypogaea, especially different cultivars of A. hypogaea (accession no. CP030984; MG814006–9; NC_037358; KX257487; KJ468094); therefore, the cloned gene was named AhCytb6. Moreover, the deduced protein (amino acid) sequence showed 93.72% similarity (with 97% query coverage) with the cytochrome b6 protein of A. hypogaea (accession no. YP_009472186) in the homology search.

**Cloning and in silico analysis of the AhCytb6 gene.** The AhCytb6 gene was 1287 base pair long (accession no. MT395343) and was comprised of 34 bp 5'-untranslated leader sequences (5'-UTR), 636 bp of an open reading frame (ORF) and 617 bp of a 3'-UTR (5'-UTR: 1–34 bp, ORF: 35–670 bp and 3'-UTR: 671–1287 bp) (Fig. S2). In genome organization study, the amplification of 636 bp AhCytb6 gene ORF was obtained from both genomic and cDNA, which revealed that the gene is intronless (Fig. S3). The ORF encodes for 211 amino acids having a molecular mass of 23.59 kD. In silico analysis revealed that the PI of the deduced protein was 10.6 and the instability index was 32.98; the protein half-life was predicted 30 h in mammalian reticulocytes (in vitro), more than 20 h in yeast (in vivo), and more than 10 h in Escherichia coli (in vivo), which showed that the protein was stable in nature. The in silico analysis predicted that the AhCytb6 peptide contained four transmembrane domains and was in the plasma membrane (Fig. S4).

**Genetic transformation and molecular confirmation of transgenic lines.** About 25 putative transgenic lines (T0) were obtained after tissue culture, out of which 17 lines showed seed germination on kanamycin,
which carried forward further for the generation of T1 transgenic lines. Integration transgenes were confirmed in all 17-transgenic lines by amplification of 1.2 kb of the uidA gene and 636 bp of the AhCytb6 gene (Fig. 1B–D and Fig. S5). All plants were found positive, and based on histochemical gus expression, five lines (L1, L4, L5, L9, and L10) were selected (Fig. 1E). Selected transgenic lines showed single gene integration and high expression of the AhCytb6 gene analyzed by southern blot and semi-quantitative RT-PCR analysis, respectively, in all selected lines (Fig. 1F, G and Fig. S5).

**Overexpression of AhCytb6 gene enhances the growth of transgenic under N₂ starvation and salt stress.** About 100% seed germination was observed under control (unstressed) conditions, and similar results were also found for the N₂ starvation condition. However, the percent of seed germination decreased
under salt stress. About 40–42% of WT and VC seeds germinated, whereas 65–80% seed germination was estimated for transgenic (L1, L4, L5, L9, and L10) lines (Fig. 2A, B). Results suggested that the N₂ starvation condition did not affect germination, while salt stress severely affects seed germination. Further, the overexpression of the AhCytb6 gene enhanced the seed germination efficiency of transgenic plants under salt stress conditions compared to WT and VC plants.

Enhanced plant growth of transgenic plants (L1, L4, L5, L9, and L10) was observed under stress conditions compared to WT and VC plants (Fig. 3). About 6–7 cm root length (RL), 0.4–0.5 cm shoot length (SL), 6–7 mg fresh weight (FW), and 0.9–1.3 mg dry weight (DW) were estimated in transgenic lines compared to WT and VC plants (RL: 3–4 cm, SL: 0.2–0.3 cm, FW: 3.9–4.1 mg, and DW: 0.3–0.5 mg) under N₂ deficit stress conditions. Similarly, higher growth parameters (RL: 2–4 cm, SL: 0.4–0.6 cm, FW: 9–12 mg, and DW: 0.7–1.4 mg) were measured in transgenic plants compared to WT and VC plants (RL: 1–1.2 cm, SL: 0.27–0.28 cm, FW: 5–7 mg, and DW: 0.4–0.5 mg) under salt stress conditions (Fig. 3A–E).

The physiological status of the transgenic plant is modulated by the ectopic expression of the AhCytb6 gene. Improved membrane stability and low electrolyte leakage were found in transgenic lines (L1, L4, L5, L9, and L10) compared to WT and VC plants under stress conditions (Fig. 4A, B). About 29–35% electrolyte leakage was found in transgenic lines, which were considerably lower than WT and VC plants (47–49%) under N₂ deficit conditions. Similarly, lower electrolyte leakage, about 18–23%, was observed in transgenic lines compared to WT and VC plants (30–32%) under salt stress conditions. High membrane stabilities, about 63–69%, and 71–78%, were estimated for transgenic lines under N₂ deficit and salt stress conditions, respectively, compared to WT and VC plants (43–50% and 63–65%, respectively). Accumulation of proline, a common physiological response indicator, and a key player in plant abiotic stress tolerance was observed in transgenic plants in N₂ deficit and salt stress conditions compared to WT and VC plants (Fig. 4C). Under control conditions, a
Figure 3. Analysis of plant growth of transgenic plants. (A) Comparative analysis of seedling growth of selected transgenic lines and control plants under N\textsubscript{2} deficit and salt stress condition. (B) Root length, (C) shoot length, (D) fresh weight and (E) dry weight of selected transgenic lines, WT and VC plants under control, N\textsubscript{2} deficit and salt stress condition. Bars represent means ± standard error, and ‘*’, ‘**’ and ‘***’ designates for significant differences at P < 0.05, P < 0.01 and P < 0.001, respectively and ‘ns’ represents no significant difference.
similar level of proline contents was observed in transgenic lines as well as WT and VC plants. Under N₂ deficit conditions, proline contents were about 0.12–0.16 µg g⁻¹ Fw in transgenic plants and about 0.035–0.045 µg g⁻¹ Fw in WT and VC. Under salt stress conditions, about 1.8–3.0 µg g⁻¹ Fw of proline contents were detected in transgenic lines and about 1.4–1.6 µg g⁻¹ Fw in WT and VC plants.

The AhCytb6 gene protects the plant from ROS buildup during stress conditions. Under control conditions, lipid peroxidation and H₂O₂ contents were similar in control and transgenic plants. Under N₂ deficit condition, transgenic lines (L1, L4, L5, L9, and L10) showed significantly lower production of MDA (2.5–4.5 µmol g⁻¹ Fw) and H₂O₂ (8–10 µmol g⁻¹ Fw) in comparison to WT and VC (5–7 µmol g⁻¹ Fw MDA and 11 µmol g⁻¹ Fw H₂O₂) plants. Similarly, transgenic lines showed significantly lower accumulation of MDA (1.5–2.5 µmol g⁻¹ Fw) and H₂O₂ (5–7 µmol g⁻¹ Fw) under salt stress condition compared to WT and VC (6–6.5 µmol g⁻¹ Fw MDA and 7 µmol g⁻¹ Fw H₂O₂) plants (Fig. 4D, E). The above results were further supported by lower in vivo localization of H₂O₂ and O₂⁻ in transgenic leaves compared to their WT and VC counterparts under stress conditions (Fig. 4F).

Ectopic expression of the AhCytb6 gene increases the photosynthesis efficiency under stress conditions. The leaf senescence assay showed a major loss of photosynthetic pigments (bleaching of leaf discs) in WT and VC plants under stress conditions compared to transgenic (L1, L4, L5, L9, and L10) lines (Fig. 5A). Higher contents of total chlorophyll (0.05–0.07 mg g⁻¹ Fw), chlorophyll a (0.03–0.05 mg g⁻¹ Fw), chlorophyll b (0.01–0.03 mg g⁻¹ Fw), and carotenoids (0.01–0.04 mg g⁻¹ Fw) were estimated in transgenic lines under N₂ deficit conditions compared to WT and VC (total chl: 0.02–0.03; chl a: 0.02–0.03; chl b: 0.001–0.005 and carotenoids: 0.003–0.006 mg g⁻¹ Fw) plants. Similarly, higher contents of photosynthesis pigments (total chl: 0.07–0.09; chl a: 0.05–0.07; chl b: 0.01–0.04 and carotenoids: 0.03–0.07 mg g⁻¹ Fw) were found in transgenic lines under salt stress conditions compared to WT and VC (total chl: 0.03–0.04; chl a: 0.01–0.03; chl b: 0.008–0.01 and carotenoids: 0.009–0.01 mg g⁻¹ Fw) plants (Fig. 5B–E). The net photosynthesis of transgenic plants was higher under the stress (5–7 µmol CO₂ m⁻² s⁻¹) environment compared to WT and VC (2–4 µmol CO₂ m⁻² s⁻¹) plants (Fig. 6A). Similarly, stomatal conductance (0.02–0.05 mol H₂O m⁻² s⁻¹) and transpiration rate (1–1.5 mmol H₂O m⁻² s⁻¹)
Figure 5. Photosynthesis efficiency analysis of transgenic plants. Comparative analysis of (A) leaf senescence and photosynthetic pigments, (B) total chlorophyll, (C) chlorophyll a, (D) chlorophyll b, (E) carotenoid in transgenic lines, WT and VC plants under control, nitrogen deficit and salt stress condition. Bars represent means ± standard error, and ‘*’, ‘**’ and ‘***’ designates for significant differences at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.
were also higher in transgenic compared to WT and VC plants under stress conditions, but it was not significant (Fig. S6). Results confirmed that transgenic plants (L1, L4, L5, L9, and L10) maintained high photosynthesis under stress conditions compared to WT and VC plants.

**Multivariate analysis of morphological, biochemical, and physiological responses of plants.** Principal component analysis (PCA) was performed to distinguish the different responses of transgenic and control plants under normal and stress conditions (Fig. 6B). A bi-plot inferred from the PCA separated plant responses in the first two-component with overall 83.38% variability (PC1: 59.84% and PC2: 23.54%). All plants (transgenic lines and control) showed comparable morphological, biochemical, and physiological responses in the unstressed conditions, as transgenic and WT plants clustered together (cnt) in the bi-plot analysis. Transgenic lines exhibited a differential response to the varying stresses. Among different stress
conditions, plants responded towards EL and H2O2 accumulation under N2 deficit conditions compared to salinity stress. Similarly, plants responded further for lipid peroxidation, analyzed by MDA quantification, under salt stress compared to N2 deficit condition. Transgenic lines L5 and L10 were inclined towards proline accumulation under salt stress compared to other lines.

Transcriptional regulation of transgenic tobacco by the AhCytb6 gene under stress condition. The effect of the overexpression of the AhCytb6 gene on the whole-transcript expression of the host plant was studied under stress conditions (N2 deficit and salt stress) using microarray (ArrayExpress ID E-MTAB-9307). The differential expression profiling of 272,410 gene-probes was done, and hierarchical cluster analysis, as well as scatter plots, were analyzed (Fig. S7). The analysis showed out of 272,410 genes, 8,704 and 24,409 genes were significantly (p < 0.05) differentially expressed (> 2 up or down-regulated) under N2 starvation and salt stress conditions, respectively. However, at a 4-fold change level (> 4 up or down-regulated), a total of 975 genes were differentially expressed, with 611 genes up-regulated and 364 genes down-regulated in the treated transgenic plant compared to WT under N2 deficit conditions. Similarly, 1360 genes were differentially expressed in the treated transgenic plant compared to WT under salt stress conditions at a 10-fold change level, out of which 1115 genes were up-regulated while 245 genes were down-regulated (Fig. 7 and Fig. S8). Some of the important differentially expressed genes are listed and grouped into different categories based on their biological activity in Table 1.

Discussion

Plant growth-promoting rhizobacteria (PGPR) is considered an attractive way for sustainable agriculture to cope up with biotic and abiotic stresses. However, due to difficulties in practical implication, handling in field conditions, and comparatively slow response, alternative ways are much needed. The gene(s) that are differentially over-expressed in host plants in the response of plant-microbe (PGPR) interaction could be potential candidates to be explored to engineer crops for future agriculture under different stress conditions. Keeping this thought in mind, we have identified and clone genes that are differentially expressed in peanut (host plant) in the response of interaction with PGPR (S. maltophilia) under the N2 starvation condition. PCR-based cDNA subtraction, commonly known as suppression subtractive hybridization (SSH), is a powerful method for selectively amplification of differentially expressed target cDNA and at the same time, non-targeted DNA amplification is suppressed. In this study, first and foremost we identified the differentially expressed gene due to interaction of S. maltophilia B101 under nitrogen starvation condition.

Figure 7. Microarray-based functional classification of host stress responsive genes. Functional classification of differentially expressed genes of AhCytb6 overexpressing transgenic tobacco plant under abiotic stress conditions. Genes differentially expressed in the AhCytb6 plant under stress conditions were normalized with the transcript of WT plants treated with the same stress.
| S. no. | Transcript probe ID | Gene name | Gene accession | Fold change (log2) |
|--------|---------------------|-----------|----------------|-------------------|
| 1.     | NtPMIa1g85328e1_st  | RuBisCO large subunit-binding protein | FH655192        | 4.36              |
| 2.     | NtPMIa1g189087e1_st | Rubredoxin RubB | FH744456        | 3.88              |
| 3.     | NtPMIa1g145438e1_st | Rubisco accumulation factor 2 | FH522410        | 3.72              |
| 4.     | NtPMIa1g60069e1_st  | Cytochrome P450 | FH898882        | 2.88              |
| 5.     | NtPMIa1g14474e2_st  | Rubisco accumulation factor 1 | FH386425        | 2.53              |
| 6.     | NtPMIa1g2806e3_st   | Cytochrome P450, family 704, subfamily B, polypeptide 1 | FH034679 | 2.48              |
| 7.     | NtPMIa1g32128e1_st  | Chlorophyll a-b binding protein 2.1 | FH443754 | – 3.75            |
| 8.     | NtPMIa1g121870e1_x_st | Chlorophyll a-b binding protein AB10 | FH174615 | – 2.38            |
| 9.     | NtPMIa1g30230e1_st  | Cytochrome P450, family 714, subfamily A, polypeptide 2 | FH558936 | – 2.36            |
| 10.    | NtPMIa1g95982e1_st  | Chlorophyll a/b-binding protein CP24 | FH977269 | – 2.06            |
| 11.    | NtPMIa1g156219e1_st | Cytochrome b (mitochondrion) | FH626006 | – 2.02            |

**Transcripts significantly differentially expressed under nitrogen deficit stress condition**

**Photosynthesis**

1. NtPMIa1g48630e1_st | Transcription factor | ET758387 | 2.86 |
2. NtPMIa1g169283e1_st | Probable WRKY transcription factor 23 | FH154173 | 2.52 |
3. NtPMIa1g31941e2_st | Heat-inducible transcription repressor | FH029374 | 2.49 |
4. NtPMIa1g100948e1_x_st | Putative transcriptional activator DEMETER | FH999805 | 2.32 |
5. NtPMIa1g73637e1_st | YABBY2-like transcription factor YAB2 | F080387 | 2.25 |
6. NtPMIa1g63257e1_x_st | Nuclear transcription factor Y subunit A-10 | ET739691 | 2.24 |
7. NtPMIa1g179140e1_st | GATA transcription factor 16 | ET049433 | 2.23 |
8. NtPMIa1g14745e1_st | Probable WRKY transcription factor 4 | FG172186 | 2.12 |
9. NtPMIa1g51042e1_st | Translation factor ILR3-like | ET854451 | 2.06 |
10. NtPMIa1g58666e1_st | GATA transcription factor 21 | FH630528 | – 2.77 |
11. NtPMIa1g84759e1_x_st | Putative transcription elongation factor SPT5 homolog | FH940966 | – 2.40 |
12. NtPMIa1g5135e1_st | NAC domain containing protein 10 | FH774009 | – 2.31 |
13. NtPMIa1g121208e1_x_st | MADS-box transcription factor FBP4 | F1070259 | – 2.24 |
14. NtPMIa1g183221e1_x_st | GATA transcription factor 8-like | FH668641 | – 2.03 |

**Transcription factors**

**Receptors/signalling /Kinases**

21. NtPMIa1g143095e1_st | Receptor lectin kinase | FH576696 | 3.41 |
22. NtPMIa1g120738e1_st | L-type lectin-domain containing receptor kinase IV.1 | FH068742 | 3.17 |
23. NtPMIa1g95764e1_st | Concanavalin A-like lectin protein kinase family protein | FH644056 | 2.48 |
24. NtPMIa1g96033e1_x_st | Mannose-binding lectin superfamily protein | FH970974 | 2.25 |
25. NtPMIa1g182212e1_x_st | Mitogen-activated protein kinase kinase kinase 5 | FH974766 | 2.21 |
26. NtPMIa1g93065e1_x_st | Putative thaumatin-like protein | FH524903 | 2.15 |
27. NtPMIa1g36616e1_st | Serine/threonine-protein kinase-like protein | FH404353 | 2.12 |
28. NtPMIa1g82319e1_st | Serine/threonine-protein kinase | FH087869 | 2.03 |
29. NtPMIa1g74319e1_x_st | LRR receptor-like serine/threonine-protein kinase | ET980645 | – 2.46 |
30. NtPMIa1g1335e2_st | Calcium-dependent protein kinase | FH408198 | – 2.44 |
31. NtPMIa1g10568e1_st | Calcium-binding protein | FH215122 | – 2.37 |
32. NtPMIa1g122388e1_st | Mitogen-activated protein kinase kinase kinase 15 | FH344502 | – 2.22 |
33. NtPMIa1g178341e1_st | Auxin-responsive family protein | FH571766 | 2.55 |

Continued
| S. no. | Transcript probe ID | Gene name | Gene accession | Fold change (log2) |
|-------|---------------------|-----------|----------------|-------------------|
| 39.   | NpMIa1g77499e1_st   | Cyclic Nucleotide-Regulated Ion Channel Family Protein | EH621839 | 2.34 |
| 40.   | NpMIa1g49198e3_st   | Auxin response factor 5 | ET050562 | 2.30 |
| 41.   | NpMIa1g24250e1_st   | Auxin-responsive protein IAA6 | ET790168 | 2.24 |
| 42.   | NpMIa1g137731e2_st  | Putative chloride channel-like protein CIC-G-Like | FH1201820 | 2.11 |
| 43.   | NpMIa1g100401e2_st  | Auxin-responsive protein IAA11 | FH974554 | 2.02 |
| 44.   | NpMIa1g69701e1_st   | Auxin efflux carrier family protein | ET860447 | – 2.93 |
| 45.   | NpMIa1g176858e1_s_st | Aquaporin PIP2 2 mRNA | ET782162 | – 2.66 |

**Biotic stress responsive**

| S. no. | Transcript probe ID | Gene name | Gene accession | Fold change (log2) |
|-------|---------------------|-----------|----------------|-------------------|
| 46.   | NpMIa1g176538e1_x_st | Disease resistance protein | FI079692 | 2.94 |
| 47.   | NpMIa1g46074e1_l_st | NBS-LRR disease resistance protein homologue | FH899274 | 2.28 |
| 48.   | NpMIa1g89587e1_st  | Pathogenesis-related thaumatin superfamily protein | ET051702 | 2.24 |
| 49.   | NpMIa1g57429e1_l_st | Plant viral-response family protein | ET737281 | 2.11 |
| 50.   | NpMIa1g33021e2_st  | Disease resistance protein (TIR-NBS-LRR) family | ET856286 | – 2.13 |

**Antioxidants**

| S. no. | Transcript probe ID | Gene name | Gene accession | Fold change (log2) |
|-------|---------------------|-----------|----------------|-------------------|
| 51.   | NpMIa1g22665e1_s_st | Superoxide dismutase 3, chloroplastic | FH372649 | 2.38 |
| 52.   | NpMIa1g10068e2_st  | Glutathione S-transferase, C-terminal-like | FH1199263 | 2.27 |

**Chaperon/Heat shock**

| S. no. | Transcript probe ID | Gene name | Gene accession | Fold change (log2) |
|-------|---------------------|-----------|----------------|-------------------|
| 53.   | NpMIa1g12414e1_l_st | Chaperonin 60 subunit beta 4, chloroplastic | ET809635 | 3.08 |
| 54.   | NpMIa1g86842e1_l_st | Heat shock 65 kDa protein | FH979702 | 2.99 |
| 55.   | NpMIa1g99174e1_l_st | Histone chaperone ASF1B | FH990555 | 2.77 |
| 56.   | NpMIa1g54227e1_s_st | Heat shock 70 kDa protein | FH595158 | 2.44 |
| 57.   | NpMIa1g97529e1_l_st | Chloroplast Heat Shock Protein 70-2 | FI272606 | 2.08 |
| 58.   | NpMIa1g742e1_s_st  | Heat shock protein DnaJ with tetrastricopeptide repeats | FH501867 | – 2.22 |

**Transporters**

| S. no. | Transcript probe ID | Gene name | Gene accession | Fold change (log2) |
|-------|---------------------|-----------|----------------|-------------------|
| 59.   | NpMIa1g35785e3_s_st | Potassium channel | ET966045 | 2.84 |
| 60.   | NpMIa1g49230e1_l_st | Peptide transporter 3 | FH083864 | 2.72 |
| 61.   | NpMIa1g51558e1_l_st | Nodulin MtN21/EamA-like transporter family protein | ET913234 | 2.49 |
| 62.   | NpMIa1g88526e1_l_st | ABC transporter family protein | FH951768 | 2.46 |
| 63.   | NpMIa1g77464e3_s_st | Plant calmodulin-binding protein-like | ET982476 | 2.38 |
| 64.   | NpMIa1g52615e1_s_st | Putative sugar transporter | EH622321 | 2.37 |
| 65.   | NpMIa1g66400e1_l_st | Auxin transport protein | FH765702 | 2.28 |
| 66.   | NpMIa1g107239e1_l_st | Nuclear Transport Factor 2 (Ntf2) Family Protein | FI022849 | 2.09 |
| 67.   | NpMIa1g12413e1_l_st | K+ transporter 5 | FH098156 | 2.02 |

**Zinc fingers/leucine zipper motifs containing proteins**

| S. no. | Transcript probe ID | Gene name | Gene accession | Fold change (log2) |
|-------|---------------------|-----------|----------------|-------------------|
| 68.   | NpMIa1g48247e2_s_st | B-box type zinc finger protein with CCT domain | FH973157 | 3.45 |
| 69.   | NpMIa1g29335e4_l_st | Homeobox-leucine zipper protein HOX27 | ET042333 | 2.64 |
| 70.   | NpMIa1g75100e1_l_st | Homeobox-leucine zipper protein HDG12 | ET046033 | 2.38 |
| 71.   | NpMIa1g205801e2_s_st | Putative DHHC-type zinc finger protein | FI045682 | 2.25 |
| 72.   | NpMIa1g12465e1_l_st | Ring zinc finger protein-like | FH486990 | 2.05 |
| 73.   | NpMIa1g93322e1_l_st | Zinc finger (C2h2 Type) Family Protein | ET858669 | 2.04 |
| 74.   | NpMIa1g150572e1_l_st | Zinc finger and hAT dimerization domain | FI059792 | – 2.39 |
| 75.   | NpMIa1g124319e1_l_st | B-box type zinc finger protein with CCT domain | FH540296 | – 2.15 |

**Unknown/hypothetical/uncharacterized**

| S. no. | Transcript probe ID | Gene name | Gene accession | Fold change (log2) |
|-------|---------------------|-----------|----------------|-------------------|
| 76.   | NpMIa1g102443e1_s_st | Uncharacterized | FI004635 | 3.65 |

Continued
| S. no. | Transcript probe ID | Gene name | Gene accession | Fold change (log2) |
|-------|---------------------|-----------|----------------|------------------|
| 77.   | NpPma1g18387e2_st   | Hypothetical protein | FIH373747 | 3.16 |
| 78.   | NpPma1g16155e1_st   | Uncharacterized | ET985839 | 3.10 |
| 79.   | NpPma1g12003e1_st   | Uncharacterized | FIH258062 | – 3.53 |
| 80.   | NpPma1g18678e1_st   | Uncharacterized | FG194168 | – 2.56 |

**Transcripts significantly differentially expressed under salt (150 mM NaCl) stress condition**

**Photosynthesis**

1. NpPma1g82906e1_st | Cytochrome P450 71A2 | FH116143 | 6.62 |
2. NpPma1g70065e1_x_st | Cytochrome P450 | FH044298 | 5.98 |
3. NpPma1g167443e1_st | Elicitor-inducible cytochrome P450 (CYP71D20) | ET820462 | 5.78 |
4. NpPma1g123263e1_st | Cytochrome b561 | EH620440 | 5.77 |
5. NpPma1g31166e2_st | Cytochrome P450, family 71, sub-family B, polypeptide 38 | FH568323 | 4.54 |
6. NpPma1g87311e1_st | SufE-like protein 2, chloroplast | FH948198 | 4.38 |

**Transcription factors**

14. NpPma1g12272e1_s_st | WRKY transcription factor | FIH228396 | 6.16 |
15. NpPma1g32161e3_x_st | Transcriptional activator | ET898480 | 3.60 |
16. NpPma1g37438e1_st | NAC domain-containing protein | ET867074 | 6.23 |
17. NpPma1g42322e2_st | NAC domain-containing protein 72 | FH231367 | 4.18 |
18. NpPma1g61499e1_x_st | myb-like transcription factor family protein | FI051254 | 3.41 |
19. NpPma1g42252e1_st | Ethylene-responsive transcription factor | FH036716 | 4.04 |
20. NpPma1g8202e1_st | BZIP transcription factor bZIP77 | FH071215 | 3.97 |
21. NpPma1g6980e1_s_st | MYC transcription factor | FG185704 | – 3.59 |
22. NpPma1g6258e1_st | AP2 transcription factor | ET046270 | – 3.46 |
23. NpPma1g43983e1_st | Basic-leucine zipper (bZIP) transcription factor | ET042023 | – 3.41 |

**Receptors/signalling /kinases**

24. NpPma1g183983e1_st | Serine/threonine protein kinase 2 | FH678658 | 3.62 |
25. NpPma1g10490e2_s_st | Serine/threonine kinase | FH213282 | 3.58 |
26. NpPma1g182617e1_st | G-type lectin S-receptor serine/threonine protein kinase | FH234969 | 3.42 |
27. NpPma1g107535e1_st | Calcium dependent protein kinase | FIH688575 | – 3.54 |

**Abiotic stress responsive**

28. NpPma1g81893e2_s_st | Abscisic acid-responsive | FH334926 | 5.29 |
29. NpPma1g25688e1_st | Hyoxia-responsive family protein | ET710946 | 5.14 |
30. NpPma1g56521e1_st | Auxin-induced protein | ET676757 | 5.03 |
31. NpPma1g687204e2_st | Cyclic nucleotide-gated ion channel | FH170343 | 4.53 |
32. NpPma1g187181e1_st | K⁺ efflux antiporter | FH733320 | 4.39 |
33. NpPma1g80304e1_st | Aquaporin | FH517170 | 4.11 |
34. NpPma1g82556e4_x_st | Water channel protein MipK | ET846459 | 4.09 |
35. NpPma1g87091e3_s_st | Early-responsive to dehydration protein | ET815593 | 3.69 |
36. NpPma1g174261e1_st | Calmodulin | FH113769 | 3.69 |
37. NpPma1g94367e1_st | Calcium binding protein | FG176020 | 3.64 |
38. NpPma1g183329e1_x_st | Sodium/calcium exchanger membrane region | FH080543 | 3.61 |

Continued
| S. no. | Transcript probe ID | Gene name | Gene accession | Fold change (log2) |
|-------|---------------------|-----------|----------------|-------------------|
| 39.   | NtPMIalg100023e1_s_st | Late embryogenesis abundant protein D-29 | FH993271 | 3.51 |
| 40.   | NtPMIalg52217e3_st | K⁺ uptake permease | FH496325 | 3.34 |
| 41.   | NtPMIalg46641e1_st | Senescence-associated gene | ET683467 | −3.95 |
| 42.   | NtPMIalg25579e1_st | Senescence-inducible chloroplast stay-green protein | FH985884 | −3.55 |

**Biotic stress responsive**

| 43.   | NtPMIalg50893e1_l_s_st | Pathogen induced protein uncharacterized | FH017913 | 5.30 |
| 44.   | NtPMIalg446e2_s_st | Pathogenesis-related transcriptional factor and ERF | FI004101 | 3.86 |
| 45.   | NtPMIalg73165e2_s_st | Pathogen induced protein | ET724700 | 3.80 |
| 46.   | NtPMIalg89450e2_st | Putative verticillium wilt disease resistance protein Vc2 | ET690367 | 3.76 |
| 47.   | NtPMIalg11236e2_st | Pathogenesis-related protein Q (PR-Q) | EH618316 | 3.35 |

**Antioxidants**

| 48.   | NtPMIalg2398e1_s_st | ACC oxidase | FH034942 | 5.83 |
| 49.   | NtPMIalg285e1_s_st | Glutathione S-transferase | FH948778 | 5.24 |
| 50.   | NtPMIalg10755e1_s_st | L-ascorbate oxidase | FH518217 | 4.16 |
| 51.   | NtPMIalg116765e2_st | Ascorbate oxidase | FI055886 | 3.39 |
| 52.   | NtPMIalg100148e1_s_st | Ascorbate peroxidase | FG199962 | 3.34 |

**Chaperon/heat shock**

| 53.   | NtPMIalg122482e1_st | Mitochondrial chaperone | EH622598 | 5.25 |
| 54.   | NtPMIalg80636e4_st | Chaperone protein chloroplastic | ET703308 | 4.89 |
| 55.   | NtPMIalg48639e1_st | Heat shock 70 kDa protein | FH643575 | 4.16 |

**Transporters**

| 56.   | NtPMIalg45198e1_s_st | Sugar transport protein | ET923251 | 5.92 |
| 57.   | NtPMIalg17929e1_s_st | ABC protein | FH628071 | 5.36 |
| 58.   | NtPMIalg36079e1_st | Amino acid transporter | FH05832 | 4.53 |
| 59.   | NtPMIalg58619e1_st | Sulfate transporter | FG143429 | 4.38 |
| 60.   | NtPMIalg123382e2_s_st | Ammonium Transporter 2 | ET797276 | 4.02 |
| 61.   | NtPMIalg170644e1_st | High affinity K⁺ transporter | FH538430 | 3.64 |
| 62.   | NtPMIalg202149e1_st | Nitrate transporter NRT1-5 | ET806108 | 3.38 |
| 63.   | NtPMIalg193197e1_s_st | Sugar phosphate exchanger, putative | FH747309 | −4.11 |

**Zinc fingers/leucine zipper motifs containing proteins**

| 64.   | NtPMIalg102701e1_st | Zinc induced facilitator | FH709466 | 6.01 |
| 65.   | NtPMIalg95486e1_st | Zinc finger protein CONSTANS | FH75799 | 5.69 |
| 66.   | NtPMIalg34050e1_st | Zinc finger CCCH domain | FH744808 | 3.35 |
| 67.   | NtPMIalg227029e1_st | Zinc finger B-box protein | FG167857 | 4.06 |
| 68.   | NtPMIalg68098e1_st | DHHC-type zinc finger family protein | FG197147 | 3.43 |
| 69.   | NtPMIalg31503e1_s_st | B-box type zinc finger protein with CCT domain | ET051218 | −3.97 |

**Unknown/hypothetical/uncharacterized**

| 70.   | NtPMIalg9436e1_st | Uncharacterized | FH583256 | 7.71 |
| 71.   | NtPMIalg110238e3_s_st | Hypothetical protein | FG133280 | 5.19 |
| 72.   | NtPMIalg197992e1_st | Uncharacterized transporter | ET761936 | 5.16 |
| 73.   | NtPMIalg191871e1_st | Uncharacterized | FH009504 | −8.35 |
| 74.   | NtPMIalg100379e1_st | Hypothetical protein | ET829011 | −6.03 |

**Miscellaneous**

| 75.   | NtPMIalg88846e1_st | Early flowering-like protein | ET778187 | 4.85 |
| 76.   | NtPMIalg36370e1_st | Early nodulin-like protein | FH240198 | 4.79 |
| 77.   | NtPMIalg35320e3_s_st | Nodulin family protein | FI036052 | 4.53 |
| 78.   | NtPMIalg51538e1_st | Nodulin/EamA-like transporter family protein | ET913234 | 3.72 |

Table 1. Selected transcripts that differentially expressed (up- or down-regulated) in AhCytb6 overexpressing transgenic tobacco plant compared with the wild type under nitrogen deficit or salt stress conditions. No sign indicates up-regulation, whereas “−” sign shows down-regulation. Fold-expression is significant at ANOVA p < 0.05.
genes were of unknown/unclassified function. Expression of a large number of uncharacterized or hypotheti-
cal genes after interaction with PGPR under the \( N_2 \) deficit condition provides a molecular insight of changes
that occurs during the interaction of \( S. \) malophilia and \( A. \) hypogaea. In contrast, the interaction between \( Cicer \)
\( arietinum \) and \( A. \) rabiei resulted in 7% genes of unknown function\(^{29}\), whereas the interaction between
\( Vitis \) \( pseudoreticulata \) with \( U. \) \( necator \) leads to the differential expression of 24% uncharacterized genes\(^{30}\).
These \( N_2 \) starvation-responsive genes were further validated by qRT-PCR, and the expression profiling of these
uncharacterized SSH clones showed that these genes were up-regulated during plant interaction with \( S. \)
\( malophilia \) (Fig. S1). An \( N_2 \) deficiency and PGPR interaction responsive clone SM409 (later on named the \( A.hCytb6 \)
gene, which shows the similarity with PSII related gene \( cytb6 \)), had higher expression (about 4-fold) among
studied clones in a transcript profiling and was selected to characterize further in a model plant tobacco. Fatafah
et al. showed that 1938 genes were differentially expressed in barley leaves after 20 days of nitrogen starvation;
when plants were resupplied with nitrogen, 62% of genes that were down-regulated were up-regulated and out
of these genes, most of the genes belong to photosynthesis\(^{31}\). The RNAseq data of Yang et al. showed that the
\( Cytb \)/ complex is upregulated in leaves of low nitrogen level tolerance verity of sugarcane; both studies indicate
the involvement of \( Cytb6 \) gene in nitrogen deficit condition\(^{32}\). Thus, this study also supports the major role of
photosynthetic related genes in the case of nitrogen starvation. In contrast, PSII related genes were downregulated
in durum wheat under nitrogen starvation conditions\(^{33}\). Thus, differential expression of gene under \( N_2 \) starvation
is due to interaction with the \( S. \) malophilia and helps plants to cope up with the nitrogen starve condition. The
genome organization study confirmed that the \( A.hCytb6 \) gene is intronless, and \( -in-silico \) analysis revealed that the
gene encodes for a transmembrane protein consisting of helix and coil motifs that is highly stable (Figs. S3–S4).

All raised transgenic lines were checked for the confirmation of transgene, and out of 17 transgenic lines
(Fig. 1), we selected five lines showing single transgene integration with the high expression for further analysis
under stress conditions. Overexpression of \( A.hCytb6 \) improved seed germination and health of the growing
seedlings under \( N_2 \) starvation and salt stress conditions where WT and VC failed to do so (Figs. 2, 3). Transgenic
seedlings grown in stress conditions exhibited higher shoot length, root length, fresh weight, and dry weight in
comparison to WT and VC (Fig. 3). The enhanced germination and growth of the transgenic plants showed that
the \( A.hCytb6 \) gene increases the tolerance against \( N_2 \) starvation and salt stress by restoring the photosynthetic
machinery and equilibrating C:N ratio, which is important during the reproductive and growth period of the
plant. In another study, Qiao et al. showed that Cytochrome b561 was differentially expressed and up-regulated
in pigeon pea after interaction with arbuscular mycorrhizal fungi under drought, which supports the role of the
\( cytb6 \) gene in legumes in the symbiotic relationship under abiotic stress condition\(^{34}\). On the other hand, Dyda
et al. showed that cytochrome b559 was down-regulated in triticale after infection with pathogenic fungus \( Micro-
dochium nivale \), which showed that gene \( cytb \) has a crucial role in plant immunity.\(^{35}\) Joaquin-Ramos et al. showed
that \( CYTb6 \) was significantly up-regulated in \( A.maranthus cruentu \) under salt stress (300 mM) which supports
the role of \( cytb6 \) under salt stress\(^{36}\). Constitutive expression of rice microRNA528 also showed enhanced growth,
elevated biomass, and tolerance to salinity stress and \( N_2 \) starvation in the transgenic plants\(^{37}\).

The physiological status of the plant determines growth and survival under harsh environmental conditions.
Abiotic stress damages the plant cell membrane, integrity of the cell membrane is essential for the stress toler-
ance of the plant. Results of EL and MSI showed that all transgenic plants overexpressing the \( A.hCytb6 \) gene had
less cell membrane injury compared to WT and VC plants (Fig. 4). Thus, increased membrane stability and a
low level of electrolyte leakage help the plant to maintain the cell homeostasis under stress conditions; similar
results were obtained by Ben-Romdhane et al.\(^{38}\). Proline is an essential osmoregulator and molecular chaperone
which helps the plant to maintain the cytosolic redox status and ROS scavenging as well as helping the plant under
stress conditions\(^{39,40}\). The transgenic plant overexpressing the \( A.hCytb6 \) gene enhanced the proline production
in transgenic plants and enabled plants to mitigate the stress conditions at the cellular level.

The ROS metabolism in the cell is regulated by redox mechanisms with the help of antioxidants, and it main-
tains the stable dynamic equilibrium in normal physiological conditions\(^{41}\). Under stress conditions, this balance
is disrupted and creates oxidative stress, which can cause damage to nucleic acids, proteins, and lipids\(^{42,43}\). When
living cells face stress, they generate free radicals like superoxide, hydrogen peroxide, and cell membranes (which
are made up of fatty acids) prone to oxidation. These free radicals cause peroxidation of the cell membrane and
generate malondialdehyde (MDA); thus, these parameters are used for the biochemical markers to measure
the stress levels\(^{44}\). Abiotic stress causes disturbance in PSII, which causes the generation of a high amount of
ROS; here overexpression of \( A.hCytb6 \) may provide stability to ETC and cause a reduction in ROS in transgenic
lines. The WT and VC plants accumulate more MDA, \( H_2O_2 \), and \( O_2 \) in comparison to transgenic plants (Fig. 4).
Thus, results confirmed the role of \( A.hCytb6 \) in ROS scavenging in plants and stress tolerance of \( N_2 \) starvation
and salt stress conditions. Recently, Yang et al. also showed that transgenic tobacco lines overexpressing chloro-
plast targeting and heme-binding genes \( A.hFC1 \) and \( A.hHEMA1 \) had less accumulation of MDA, \( H_2O_2 \) content
in comparison to the wild-type under 200 mM salt stress conditions\(^{45}\). This finding also supports the role of
chloroplast targeting and cytochrome-related genes in plant defense mechanisms other than photosynthesis.

Salt stress can cause damage to the chlorophyll pigment-protein complex and degrades the enzyme chloro-
phyllase, and nitrogen starvation causes chlorosis in leaves\(^{46,47}\). Chlorophyll contents observed under salt and
\( N_2 \) deficit condition showed that the transgenic plants were able to retain more chlorophyll contents and carot-
enoids in comparison to the WT and VC counterparts (Fig. 5). A higher concentration of carotenoid content in
transgenic lines is an indicator of better photosynthetic efficiency as well as reduced oxidative stress in stressed
conditions because carotenoid also plays a protective role against ROS\(^{48}\). It is quite evident that \( A.hCytb6 \) protects
plants from the loss of chlorophyll and helps the information of vital pigments via improving performance in
photosynthesis. Under low nitrogen, plants reduce their photosynthesis to reduce energy loss; we found that
the net photosynthesis rate was significantly higher in the transgenic plants in \( N_2 \) starvation in comparison to
WT and VC. Electron transport is very much affected during photosynthesis by high salt concentration and/or

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nitrates, which deteriorates the photosynthetic performance of plants\(^2\). In our study, transgenic lines overexpressing the AhCytb6 gene has a higher net photosynthesis rate, stomatal conductance, and transpiration rate than WT and VC show that the AhCytb6 gene plays a key factor in PSII and enhances photosynthesis efficiency and yield of the plant under stress (Fig. 6A and Fig. S6). Similar activity of the OsPGK2-P gene was also reported in transgenic tobacco under salt stress\(^5\).

Microarray analysis of the plant overexpressing the AhCytb6 gene showed differential expression of the gene compared to the WT plant in similar stress conditions. Our results showed that the ectopic expression of AhCytb6 influenced the expression of genes belonging to metabolism (28%), transporters (9%), photosynthesis (7%), abiotic stress-responses (7%), receptor/kinase/signaling (7%), uncharacterized (6%), transcription factors (5%), abiotic stress (3%), antioxidant (1%), and chaperon/heat shock protein (1%). Besides this, miscellaneous (21%) and housekeeping (3%) were also differentially expressed under salt stress. Results coincided with the study of Passricha et al.\(^3\), where transporters, kinases, and genes related to abiotic stress were also differentially expressed in transgenic rice overexpressing the PsLeaRLK gene under salt stress. Under N\(_2\) starvation conditions metabolism (19%), transporter (4%), photosynthesis (3%), abiotic stress-responsive (5%), receptor/kinase/signaling (8%), uncharacterized (6%), transcription factors (4%), abiotic stress (2%), and chaperon/heat shock protein (3%) along with miscellaneous (34%) and housekeeping (8%) were also differentially expressed.

Transgenic tobacco overexpressing wheat microRNA TaMIR444a led to the differential expression of 1733 genes in compression of WT under N\(_2\) starvation. These genes belonged to unknown, transcription, transportation, abiotic and biotic stress, signaling, metabolism, and a miscellaneous category\(^5\). Microarray data show that overexpression of the AhCytb6 gene affects the plant response in nitrogen starvation and salt stress at the molecular level, these changes cumulatively support a plant under stress condition, and transgenic lines perform better than the wild-type counterparts do. Overexpression of abiotic and biotic stress-related genes and heat shock proteins show that this gene can play important role in priming plant immunity under biotic and abiotic stresses. In a study by Luo et al., nitrogen availability is directly proportional to the differential overexpression of photosynthesis-related genes\(^5\). In contrast to this, due to the overexpression of the Cytb6 gene, 3% of photosynthetic genes were differentially expressed under N\(_2\) starvation, showing the role of this gene in N\(_2\) assimilation, which follows a recent study of Iqbal et al.\(^4\). Overexpression of transcription factors like WRKY, GATA, YAB2 under N\(_2\) starvation and WRKY, NAC, MYB, under salt stress show that the Cytb6 gene plays a major role in C–N metabolism and in salt stress that starts at the transcriptional level. Rubisco, which is an indicator for total N\(_2\) level in plants and leaves, also up-regulates in transgenic plants under salt and N\(_2\) starvation showing the balancing role of Cytb6 in the C–N cycle in stress conditions. Similar results were observed by Xin et al.\(^5\). Based on the above results, we hypothesized a model that summarized the probable role of AhCytb6 in plant-microbe interaction and abiotic stress tolerance (Fig. 8).

**Conclusion**

In this study, we reported the changes at the molecular level in the host plant after plant-microbe interaction. AhCytb6 is a photosynthetic gene differentially expressed after interaction between *Arachis hypogaea*– *Stenotrophomonas maltophilia* under N\(_2\) stress. This gene plays a significant role in plant-microbe interaction, and its role is functionally validated by ectopic overexpression in transgenic tobacco plants. Morphology, physiology, biochemical, and genetic parameters were analyzed under N\(_2\) starvation and salt stress conditions and compared to their wild-type counterparts. We observed that transgenic plants perform better under stress conditions than WT and VC. Overexpression of this gene enhanced endurance against N\(_2\) starvation and salt stress. Microarray analysis of transgenic plants showed that this gene also affects the transcript expression of different stress-responsive genes and transcription factors. Overall results reveal roles of AhCytb6 in stress tolerance under N\(_2\) deficit and salt stress other than photosynthesis. This gene could be further explored for the development of genetically modified crops for sustainable agriculture under N\(_2\) deficits and/or salt-affected areas.

**Materials and methods**

**PGPR treatment to the peanut plant.** Peanut (*Arachis hypogaea*) seeds (cultivar GG-20) were obtained from the Junagadh Agricultural University, Junagadh (Gujarat), which also have the voucher specimen for the identification of plants. Procure peanut seeds were surface sterilized, germinated, and transferred to previously optimized hydroponics conditions\(^1\). Briefly, seven days old seedlings were transferred 300 ml ½ MS media supplemented without nitrogenous component. Plants were treated with plant growth-promoting rhizobacterium *S. maltophilia* BJ01, which has proven ability to provide plant tolerance under N\(_2\) deficit conditions\(^1\). Plants were grown under the N\(_2\) starvation condition with (T) or without bacterial inoculation (C) for 21 days. Plant leaves were collected after 21 days and stored at –80°C for further experiments.

**Suppression subtractive hybridization (SSH) and dissemination of differentially expressed gene(s).** Total RNA was isolated from control and treated plants by guanidine isothiocyanate (GITC) method, and mRNA was purified using Poly AT tract mRNA isolation kit following the manufacturer’s instruction (Promega, USA). Total 2 µg mRNA was used for single stranded cDNA synthesis using 1 mM primer (5′-TTT TGT ACA AGC TTG TGG-3′ containing Rsa I restriction sites; GTAC), 1 mM deoxynucleotides (dNTPs), and 20 units of avian myeloblastosis virus (AMV) reverse transcriptase. Immediately after completion of single stranded cDNA, proceed for the double stranded cDNA synthesis at 16°C in thermal cycler using 0.2 mM dNTPs mix, a second strand enzyme cocktail (containing DNA polymerase I, RNase H and DNA ligase) and 6 unit of T4 DNA polymerase. The cDNA, synthesized from the treated plant was considered as a ‘tester’ while the cDNA of the control plant was used as a ‘driver.’
Suppression subtractive hybridization (SSH) was performed with a PCR-Select cDNA Subtraction Kit according to the user manual (Clontech, USA). In brief, the blunt end was created in driver and tester double-stranded cDNAs by Rsa I restriction digestion. The tester cDNA was purified and subdivided into two sets, and each was ligated with different adaptors at 16 °C for 12 h. However, driver cDNAs were not ligated with any adapters. Hybridization of tester and driver cDNA was carried out in two steps; in the first step, digested driver cDNA was added to individual adapter-ligated tester cDNA, denatured at 98 °C for 90 s and allowed for hybridization at 68 °C for 8 h. In the second step, both hybridized products were mixed, and the fresh denatured driver cDNA was added and allowed for hybridization again. Differentially expressed cDNAs were exponentially amplified using adapter-specific primers, cloned in pGEM-T easy vector (Promega, USA) and transformed into E. coli DH5α cells. Positive clones were selected, confirmed, sequenced (at M/s Macrogen Inc., South Korea), and analyzed by bioinformatics tool.

Selection and transcript profiling of differentially expressed genes. Differentially expressed genes obtained by SSH were categorized, and representative primer sets were designed for each category (Table S1). Total RNA was isolated (from control and treated plants), and cDNA was synthesized from 5 µg of total RNA using the ImProm-II Reverse Transcription System (Promega, USA). Quantitative real-time (qRT)-PCR reaction was performed with Power SYBR Green PCR Master Mix (Invitrogen, USA) in a Bio-Rad CFX96 detection system (Bio-Rad, USA). The specificity of qRT-PCR was determined by melt curve analysis followed by 1% agarose gel electrophoresis. The relative fold expression of genes was calculated by the $2^{-\Delta\Delta CT}$ method, while actin was used as the housekeeping gene.

A clone SM409 (538 bp), which was classified in the unknown category (showing resemblance with unknown mRNA from NCBI database), and showed about 4-fold up-regulation in the treated plant (compared to the control under stress conditions), was selected for the further study.

Cloning of gene and bioinformatics analysis. Differentially expressed gene SM409 was made full by rapid amplification of cDNA ends, cloned in pGEM-T easy vector (Promega, USA), transformed into E. coli DH5α cells, and sequenced (at M/s Macrogen Inc., South Korea). The contiguous sequences obtained through RACE (3’RACE and 5’RACE) were assembled to obtain the full-length gene sequence. The gene-specific primer (Table S2) was designed, and a full-length gene was amplified from the cDNA of A. hypogaea using proofread (Pfu) polymerase, cloned in pGEM-T easy vector and sequenced (M/s Macrogen Inc., South Korea).
sequence was analyzed using different bioinformatics tools available at the ExPASY portal. Based on different bioinformatics analyses, the SM409 clone sequence was named as the \textit{AhCytb6} gene.

**Genetic transformation of tobacco and generation of transgenic plants for the functional analysis of \textit{AhCytb6} gene.** The complete coding region of the \textit{AhCytb6} gene was amplified (Table S2) and cloned into the pTiC58 vector down-stream to the 35S promoter. Recombinant pTiC58 \textit{pAHCytb6} was digested with enzyme 	extit{PstI}, expression cassette (35S:AhCytb6:35S-ter) was obtained and cloned in pCAMBIA2301 vector. The resultant plant expression vector pCAMBIA2301:35S:AhCytb6 was mobilized into \textit{Agrobacterium tumefaciens} strain EHA105 for the genetic transformation. \textit{Agrobacterium}-mediated genetic transformation of \textit{Nicotiana tabacum} cv. Petit Havana with the \textit{AhCytb6} gene was done using the leaf disc transformation method\(^{49}\). After genetic transformation, leaf disc was regenerated as per standard tissue culture protocol, putative transgenic lines (\(T_0\)) were screened on kanamycin (50 mg L\(^{-1}\)) for the selection, positive plants were transferred in the greenhouse under controlled condition, and matured seeds (\(T_0\)) were collected\(^{50,61}\).

**Analysis of transgenic lines under different abiotic stress condition.** Transgenic seeds were germinated on kanamycin (50 mg L\(^{-1}\)), and \(T_1\) transgenic lines were obtained. Transgene integration was confirmed by PCR amplification of \textit{uidA} (GUS) and \textit{AhCytb6} gene (Table S2); however, transgene event (copy number) was checked by southern blot analysis. Transgenic lines were subjected for histochemical GUS analysis, and five lines (L1, L4, L5, L9, and L10) were selected, and overexpression of the \textit{AhCytb6} gene was analyzed by semi-quantitative reverse transcriptase PCR (Table S2). The selected transgenic lines were studied for morphological, biochemical and physicochemical analyses, and compared with wild-type (WT: untransformed tobacco plant) and vector control (VC: transgenic lines transformed with pCAMBIA2301 vector) plants under different abiotic stress treatments. Germination efficiency (% germination) of transgenic lines were studied under \(N_2\) starvation and NaCl (150 mM) stress conditions.

For the morphological study, seeds (transgenic lines, WT and VC) were germinated on MS media supplemented with kanamycin (50 mg L\(^{-1}\)), and 3 days old equal size seedlings were transferred to different petri-plates (containing MS media) and subjected to \(N_2\) starvation and NaCl (150 mM) stress conditions for 21 days (8 h dark/16 h light cycle at 25 \(^\circ\)C). Growth parameters were measured and documented\(^{52}\). For stress treatments, 21 days-old seedlings (grown on MS media supplemented with kanamycin) were transferred to hydroponics (containing 1/2 strength of MS media without \(N_2\) source) and grown further 21 days under \(N_2\) starvation conditions. In a parallel set of experiment, forty-two days old plants grown under normal conditions (1/2 MS media without any stress) were subjected to NaCl (150 mM) stress conditions for 24 h. Plants (transgenic lines: L1, L4, L5, L9, and L10; WT and VC) grown under control (without any stress) or different stress conditions (\(N_2\) deficit and NaCl stress) were harvested and studied for different morphological, biochemical and physiological analyses.

Leaves disc (~ 8 mm) of plants (42 days) grown (as above) under control (unstressed) conditions were subjected to different stresses (\(N_2\) deficit and NaCl stress) conditions for 7-days. Leaf senescence was documented, whereas chlorophyll and carotenoids were measured\(^{52}\). Comparative physio-biochemical analyses, including, electrolyte leakage, membrane stability index, proline, lipid peroxidation (MDA) content and \(H_2O_2\) content were performed for all harvested plants\(^{53-68}\). The \textit{in vivo} localization of hydrogen peroxide (\(H_2O_2\)) and superoxide radicals (\(O_2^-\)) was done by histochemical staining\(^{69}\). Photosynthesis parameters, including net assimilation rate, stomatal conductance, and transpiration rate, were measured by portable photosynthesis (LI6400XT, LI-COR Biosciences, USA) system\(^{55}\).

**Expression profiling of transgenic plants by microarray.** A transgenic plant that performed better compared to other lines was selected for the differential transcript expression profiling\(^{52,70}\). Forty-two days old plant grown under \(N_2\) starvation (21 days) stress condition and a plant (42-days old) subjected to NaCl (150 mM for 24 h) stress were used for microarray analysis along with corresponding control plants. Total RNA was extracted from treated and WT (unstressed) plants and converted to first strand cDNA followed by second strand cDNA synthesis. \textit{In vitro} transcription was performed and a cRNA was synthesized and finally converted to single-stranded cDNA. Single-stranded cDNA was fragmented, labeled and hybridized with a whole gene tobacco array, which was comprised of 272,410 gene probes. Hybridization was performed at 42 \(^\circ\)C for 16 h, according to the user manual (Affymetrix, USA). After hybridization, the array chip was washed and stained in the fluidics module (Fluidics Station 450, Affymetrix, USA), scanned (Scanner 3000 7G, Affymetrix, USA), and analyzed using expression console (version 1.1) and transcriptome analysis console (version 3.0) software (Affymetrix, USA).

**Statistical analysis.** All experiments were performed in triplicates, and each set of experiments contained five plants (except microarray, which was performed in duplicate). Statistical analysis was performed by Graph-Pad Prism software. All data were subjected to analysis of variance (ANOVA) followed by Dunnnett test to compare all column vs WT in each condition. Values are expressed as the mean ± SE, and p value < 0.05 is considered as statistically significant.

**Data availability** All datasets presented in this study are included in the article and supplementary data. Microarray data are available in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-9307.
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**Author contributions**

Conceived and designed the experiments: A.M.; Performed the experiments: A.A. and V.S.; Analyzed the data: A.A., V.S. and A.M.; Wrote the manuscript: A.A., V.S., and AM.

**Competing interests**

The authors declare no competing interests.
