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Co-expression of *NCED* and *ALO* improves vitamin C level and tolerance to drought and chilling in transgenic tobacco and stylo plants

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Summary

Abscisic acid (ABA) regulates plant adaptive responses to various environmental stresses, while L-ascorbic acid (AsA) that is also named vitamin C is an important antioxidant and involves in plant stress tolerance and the immune system in domestic animals. Transgenic tobacco (*Nicotiana tabacum* L.) and stylo [*Stylosanthes guianensis* (Aublet) Swartz], a forage legume, plants co-expressing stylo 9-*cis*-epoxycarotenoid dioxygenase (SgNCED1) and yeast D-arabinono-1,4-lactone oxidase (ALO) genes were generated in this study, and tolerance to drought and chilling was analysed in comparison with transgenic tobacco overexpressing *SgNCED1* or *ALO* and the wild-type plants. Compared to the *SgNCED1* or *ALO* transgenic plants, in which only ABA or AsA levels were increased, both ABA and AsA levels were increased in transgenic tobacco and stylo plants co-expressing *SgNCED1* and *ALO* genes. Compared to the wild type, an enhanced drought tolerance was observed in *SgNCED1* transgenic tobacco plants with induced expression of drought-responsive genes, but not in *ALO* plants, while an enhanced chilling tolerance was observed in *ALO* transgenic tobaccos with induced expression of cold-responsive genes, but not in *SgNCED1* plants. Co-expression of *SgNCED1* and *ALO* genes resulted in elevated tolerance to both drought and chilling in transgenic tobacco and stylo plants with induced expression of both drought and cold-responsive genes. Our result suggests that co-expression of *SgNCED1* and *ALO* genes is an effective way for use in forage plant improvement for increased tolerance to drought and chilling and nutrition quality.

Introduction

Plants are often exposed to multiple environmental stresses, such as drought, salinity and extreme temperatures (cold and heat), during a plant's life cycle. These abiotic stresses result in both general and specific effects on plant growth and development and are the major cause of reducing crop productivity and quality (Mahajan and Tuteja, 2005). Photosynthesis is greatly inhibited under abiotic stresses, which lead to elevated accumulation of reactive oxygen species (ROS) and oxidative damage on membranes and thus affecting cell viability. Plants have evolved adaptation mechanisms in response to stress conditions. Abscisic acid (ABA) is a phytohormone critical for diverse physiological and developmental processes and plays an important role in integrating various stress signals and regulating downstream stress responses (Fujita *et al.*, 2011; Roychoudhury *et al.*, 2013). Abscisic acid level is triggered in response to various stress signals, mainly due to the induction of the genes responsible for ABA synthesis (Xiong and Zhu, 2003). 9-*cis*-Epoxycarotenoid dioxygenase (NCED) is the key enzyme involved in ABA synthesis in higher plants (Nambara and Marion-Poll, 2005; Qin and Zeevaart, 1999). *NCED* expression is induced by water-deficit, salinity and chilling stresses (Iuchi *et al.*, 2000; Qin and Zeevaart, 1999; Yang and Guo, 2007). Overexpression of *NCED* gene resulted in ABA accumulation and increased tolerance to drought and salinity in transgenic plants (Iuchi *et al.*, 2001; Qin and Zeevaart, 2002; Zhang *et al.*, 2008, 2009), while Arabidopsis *NCED3* knockout mutant exhibit drought-sensitive phenotype with reduced ABA level (Iuchi *et al.*, 2001).

Antioxidant defence system including both antioxidant enzymes and nonenzymatic antioxidants plays an important role in abiotic stress tolerance. It functions to scavenge the elevated ROS, including superoxide radicals, hydrogen peroxide (H_2O_2) and hydroxyl radicals, in plant cells resulted from abiotic stresses for protection against oxidative damages on cell membranes. Superoxide radicals are usually detoxified by superoxide dismutase (SOD), while H_2O_2 is scavenged by catalase (CAT) and the ascorbate–glutathione cycle that includes ascorbate peroxidase (APX), glutathione reductase (GR), ascorbate (AsA) and glutathione (GSH) (Foyer and Noctor, 2009; Gill and Tuteja, 2010). Numerous studies indicate that activities of antioxidant enzymes are correlated with plant tolerance to abiotic stresses such as drought and chilling stress (Gill and Tuteja, 2010; Guo *et al.*,

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2006; Lu *et al.*, 2013). Ascorbic acid is an important component of the plant antioxidant system, regulating ROS levels, plant defence gene expression and responses to environmental stresses (Chen and Gallie, 2005; Conklin and Barth, 2004; Hemavathi *et al.*, 2010; Smirnoff and Wheeler, 2000). Ascorbic acid is synthesized through multiple biosynthetic pathways in plants (Gallie, 2013), while the major pathway is Smirnoff–Wheeler pathway (Wheeler *et al.*, 1998). Overexpression of D-galacturonic acid reductase, GDP-L-galactose phosphorylase, L-galactono-1,4-lactone dehydrogenase and GDP-L-galactose guanyltransferase genes resulted in increased AsA levels (Agius *et al.*, 2003; Bulley *et al.*, 2009, 2011; Liu *et al.*, 2013; Tokuna *et al.*, 2005) and tolerance to paraquat and salinity stresses (Liu *et al.*, 2013). D-Arabinono-1,4-lactone oxidase (ALO) is a key enzyme for synthesis of D-erythroascorbic acid in yeast. This enzyme can use L-galactono-1,4-lactone as efficiently as D-arabinono-1,4-lactone to produce AsA (Huh *et al.*, 1994; Lee *et al.*, 1999). Overexpression of *ALO* resulted in elevated AsA levels and tolerance to paraquat and high light-induced oxidative stress and aluminium toxicity (Chen *et al.*, 2014). Ascorbic acid is also essential for domestic animals. Although domestic animals can synthesize AsA in their liver, the amount of AsA produced by the animals may be insufficient to meet its requirements under specific physiological conditions. Lactating dairy cattle may predispose AsA deficiency due to the huge use of glucose and galactose, precursor of AsA synthesis, for production of milk (Matsui, 2012). It is hypothesized that an elevated AsA level will improve both tolerance to abiotic stresses and nutrition quality in forage plants.

Stylo is an important forage legume and cover crop in tropical and southern subtropical regions with great tolerance to soil acidity and good adaptation to infertile soil (De La Rue *et al.*, 2003; Jiang *et al.*, 2005), but it is sensitive to cold. Low temperature in winter is the major factor limiting its growth and survival in subtropical regions. Stylo seedlings are damaged upon exposure to low temperature at 10 °C in growth chamber (Zhou *et al.*, 2005a). Chilling tolerance of stylo is associated with regulation of antioxidant system under low temperature. Higher activities of SOD, CAT and APX and contents of AsA and GSH were observed in chilling tolerant mutants of stylo as compared to the wild type under low temperature conditions (Lu *et al.*, 2013). Chilling tolerance of stylo is increased by the exogenous application of ABA through induced antioxidant defence system (Zhou *et al.*, 2005a), with the involvement of signal molecules nitric oxide (NO) (Zhou *et al.*, 2005b) and Ca^{2+} (Zhou and Guo, 2009). In addition, enhancing drought tolerance is important for a cover crop. However, there is no investigation on improvement of abiotic stress tolerance and vitamin C content in stylo.

Although *Agrobacterium*-mediated transformation of stylo was reported using *bar* gene as selective marker (Sarra *et al.*, 1994), the transformation efficiency was low, and thus, microparticle bombardment protocol was examined later (Quecini *et al.*, 2006). It is important to establish an effective transformation protocol in stylo for its improvements by transgenics. The objective of this study was to generate transgenic stylo plants with improvements in abiotic stress tolerance and vitamin C content by co-expressing *SgNCED1* and *ALO* genes, using *Agrobacterium*-mediated transformation. In addition, transgenic tobacco plants overexpressing *SgNCED1* or *ALO* and co-expressing *SgNCED1* and *ALO* were analysed for understanding effectiveness of *SgNCED1* and *ALO* genes.

Results

Analysis of transgenic tobacco plants co-expressing *SgNCED1* and *ALO*

Transgenic tobacco plants co-expressing *SgNCED1* and *ALO* under control of the CaMV35S promoter (Figure 1a) were generated after selection on basta-containing medium. DNA hybridization showed that *bar* gene was integrated into the genomes of transgenic plants (lines NA1 and NA2), whereas no cross-hybridization was observed in the wild type (Figure 1b). Transcripts of *SgNCED1* and *ALO* genes in two homozygous plant lines (T_2) were further analysed, in comparison with the transgenic tobacco plants overexpressing *SgNCED1* (lines N16 and N61) or *ALO* (lines A53 and A75) under control of the CaMV35S promoter (Chen *et al.*, 2014; Zhang *et al.*, 2009). Real-time quantitative RT-PCR data showed that *SgNCED1* transcripts could be detected in lines NA1, NA2, N16 and N61, while *ALO* transcripts could be detected in lines NA1, NA2, A53 and A75. In addition, higher levels of *SgNCED1* transcripts were observed in lines NA1 and NA2 than in lines N16 and N61 (Figure 1c).

In consistent with previous observations (Chen *et al.*, 2014; Zhang *et al.*, 2009), higher levels of ABA were observed in lines N16 and N61, while higher levels of AsA were observed in lines A53 and A75 than in the wild type (Figure 1d,e). Both ABA and AsA levels were higher in transgenic lines NA1 and NA2 than in the wild-type plants; compared to that, ABA level was not affected in lines A53 and A75, and AsA level was not affected in lines N16 and N61 (Figure 1d,e).

Analysis of plant tolerance to drought and chilling stresses

Relative water content and ion leakage were at about 95% and 10%, respectively, in leaves of all tobacco plants under normal growth conditions (data not shown). Relative water content was decreased from 30% to 38%, and ion leakage was increased from 39% to 41% in the wild-type and transgenic lines A53 and A75 after withholding irrigation, and they showed no significant difference between the wild-type and transgenic lines A53 and A75. Transgenic lines N16, N61, NA1 and NA2 had significantly higher levels of RWC and lower levels of ion leakage than the wild type (Figure 2a,b). Moreover, MDA levels showed the similar pattern to ion leakage in response to withholding irrigation. No significant difference in MDA levels between the wild-type and transgenic lines A53 and A75 was observed, but lower levels of MDA were observed in transgenic lines N16, N61, NA1 and NA2 as compared to the wild-type plants (Figure 2c). When the wild-type and transgenic lines A53 and A75 became died after withholding irrigation, transgenic lines N16, N61, NA1 and NA2 were survived after the recovery of water supply. The results indicated that drought tolerance was increased in transgenic plants overexpressing *SgNCED1* or co-expressing *SgNCED1* and *ALO*, but not affected in transgenic plants overexpressing *ALO* gene alone.

The maximum photochemical efficiency (F_v/F_m) was usually 0.85 in tobacco plants under normal growth conditions (Sambe *et al.*, 2015). It was decreased from 0.26 to 0.27 in the wild type and N16 and N61 after 3 days of chilling stress at 3 °C, while higher levels of F_v/F_m were observed in transgenic lines A53, A75 NA1 and NA3 than in the wild type (Figure 3a). Similar to MDA, ion leakage showed no significant difference between the wild-type and transgenic lines N16 and N61, and lower levels were

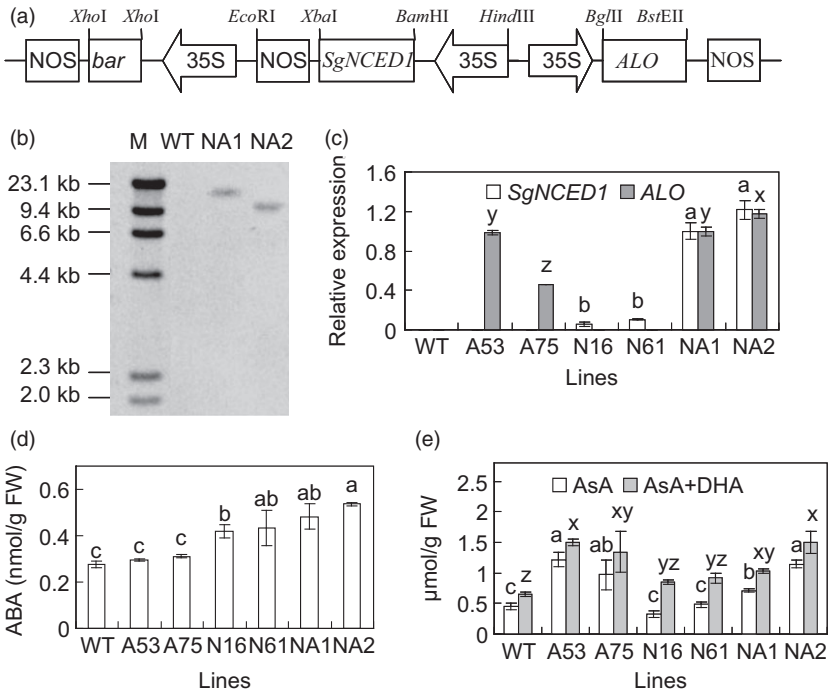

Figure 1 Map of the construct (pZG103-*SgNCED1-ALO*) driven by CaMV 35S (a) and analysis of transgenic tobacco plants overexpressing *ALO* (A53 and A75) and *SgNCED1* (N16 and N61) or co-expressing *SgNCED1* and *ALO* (NA1 and NA2) in comparison with the wild-type control (WT). Fifteen micrograms of DNA was digested with *Eco*RI for DNA hybridization using digoxigenin labelled *bar* as probe (b). Relative expression of *SgNCED1* and *ALO* was measured by quantitative RT-PCR (c). Abscisic acid (d) and ascorbic acid (e) were measured using enzyme-linked immunosorbent assay and HPLC, respectively. Means of three repeats and standard errors are presented; the same letter above the column indicates no significant difference at $P < 0.05$.

observed in the transgenic lines A53, A75, NA1 and NA3 than in the wild type after chilling treatment (Figure 3b,c). The results indicated that chilling tolerance was increased in transgenic plants overexpressing *ALO* or co-expressing *SgNCED1* and *ALO*, but not affected in transgenic plants overexpressing *SgNCED1* alone.

Analysis of transgenic stylo plants co-expressing *SgNCED1* and *ALO*

Our preliminary experiment data had shown that callus could not been induced when cotyledons of stylo were placed on callus induction medium containing 0.6 mg/L of basta for 5 weeks (data not shown). Thus, 0.6 mg/L of basta was used as selection pressure for transformants in this study. Transgenic stylo plants co-expressing *SgNCED1* and *ALO* genes were further generated using cotyledons from stylized 1-week-old seedlings as explants and *bar* as selective marker gene. After the regenerated plants were analysed by PCR (data not shown), PCR-positive plants were further analysed by DNA blot hybridization. The data showed that *bar* gene was integrated into the genomes of the 15 transgenic plants; among them, 11 plants had one copy of transgene, three plants had two copies, and one plant had four copies, whereas no cross-hybridization was observed in the wild type (Figure 4a). Real-time quantitative RT-PCR data showed that *SgNCED1* and *ALO* transcripts could be detected in most of the transgenic lines (Figure 4b). Three lines (2, 15 and 17) were selected for the analysis of ABA and AsA levels. Levels of ABA, AsA and total AsA (AsA + DHA) were all significantly increased in transgenic lines as compared to the wild type. Compared to the wild type, 2.31- to 3.38-fold higher levels of AsA were observed in transgenic lines (Figure 5).

Evaluation on tolerance to drought and chilling in transgenic stylo plants

Compared to the wild type, transgenic stylo plants had higher levels of F_v/F_m after 5 days of chilling stress at 6 °C (Figure 6a). In consistence, lower levels of ion leakage and MDA were observed in transgenic plants than in the wild type after chilling treatment (Figure 6b,c). Transgenic plants were survived after 5 days of recovery at room temperature, when most of the wild-type plants were died (Figure 6d). The results suggest that transgenic plants had increased chilling tolerance.

Drought tolerance was also assessed by measuring RWC, ion leakage and MDA. RWC and ion leakage were at about 95% and 5%, respectively, in stylo leaves under normal growth conditions (Zhou *et al.*, 2005a). Relative water content was decreased to 21.4% in the wild type, while 45.6 to 55.7% RWC was maintained in transgenic plants after 7 days of withholding irrigation (Figure 7a). In addition, lower levels of ion leakage and MDA were observed in transgenic plants than in the wild type (Figure 7b,c). In contrast to the wild-type plants which could not survive after the drought treatment, most of the transgenic plants survived 5 days after recovery by resupplying water (Figure 7d). The results suggest that transgenic plants had increased drought tolerance.

Analysis of antioxidant enzyme activities and expression of abiotic stress response genes in transgenic tobacco and stylo plants

Compared to those in the wild type, SOD, CAT and APX activities were not altered in *ALO* transgenic tobacco plants, while they were increased in transgenic tobacco overexpressing *SgNCED1* or co-expressing *SgNCED1* and *ALO* (Figure 8a–c). Similarly, higher levels of SOD, CAT and APX activities were also observed in transgenic stylo plants than in the wild type (Figure 8d–f).

Eight abiotic stress-responsive genes were analysed in transgenic lines in comparison with the wild type. *P5CS1* (encoding delta-pyrroline-5-C synthase), *TIP* (encoding tonoplast intrinsic protein) and *ERD10B* (encoding dehydrin) are drought- and ABA-responsive genes (Yamaguchi-Shinozaki and Shinozaki, 2006). Their transcript levels were higher in transgenic plants expressing *SgNCED1* or co-expressing *SgNCED1* and *ALO* than in the wild

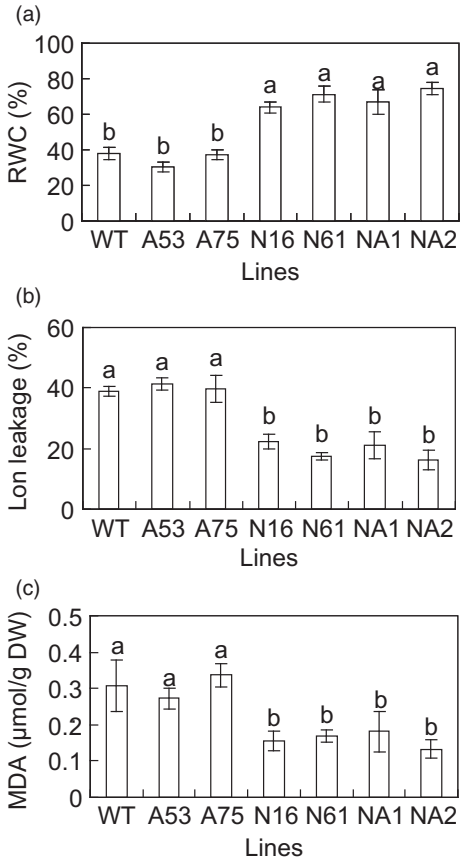

Figure 2 Assessment of drought tolerance in transgenic tobacco in comparison with the wild-type plants (WT). Relative water content (RWC, a), ion leakage (b), and malondialdehyde (MDA, c) were measured when WT plants showed wilting after withholding irrigation. Means of three independent samples and standard errors are presented. The same letter above the columns indicates no significant difference at $P < 0.05$.

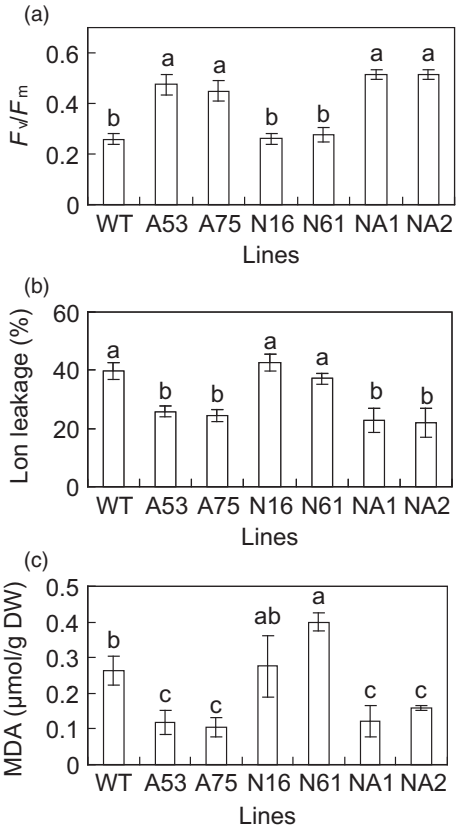

Figure 3 Assessment of chilling tolerance in transgenic tobacco in comparison with the wild-type plants. The maximum photochemical efficiency (F_v/F_m, a), ion leakage (b), and malondialdehyde (MDA, c) were measured after 3 days of chilling treatment at 3 °C. Means of three independent samples and standard errors are presented. The same letter above the columns indicates no significant difference at $P < 0.05$.

type, while *NtP5CS* transcript level was lower and *NtTIP* and *NtERD10B* levels showed no significant difference in transgenic plants expressing *ALO* as compared to the wild type (Figure 9a–c), indicating that these genes were induced by ABA as a result of expression of *SgNCED1*. *NtCOR15a*, *NtDREB1*, *NtDREB2*, *NtDREB3* and *NtDREB4* are cold-responsive genes. NtDREB1, 2, 3 and 4 belong to C-repeat binding factor (CBFs)/DREB1s as they had the conserved PKKP/RAGR×KF×ETRHP and DSAWR, two signature sequences of CBFs located immediately upstream and downstream of the AP2 domain, respectively (He *et al.*, 2015). Compared to the higher levels of *NtCOR15a* in all transgenic plants (Figure 9d), levels of *NtDREB1*, *NtDREB2*, *NtDREB3* and *NtDREB4* transcripts were higher in transgenic plants expressing *ALO* or co-expressing *SgNCED1* and *ALO* than in the wild type, but they were not altered in *SgNCED1* transgenic plants (Figure 9e–h). The results indicate that expression of *NtCOR15a*, *NtDREB1*, *NtDREB2*, *NtDREB3* and *NtDREB4* was induced by AsA as a result of expression of *ALO*.

Discussion

Up-regulation of ABA synthesis by overexpressing *NCED* gene usually results in enhanced tolerance to drought and salinity in transgenic plants through induced stomata closure and antioxidant enzyme activity (Iuchi *et al.*, 2001; Qin and Zeevaart, 2002; Zhang *et al.*, 2008, 2009). It has been well documented that antioxidant protection system plays an important role in drought and chilling tolerance (Guo *et al.*, 2006; Lu *et al.*, 2013). Abscisic acid levels and drought tolerance were increased in transgenic tobacco plants overexpressing *SgNCED1* or co-expressing *SgNCED1* and *ALO* genes. Activities of SOD, CAT and APX and transcript levels of *NtP5CS1*, *NtTIP* and *NtERD10B*, ABA- and drought-responsive genes were also increased in the above two types of transgenic tobacco, but not in *ALO* transgenic tobacco. The results indicated that the increased activities were associated with elevated ABA levels as a result of expression of *SgNCED1* gene that induces expression of antioxidant enzyme-encoding genes (Zhang *et al.*, 2009) and other ABA-responsive genes. Nevertheless, the ABA-induced antioxidant enzyme activities and expression of ABA-responsive genes were associated with the elevated drought tolerance in transgenic plants overexpressing *ALO* or co-expressing *SgNCED1* and *ALO* genes. However, chilling tolerance was not altered in *SgNCED1* transgenic tobacco plants, and expression of some cold-responsive genes, such as *NtDREB1, 2, 3* and *4*, was neither altered. On the contrary, exogenous application of ABA increases chilling tolerance of stylo with induced activities of SOD, CAT, APX and GR (Zhou *et al.*, 2005a). The differential effect of ABA on chilling tolerance in tobacco and stylo might be resulted from the difference in sensitivity to ABA among plant species.

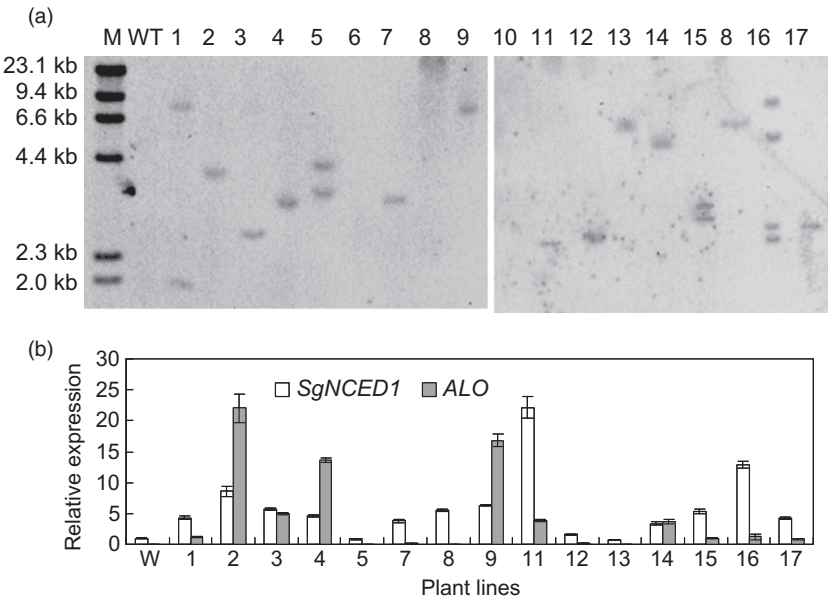

Figure 4 Analysis of transgenic stylo plants co-expressing *SgNCED1* and *ALO* in comparison with the wild-type control (WT). Fifteen micrograms of DNA was digested with *Eco*RI for DNA hybridization using digoxigenin labelled *bar* as probe (a). Relative expression of *SgNCED1* and *ALO* was measured using quantitative RT-PCR (b).

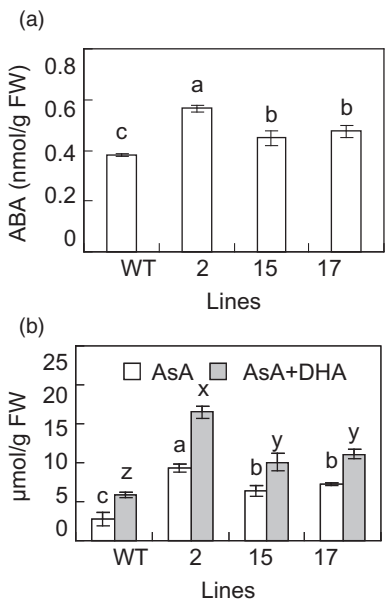

Figure 5 Abscisic acid (ABA) and ascorbic acid (AsA) levels of transgenic stylo plants co-expressing *SgNCED1* and *ALO* (lines 2, 15 and 17) in comparison with the wild-type control (WT). ABA (a) and AsA (b) were measured using enzyme-linked immunosorbent assay and HPLC, respectively. Means of three repeats and standard errors are presented; the same letter above the column indicates no significant difference at $P < 0.05$.

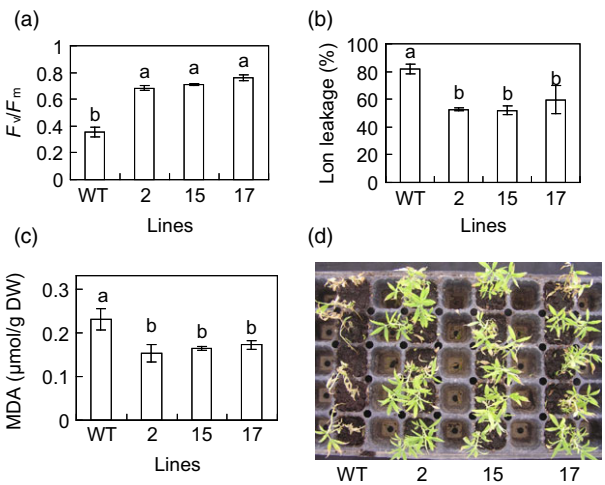

Figure 6 Assessment of chilling tolerance in transgenic stylo in comparison with the wild-type (WT) plants. The maximum photochemical efficiency (F_v/F_m, a), ion leakage (b), and malondialdehyde (MDA, c) were measured after 3 days of chilling treatment at 3 °C. (d) Photography was taken 5 days after recovery at room temperature. Means of three independent samples and standard errors are presented. The same letter above the columns indicates no significant difference at $P < 0.05$.

An elevated AsA levels and chilling tolerance were observed in transgenic tobacco plants overexpressing *ALO* or co-expressing *SgNCED1* and *ALO* genes in this study, but not in *SgNCED1* transgenic tobacco, indicating that the increased chilling tolerance was associated with elevated AsA levels due to the expression of *ALO* gene. In addition, expression of *NtCOR15a* and four *NtDREB* genes which belong to *CBFs/DREB1s* type (He *et al.*, 2015) was induced by AsA as a result of expression of *ALO*. It is interesting that AsA up-regulates expression of cold-responsive genes apart from being an antioxidant. Many defence genes have been found to be up-regulated by AsA deficiency in *vtc1* mutant of Arabidopsis (Pastori *et al.*, 2003). The results revealed that AsA plays an important role in chilling tolerance. Multiple abiotic stresses induce the production of ROS and lead to oxidative damage to plant cells. Ascorbic acid is an abundant antioxidant in plant cell and functions to scavenging to avoid accumulation of ROS under stress conditions (Foyer and Noctor, 2009; Gill and Tuteja, 2010). Exogenous application of AsA increases tolerance to drought, chilling, salinity and Al-induced oxidative damages in rice and tomato seedlings (Guo *et al.*, 2005; Shatlata and Neumann, 2001). Up-regulation of AsA synthesis results in enhanced tolerance to salinity, high light- and

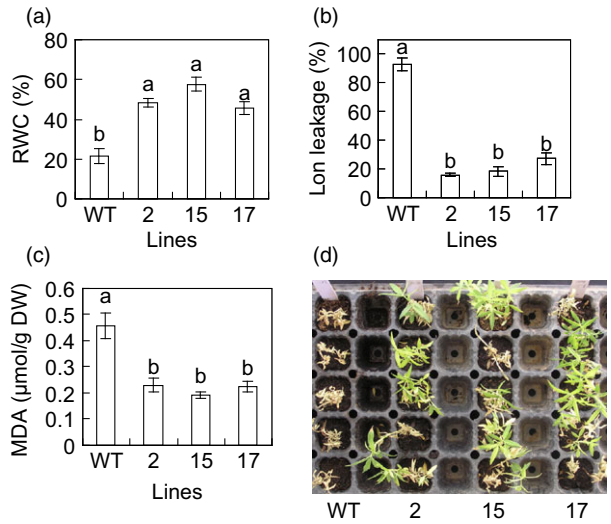

Figure 7 Assessment of drought tolerance in transgenic stylo in comparison with the wild-type (WT) plants. Relative water content (RWC, a), ion leakage (b), and malondialdehyde (MDA, c) were measured when WT plants showed wilting after withholding irrigation. (d) Photography was taken 5 days after recovery under irrigation conditions. Means of three independent samples and standard errors are presented. The same letter above the columns indicates no significant difference at $P < 0.05$.

MV-induced oxidative stress and aluminium toxicity (Chen *et al.*, 2014; Liu *et al.*, 2013). However, drought tolerance was not altered in *ALO* transgenic plants, which might be resulted from that the elevated AsA levels were not high enough to increase drought tolerance in *ALO* transgenic tobacco, or expression of some drought- and ABA-responsive genes, such as *NtP5CS*, *NtTIP* and *NtERD10B,* was not altered or even down-regulated by AsA.

Based on the report that *bar* is an effective selection marker (Sarra *et al.*, 1994), transgenic stylo plants co-expressing *SgNCED1* and *ALO* genes were obtained in this study using basta as selection pressure. Similar to the transgenic tobacco, transgenic stylo plants showed elevated ABA and AsA levels and tolerance to drought and chilling with induced antioxidant enzyme activities. In addition, AsA is critical to the effectiveness of the immune system in domestic animals. The antioxidant properties of AsA may enhance immune function in dairy cows (MacLeod *et al.*, 2003) and may also impart improved quality to beef (Yin *et al.*, 1993). Our result suggests that co-expression of *SgNCED1* and *ALO* genes is an effective way for use in forage plant improvement for increased tolerance to drought and chilling and nutrition quality.

Material and methods

Generation of transgenic plants

The construct pZG103-*SgNCED1-ALO* (Figure 1a) was formed using pCAMBIA3301 as basic plasmid, in which coding sequence of *SgNCED1* (Zhang *et al.*, 2008) driven by CaMV 35S promoter with a NOS terminator was inserted, while β-glucuronidase gene (*uidA*) was replaced by coding sequence of *ALO* (Chen *et al.*, 2014). Transgenic tobacco plants were generated as previously described (Zhang *et al.*, 2008), but using *Agrobacterium tumefaciens* strain EHA105 harbouring the construct pZG103-*SgNCED1-ALO* and 0.6 mg/L basta instead of kanamycin for selection. For transformation of stylo, seeds of a chilling tolerant

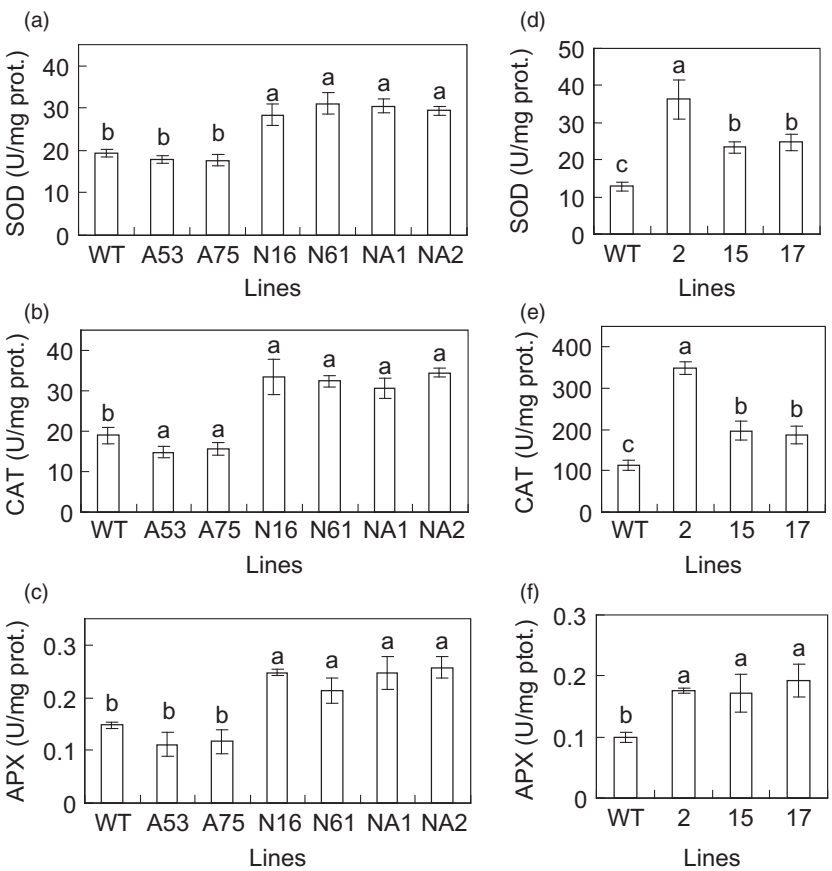

Figure 8 Analysis of antioxidant enzyme activities in transgenic tobacco (a–c) and stylo plants (d–f). Enzyme activities were measured from plant leaves under normal growth conditions. Means of three independent samples and standard errors are presented. The same letter above the columns indicates no significant difference at $P < 0.05$.

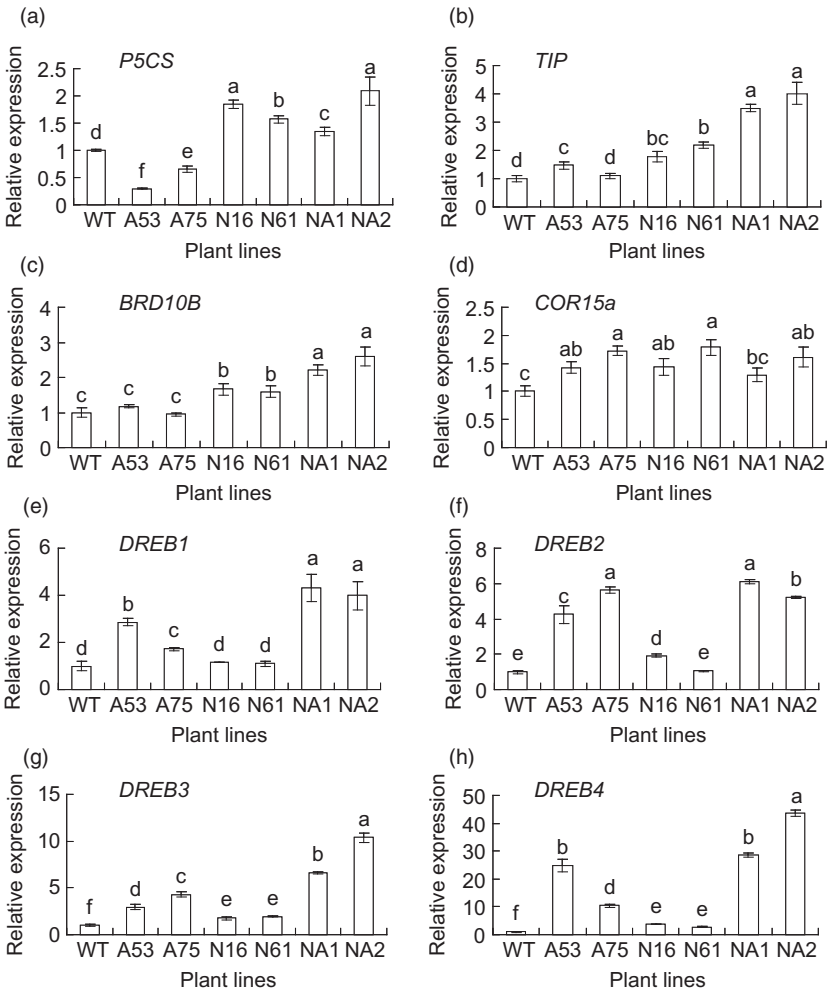

Figure 9 Analysis of transcript levels of *NtP5CS* (a), *NtTIP* (b), *NtERD10B* (c), *NtCOR15a* (d), *NtDREB1* (e), *NtDREB2* (f), *NtDREB3* (g), *NtDREB4* (h) in the transgenic tobacco plants in comparison with the wild-type control (WT). Relative expression was measured using qRT-PCR using *actin* was as a reference gene. The same letter above the columns indicates no significant difference at $P < 0.05$.

mutant line 4-1-3-8 (Lu *et al.*, 2013) were incubated in heat water (80 °C) for 3 min and then were sterilized for 30 min in 3.5% (v/v) aqueous solution of sodium hypochlorite, followed by five rinses with sterile, distilled water. After germinated for 7–10 days on 1/2 strength MS medium without phytohormones, cotyledons were cut into two parts and were incubated in suspension cells of *A. tumefaciens* strain EHA105 harbouring the construct pZG103-*SgNCED1-ALO* for 10 min with gentle shaking. Excess bacteria were removed with sterilized paper towel after the incubation; the explants were transferred onto solid coculti-vation medium containing MS salts, sucrose (30 g/L), α-naphthylacetic acid (NAA, 0.5 mg/L) and 6-benzylaminopurine (BAP, 2 mg/L) and placed in the dark at 25 °C for 3 days. The infected explants were then transferred onto the callus induction medium containing MS salts, sucrose (30 g/L), agar (6.5 g/L), NAA (0.5 mg/L), BAP (2 mg/L), cefazolin sodium (200 mg/L), carbenicillin (200 mg/L) and agar (6.5 g/L), pH 5.8, for 1 week of recovery. The explants were transferred onto selection medium containing MS salts, sucrose (30 g/L), agar (6.5 g/L), NAA (0.5 mg/L), BAP (2 mg/L), cefazolin sodium (200 mg/L), carbenicillin (200 mg/L), basta (0.6 mg/L) and agar (6.5 g/L), pH 5.8, for 5 weeks of selection. Basta-resistant calli were transferred onto regeneration medium containing MS salts, sucrose (30 g/L), agar (6.5 g/L), BAP (2 mg/L), cefazolin sodium (200 mg/L), carbenicillin (200 mg/L), basta (0.6 mg/L) and agar (6.5 g/L), pH 5.8, for regeneration at 25 °C under light of 80 μmol/m^2/s with 16/8-h (day/night) photoperiod for 8–10 weeks. The regenerated plantlets were transferred onto rooting medium (phytohormone-free MS basal medium) for rooting. The rooted plants were subsequently transplanted to soil in plastic pots and grown in greenhouse. The positive transgenic plants (T_0) were selected from the regenerated basta-resistant plants using PCR, with primers ZG1409 (CAGCTGCCAGAAACCCACGT) and ZG1410 (CTGCACCATCGTCAACCACT), and DNA blot hybridization and allowed to harvest seeds. By selection with resistance to basta (60 mg/L) at seedlings stage of T_1 and T_2 plants, homozygous transgenic lines were harvested for investigation.

Real-time quantitative RT-PCR

Total RNA was isolated from 0.1 g leaves using HiPure Plant RNA Mini Kit (Magen, Guangzhou, China) according to the manufacturer's protocol. First-strand cDNA was synthesized from 1 μg of total RNA, using the PrimeScript RT reagent Kit with gDNA Eraser (Takara Bio Inc., Dalian, China). PCR solutions (10 μL) contained 15 ng of cDNA, 200 nM each for forward and reverse primers and 5 μL SYBR Premix *Ex Taq* (Takara). Real-time quantitative RT-PCR (qRT-PCR) was conducted in Mini Option real-time PCR System (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. A negative control without cDNA template was always included. Three technical and two biological replicates were performed in each experiment. Parallel reactions to amplify *actin1* were used to normalize the amount of template. Primers

used for qRT-PCR were listed in Table S1. All primers were designed using the software tool Beacon Designer (Premier Biosoft International, Palo Alto, CA, USA). The primer specificity was validated by melting profiles, showing a single product-specific melting temperature.

DNA blot hybridization

Genomic DNA was extracted from 1 g of leaves using the hexadecyltrimethylammonium bromide method. After digested with *EcoR*I overnight, DNA samples (10 μg) were separated by electrophoresis on 0.8% agarose gel, followed by transferring to Hybond XL nylon membrane (Amersham; GE Healthcare Limited, Buckinghamshire, UK). DNA probe specific to *bar* gene was labelled using a PCR digoxigenin probe synthesis kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's protocol. After hybridization, the DNA filter was washed sequentially with 2× SSC, 0.1% SDS; 1× SSC, 0.1% SDS for 10 min at room temperature; and 0.5× SSC, 0.1% SDS for 15 min at 65 °C. Detection of the signals was carried out using a Lumivision PRO (TAITEC, Saitama, Japan).

Measurements of ABA and AsA

Abscisic acid was determined using enzyme-linked immunosorbent assay kit (made by the China Agricultural University), following the manufacturer's instruction as described previously (Yang and Guo, 2007). Ascorbic acid was determined by HPLC system (Model HP1100; Alltech Associates, Inc., Deerfield, Illinois) as described previously (Chen *et al.*, 2014). Leaves (0.2 g) were extracted in 2 mL of 10% trichloroacetic acid. The homogenates were centrifuged at 15 000 ***g*** for 15 min. The supernatants (20 μL) were passed through TSK-Gel C_{18} column (4.5 × 250 mm) and eluted with 0.1% H_2SO_4 at a rate of 0.8 mL min^{-1} at room temperature. Detection was performed at 240 nm. One dominant peak at the same retention as the standard AsA was calculated.

Assessment of abiotic stress tolerance

Seeds of the homozygous transgenic tobacco (T_3) and stylo (T_2) in comparison with the wild-type plants were sown in 50 pot containing a mixture of peat and perlite (3:1, v/v) in a greenhouse at temperatures of 30/25 °C (day/night) under light of 800 μmol/m^2/s. One tobacco plant was kept in one pot and grown for 2 months, while two stylo plants were kept in each pot and allowed to grow for 1 month. For drought treatment, plants were continuously withheld irrigation until the wild-type plants showed serious wilting. For chilling treatment, plants were moved to a growth chamber under 800 μmol/m^2/s at 3 °C for 3 days (for tobacco) or 6 °C for 5 days (for stylo). The third leaf from the top was sampled and used to measure relative water content (RWC), ion leakage, MDA and F_v/F_m as described previously (Guo *et al.*, 2006; Lu *et al.*, 2013). Photography was taken 5 days after resuming irrigation or recovery at room temperature.

Determination of antioxidant enzyme activity

The third leaf (0.2 g) from the top was sampled from 2-month tobacco seedlings which were ground in 5 mL of 50 mM phosphate buffer solution (pH 7.8) for extraction of SOD and CAT, or in 5 mL of 50 mM phosphate buffer (pH 7.0, containing 1 mM AsA and 1 mM EDTA) for extraction of APX (Guo *et al.*, 2006). After centrifugation at 13 000 ***g*** for 15 min, the supernatants were recovered for determinations of SOD, CAT and APX as previously described (Guo *et al.*, 2006). One unit of SOD activity was defined as the amount of enzyme required for the inhibition of photochemical reduction of p-nitro blue tetrazolium chloride (NBT) by 50%. One unit of CAT and APX was defined as the amount of enzyme required for catalyszing the conversion of one μmol H_2O_2 (extinction coefficient 0.0394 mM^{-1}/cm) or AsA (extinction coefficient 2.8 mM^{-1}/cm) within 1 min. Protein contents in the enzyme extracts were determined using Coomassie brilliant blue G-250 (Bradford, 1976).

Statistical analysis

All the measurements were repeated three times from different individual plants. All data were subjected to analysis of variances according to the model for completely randomized design using an SPSS programme (SPSS Inc, Chicago, IL, USA). Differences among means of treatments or plant lines were evaluated by Duncan's test at 0.05 probability level.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Table S1 Primer sequences used for quantitative RT-PCR and the accession numbers of the analysed genes.