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Xylem functionality controlling blossom-end rot incidence in transgenic *ALC::NCED* tomato plants

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ABSTRACT

Fruit susceptibility to the physiological disorder known as blossom-end rot (BER) is an important limitation in tomato production. Abscisic acid (ABA) is known to reduce leaf transpiration, which can enhance plant water use efficiency (WUE), as well as increase fruit xylem functionality, Ca^{2+} uptake and oxidative stress defenses, which has been suggested to reduce BER incidence. However, the role of ABA on most of these factors determining fruit susceptibility to BER remains poorly understood. ABA production is mainly regulated by the expression of *9-cis-epoxycarotenoid dioxygenase* (*NCED*) genes. Manipulation of *NCED* gene expression by the *alcohol inducible* promoter (*ALC*) could be an alternative approach to stimulate ABA production and its beneficial effects on inhibiting BER incidence. The objectives of this study were to use *ALC::NCED* transgenic tomato plants to decrease BER incidence and investigate which mechanisms were involved in BER. In this study, two transgenic tomato lines (1 and 2) were developed with the *ALC::NCED* construct. This construct allows the inducible activation of the *ALC* promoter by treating the plants with ethanol vapor that drives *NCED* expression and ABA synthesis. According to the results, after full bloom, weekly spraying transgenic plants with ethanol (2%) decreased BER incidence in both transgenic lines, compared to the wild type 'New Yorker' plants. The transgenic line 1 had higher *NCED* expression in response to ethanol than the transgenic line 2 and wild type 'New Yorker'. At 15 and 30 days after pollination, transgenic lines 1 and 2 had higher number of functional xylem vessels which helped to increase Ca^{2+} concentration in the distal end of the fruit, compared to the wild type fruit. In response to higher *NCED* expression, WUE and antioxidant content in leaves and fruit were higher in both transgenic lines, compared to the wild type, helping to explain the lower BER incidence. Therefore, our study shows that stimulating *NCED* expression with an inducible system increases the number of functional xylems and Ca^{2+} uptake into the fruit, improving plant WUE and reducing BER incidence.

1. Introduction

Blossom-end rot (BER) is a physiological disorder characterized by a water-soaked tissue evolving into necrotic tissue, caused by a rupture in the cell wall and membranes in the distal part of fleshy fruits, like tomatoes, peppers, and watermelons (Ho et al. 1993). For many years, BER incidence has been known to be related to calcium (Ca^{2+}) concentration and distribution in leaves and fruit, but recently, new studies proposed that other factor could also determine fruit susceptibility to BER, such as the number of functional xylems, Ca^{2+} uptake, and oxidative stress in the fruit (De Freitas et al. 2014, Riboldi et al. 2019).

Water transport, nutrients, and other metabolites move through plants mainly via transpiratory flow from the roots to the aerial parts, driven by high-growth and intense transpiration zones in meristems and leaves (Ho et al. 1993). Developing fruit tend to have higher transpiration rates at early stages of growth and development, when xylems are the most important vessels for fruit sap uptake (Brüggenwirth et al., 2016, Winkler and Knoche, 2021).

Ca^{2+} translocation occurs exclusively via xylem and is determined by transpiration and growth rates of different plant organs (Thor et al. 2019). As Ca^{2+} transport is coordinated by transpiratory flow and carried to the fruit via xylem, it is necessary that its xylem vessels remain functional, mainly in the distal portion of the fruit (De Freitas et al. 2014, Riboldi et al. 2018a), inhibiting BER development.

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During early stages of growth and development, fruit present a higher number of functional xylems, which decreases toward fruit maturation possibly due to the accumulation of substances, such as callose, leading to physical blockage (Grimm et al. 2017), embolism, and loss of the hydrostatic gradient between the peduncle and distal ends of the fruit (Bondada et al. 2005). Indeed, studies have found a decrease in xylem flow in the developing sweet cherry (Winkler et al. 2020), grape, orange, pear (Song et al. 2018), tomato (Riboldi et al. 2018a), and apple (Miqueloto et al. 2014).

The decrease in xylemic flow starts from minor veins in the distal portion of the fruit towards the major bundles at the proximal end, and at maturity, xylem transport is restricted to a small portion of tissue located between the receptacle at the pedicel end and the cavity just proximal to the seeds, making it impossible for water and particularly Ca^{2+} to reach the distal portion of the fruit (Grimm et al. 2017). Furthermore, studies in tomatoes revealed that cultivars with long shaped fruit, like 'San Marzano' and 'Banana Legs', are more prone to lose xylem functionality during fruit growth and have increased BER incidence than round shaped genotypes (Riboldi et al. 2018a; Riboldi et al. 2020). These studies have suggested that the higher the rate of fruit xylem functionality loss, the lower fruit Ca^{2+} uptake and the higher fruit susceptibility to BER.

One approach that has being used to increase and/or maintain higher xylem functionality during fruit growth and development is the application of abscisic acid (ABA) (De Freitas et al. 2014). ABA is a well-studied plant hormone, and its concentration rapidly increases during stress conditions. ABA signaling induces stomata closure, decreasing plant water loss via transpiration, leading to an increased plant water use efficiency (WUE) (Lamarque et al. 2020), in ABA over-expressing lines, which can also help mitigate the negative effects of drought and high temperatures on fruit production (Medrano et al. 2015, Chai et al. 2016).

During plant stress conditions, there is a decrease in auxin and an increase in ABA biosynthesis, inhibiting growth and changing plant morphology, such as increasing the number of xylem vessels to overcome the restrictive environment (Popko et al. 2010). Many genes have been shown to control plant xylem vessel differentiation in response to ABA, including *CELLULOSE SYNTHASE, LACCASE, XYLEM CYSTEINE PEPTIDASE*, as well as genes encoding transcription factors *MYB46, MYB83, VND2, VND3*, and *VND7* (Ramachandran et al. 2021). Thus, ABA plays an important role in controlling xylem differentiation. However, there are no studies identifying genes that could maintain xylem differentiation in the fruit under high ABA biosynthesis.

Although exogenous ABA applications have been shown to increase plant resistance to abiotic stresses, an interesting alternative to external ABA application is the manipulation endogenous ABA concentration within plant tissues. ABA content can be controlled by manipulating expression of genes coding for enzymes involved in ABA biosynthesis, mainly its rate-limiting enzyme 9-cis-epoxycarotenoid dioxygenase (NCED) (Huang et al. 2018). Previous studies had success on controlling *NCED* expression using either a constitutive promoter (Thompson et al. 2000, Qin and Zeevart 2002) or an inducible system, such as the tetracycline (Thompson et al. 2000), dexamethasone (Qin and Zeevart 2002), rbcS3C promoter (Tung et al. 2008), ecdysone receptor (EcR) and methoxyfenozide (MOF) (Martínez-Andújar et al. 2011), stress promoter RD29A and RD29B genes (Estrada-Melo et al. 2015), all of which resulted in higher ABA synthesis, as well as morphological and physiological changes.

However, those approaches present some technical problems to study BER. First, constitutive promoters, like Cauliflower Mosaic virus 35S (CaMV 35S), directs gene expression uniformly in most tissues, cells at all stages of plant growth and development (Dutt et al. 2014), and a varied expression effects result from its interaction with environmental factors (Schnurr and Guerra 2000) and the physiological state of the plant's development (Pretová et al. 2001). In addition, the ability of constitutive promoters to direct high levels of transgene expression can be a limiting factor when temporal and spatial gene expression patterns are required to achieve manipulation of specific plant organs or developmental stages (Dutt et al. 2014).

Inducible promoters, triggered by physical or chemical factors, have been shown to be a powerful tool to regulate the expression of genes at certain stages of plant or tissue development (Gatz and Lenk 1998). In Arabidopsis, an example is *RD29* genes, in which two genes, *RD29A* and *RD29B*, are stress induced, where *RD29A* is induced by drought and cold and *RD29B* by salt stress (Msanne et al. 2011).

However, as discussed above, BER and *NCED* are both induced by drought. Therefore, a new study was necessary to trigger *NCED* gene expression independent on inducing BER directly, such as during drought conditions. Based on those difficulties, different promoters could be used to achieve the best results. The ethanol-inducible system is derived from the fungus *Aspergillus nidulans* in which the alcR gene encodes transcription factor ALCR that controls the activation of several structural genes, such as alcA (Felenbok et al. 1988). In the absence of ethanol, the ALCR protein is inactive, but when ethanol is added, ALCR and ethanol interact to form an activated ALCR, which then binds to the promoter of the target gene such as alcA, inducing the expression of the gene (Tomsett et al. 2004).

This system has been successfully used to control different genes in tobacco, *Arabidopsis*, potato, oilseed rape, tomato, and rice (Caddick et al. 1998, Roslan et al. 2001, Runzhi et al. 2005). As ethanol is less expensive, readily available, non-toxic in moderate amounts and can be easily supplied to the plants, this system is considered to have a great potential for field application (Corrado and Karali 2009).

In this way, the manipulation of *NCED* gene expression using the alcohol-inducible promoter (*ALC*) (Caddick et al. 1998, Roslan et al. 2001, Tomsett et al. 2004, Randall 2021) could be used to stimulate ABA biosynthesis and responses in plants before drought stress occurs in a commercial setting. This approach would allow the control *NCED* expression and subsequent ABA generation during an effective timeframe, triggering preemptive xylem development and functionality, decreasing BER incidence.

Therefore, the objective of this study was to use *ALC::NCED* transgenic tomato plants to manipulate *NCED* expression and ABA biosynthesis in order to increase xylem functionality, fruit Ca^{2+} uptake, as well as improve plant WUE and diminish oxidative stress responses, reducing losses due to BER incidence.

2. Material and methods

2.1. Plant material

This study was carried out with two *ALC::NCED* transgenic lines and wild type 'New Yorker' tomato plants. Transgenic lines were obtained and selected for this experiment using the *ALC::NCED* construct that contains the alcohol (*ALC*) responsive promoter (Roslan et al. 2001), controlling *NCED* gene expression. This gene codes for 9-cis-epoxycarotenoid dioxygenase (NCED), which is a key enzyme responsible for ABA biosynthesis (Estrada-Melo et al. 2015)

Tomato plant transformation was accomplished according to the protocol described by Liang et al. (2014). The 2222 bp coding region of *SlNCED1* cDNA (GenBank Accession No. AJ439079.2) was amplified from tomato (*Solanum lycopersicum* Mill. L. cv. New Yorker) and placed in the pGSA1403 backbone, with the 35s promoter replaced by the *ALC* promoter derived from the *Aspergillus nidulans alcA* gene (Fig. 1) (Caddick et al. 1998; Roslan et al. 2001; Romero et al. 2003). Plasmid vector for binary map *ALC::NCED*, based on pGSA1403, was generated using Serial Cloner v.2.6 (http://serialbasics.free.fr/Serial_Cloner.html). The 3×35s promoter was replaced with the alcA promoter and the complete coding sequence of SlNCED1 from tomato was included. This construct was then used to transform 'New Yorker' tomato plants at the University of California Plant Transformation Facility, Davis, California. Seeds were collected from self-

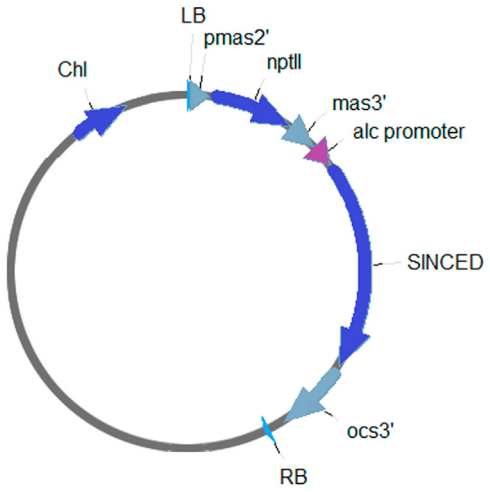

Fig. 1. Plasmid map for binary vector ALC::NCED, based on pGSA1403. The 335s promoter was replaced with the alcA promoter and the complete coding sequence of SlNCED1 from tomato was included.

pollinated transgenic plants and T1 seedlings were selected on MS medium containing 20 mg L^{-1} hygromycin and the presence of the transgene was confirmed by PCR. Two T1 transgenic lines showing the strongest *NCED* expression in response to alcohol induction were selected for further study. These plants were sprayed daily with 2% ethanol solution (the *ALC* inducer) for 10 days and the resulting plant architecture was compared with wild-type plants sprayed with alcohol and H_2O-sprayed transgenic plants. Three mature leaves of each plant middle third were selected for the measurement of leaf size.

Total RNA was extracted from leaf tissues and semi-quantitative RT-qPCR was used to compare the abundance of *NCED* transcripts using primers forward 5′- CAGCTAAAATCCACCATGATAGC-3′ and reverse 5′- AATACCAAATCGGGAAACTTTGT -3′, using the 26S reference gene–Forward AGCTCGTTTGATTCTGATTTCCG, and reverse GATAGGAAGAGCCGACATCGAAGG.

2.2. Growth conditions and plant treatments

The study was carried out in a greenhouse. The tomato seeds were sown in trays with sterile UC soil mix, composed of 33% peat, 33% sand, and 33% redwood compost. Later, the seedlings were transplanted to individual 10 L pots containing the same soil mix. Plants were fertilized everyday with 1% Hoagland nutrient solution. Every 30 days, during growing and fructification time, 10 g of slow release fertilizer was applied in each pot as well, containing: N (43 kg ha^{-1}), P_2O_5 (21.2 kg ha^{-1}), K_2O (32 kg ha^{-1}), MgO (5.3 kg ha^{-1}), S (13.3 kg ha^{-1}), Fe (1.1 kg ha^{-1}), Cu (0.13 kg ha^{-1}), Mn (0.16 kg ha^{-1}), Zn (0.05 kg ha^{-1}), B (0.05 kg ha^{-1}), Mo (0.04 ka ha^{-1}), but without Ca (Osmocote, OH, USA).

At full bloom, 40 days after transplanting, flowers were tagged and manually pollinated. Wild type and transgenic plants were then sprayed weekly (five weeks) with 200 mL of ethanol 2% (v/v) to activate the *ALC* promoter and *NCED* expression. Fruits were harvested and evaluated at 15 days after pollination (DAP), which is the time that BER symptoms develop in the fruit (De Freitas et al. 2014, Riboldi et al. 2019).

BER incidence was determined by the percentage of fruit affected by BER symptoms. Plant and fruit dry weight were determined by drying the samples at 65 °C until constant weight. Plant dry weight was determined at full bloom. Fruit length and diameter were determined using a caliper. Fruit fresh and dry weight were determined using the average fruit weight per plant.

2.3. Gene expression

RNA was extracted from samples with the Trizol Extraction Kit (Invitrogen^{tm}, MA, USA) and total RNA was quantified by spectrophotometry (Nanodrop-Thermo Scientific, DE, USA). Total RNA was then treated with DNase (RNeasy® cleanup kit – QIAGEN, MD, USA) to remove most of the genomic DNA. cDNA synthesis was accomplished using 5 μg of RNA with the iScript^{tm} cDNA Synthesis kit, according to the manufacturer's protocol (BIO-RAD, CA, USA). RT-qPCR analysis was performed on the Applied Biosystems 7300 RT-PCR System (Applied Biosystem, CA, USA). Expression analysis was accomplished using the housekeeping gene *26S*. RT-qPCR reaction was performed using 2xAll-in-One^{TM} qPCR Mix (Applied Biosystems, MA, USA). The 26S primers were forward-AGCTCGTTTGATTCTGATTTCCG, reverse-GATAGGAAGAGCCGACATCGAAGG, whereas the NCED primers were forward-CAGCTAAAATCCACCATGATAGC, reverse-AATACCAAATCGGGAAACTTTGT.

2.4. Xylem functionality

Xylem functionality was determined in developing fruit as previously described by Ho et al. (1993) and Riboldi et al. (2020). Fruits were harvested at 15 days after pollination and 30 DAP and held in sealed plastic bags for 20 min with 100 mL of water to reduce transpiration until the peduncle of each fruit was immersed in a solution of 1% Safranin-O (Sigma-Aldrich, MO, USA) at 20 °C under ≤ 20% relative humidity. After 24 h, fruits were cut into three equal sections at a 90° angle to the peduncle axis. The number of stained vascular bundles (functional xylem vessels) was counted in the placenta and pericarp tissues at the cut surfaces of the blossom-end and peduncle-end regions of each fruit.

2.5. Plant water use efficiency

Every day, for up to 10 days after pollination, water in the soil was monitored using a soil moisture meter DSMM500 (General, Taiwan), repeated 5 times. Plants were irrigated until soil saturation, and the percentage of soil water content was measured early in the morning on the next day. The percentage of water lost, average of 5 replicates, was calculated as the difference of water lost percentage between the fist and the last day of measurement. During the evaluation period, plants were not irrigated to trigger BER. Relative water content was calculated based on plant and fruit dry weight, determined when plants reached maximum height and fruit reached the full red-ripe stage. Plants and fruit were oven dried at 65 °C until constant weight. Relative water content was then calculated as the weight differences between fresh and dry samples, multiplied by 100 and divided by the initial fresh weight. Fruit diameter and length were determined with a digital caliper. The plant water use efficiency was calculated as plant dry weight accumulated from 1 to 15 DAP, divided by the weight of water lost from each plant from 1 to 15 DAP.

2.6. Ca^{2+} analysis

Ca^{2+} analysis was performed in the distal fruit tissue at 15 DAP. Samples were oven dried at 65 °C until constant weight. A total of 500 mg of dry sample was added to 6 mL of nitroperchloric acid, an acid mixture of nitric acid (HNO_3) and perchloric acid ($HClO_4$), in a 3:1 proportion. Digestion was performed in a plaster block at 240 °C with 15 mL of distilled water. Nutrient quantification was performed by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) (Meyer and Keliher 1992) at UC Davis Analytical Laboratory. The results were expressed as g of Ca^{2+} per kg of tissue dry weight.

2.7. Leaf chlorophyll and carotenoids extraction

Fully expanded mature leaves were collected from the lower middle third position on each plant for chlorophyll A and B and carotenoid analyses. Pigments were extracted with an 80% (v/v) aqueous acetone solution. Approximately, 0.1 g of leaves were ground in liquid nitrogen. Then, 10 mL of the acetone solution was added, mixed, and centrifuged for 10 min at 3000 x *g*. Later, 1.5 mL of the supernatant was added to 1.5 mL of acetone solution. Absorbance of each sample was then determined at 663, 646 and 440 nm. Absorbance values at each wavelength were used to determine each pigment content based on the method described by Lichtenthaler (1987).

2.8. Antioxidant capacity

The diphenyl-1-picrylhydrazyl (DPPH) assay was accomplished according to the method described by Brand-Williams et al. (1995), with modifications. Stock solution was prepared by dissolving 20 mg DPPH (Sigma-Aldrich, MO, USA) in 100 mL ethanol (100%). Fruit extracts (300 μL) were allowed to react with 300 μL of DPPH solution and 3.2 mL of pure ethanol for 1 h in the dark. Then, absorbance was taken at 515 nm. Standard curve was linear between 5 and 30 μM of Trolox (Sigma-Aldrich, MO, USA). Results were expressed in Trolox equivalent antioxidant capacity (TEAC) per gram of fresh weight (FW).

2.9. Experimental design

The experiment followed a randomized block design with three treatments (wild type and 2 transgenic lines) containing six blocks and two plants per block. Data were subjected to analysis of variance (ANOVA) and the averages were compared by Tukey test at 5%.

3. Results

Blossom-end rot incidence was markedly different between the wild type 'New Yorker' and the transgenic ALC::NCED lines 1 and 2 (Fig. 2). Wild type plants had 52.6% of BER incidence (Fig. 2), whereas both transgenic lines had less than 20% of BER incidence (Fig. 2). *NCED* gene expression was higher in the transgenic line 1 and 2, after ethanol induction compared to NY (Fig. 3).

There was no difference in xylem functionality in the proximal fruit tissue among genotypes at 15 DAP (Fig. 4). However, xylem functionality in the distal fruit tissue was different between wild type and transgenic lines at 15 DAP (Figs. 4 and 5A). Wild type fruit had a lower density of stained xylems compared to lines 1 and 2. At 30 DAP, line 1 presented a higher number of functional xylems compared to line 2 and NY in the proximal fruit tissue. On the other hand, in the distal fruit tissue lines 1 and 2 presented higher xylem functionality compared to the wild type NY (Figs. 4 and 5B). Fruit number, fresh and dry weights; diameter and length were higher in wild type plants compared to the transgenic lines (Table 1).

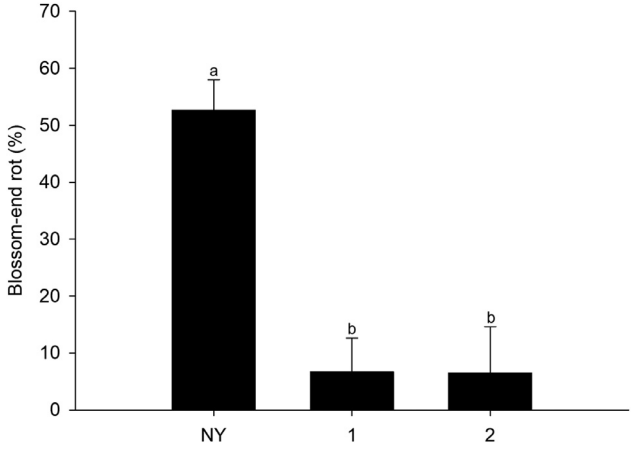

Fig. 2. Blossom-end rot (BER) incidence in wild type 'New Yorker' (NY) and ALC::NCED transgenic tomato lines 1, 2. Wild type (NY) and ALC::NCED transgenic plants were sprayed weekly with 2% ethanol, starting at 40 days after transplanting. Fruit were harvested at 15 DAP. Averages followed by different letters (n=6) are statistically different according to Tukey's test (5%). Data shown mean ± standard deviation.

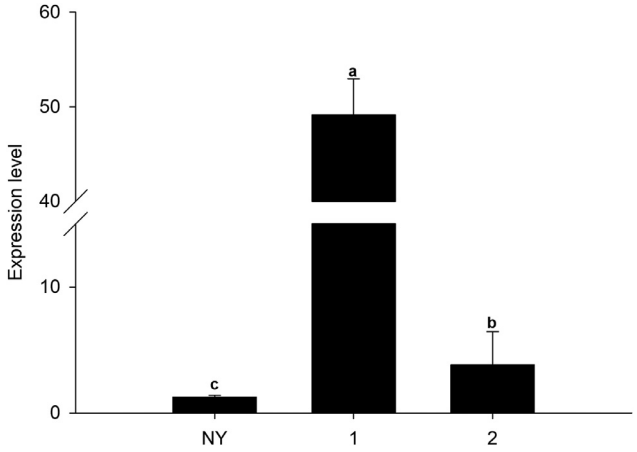

Fig. 3. Relative NCED expression in leaves of wild type 'New Yorker' (NY) and ALC::NCED transgenic tomato lines 1, 2. The data were plotted relative to the wild type (NY) plant and normalized by reference gene 26S. Wild type (NY) and ALC::NCED transgenic plants were sprayed weekly with 2% ethanol, starting at 40 days after transplanting. Fully expanded leaves harvested 1 day after ethanol treatment. Averages followed by different letters (n=6) are statistically different according to Tukey's test (5%). Data shown mean ± standard deviation.

Wild type plants had higher water loss than transgenic lines, which resulted in severe water stress symptoms, reaching almost 90% of soil water lost among the 14 days of water monitoring (Fig. 6A). Both transgenic lines showed 60% of water loss during the evaluation period (Fig. 6A). Transgenic Line 1 plants had higher relative water content than wild type and transgenic line 2 plants (Fig. 6B). There were no differences in plant dry weight among genotypes (Fig. 6C). Transgenic line 1 plants had higher WUE than transgenic line 2 and wild type plants (Fig. 6D).

Ca^{2+} concentration in the distal fruit tissue was higher in transgenic lines 1 and 2 than wild type (Fig. 7). Transgenic lines 1 and 2 showed an average of 12% more Ca^{2+} concentration in the distal fruit tissue, compared to wild type (Fig. 7). Chlorophyll A, chlorophyll B, and carotenoid contents were lower in wild type leaves compared to transgenic lines 1 and 2 leaves (Table 2). Antioxidant capacity in leaves (Fig. 8A) and distal fruit tissues (Fig. 8B) were lower in wild type plants, compared to transgenic line 1 and 2 plants (Figs. 8A and B).

4. Discussion

Abscisic acid is known to be an important signaling molecule involved in many plant responses to stress conditions (Gilroy et al. 2014). ABA biosynthesis is mainly regulated by the increase in *NCED* expression that catalyzes a rate-limiting step in ABA production (Thompson et al. 2000, Gavassi et al. 2021). Indeed, many studies have shown that *NCED* expression is highly correlated to ABA concentrations in petunia (Estrada-Melo et al. 2015), rubber tree (Woraathasin et al. 2021), tomato (Thompson et al. 2000), *Arabidopsis thaliana* (Wan and Li 2006), and tobacco plants (Zhang et al. 2008).

Our study shows a potential molecular approach to manipulate the internal plant *NCED* expression, increasing ABA biosynthesis and leading to higher fruit xylem functionality and Ca^{2+} concentration in distal tissues, as well as improving plant WUE and reducing fruit susceptibility to BER. The *ALC::NCED* transformed plants showed a great

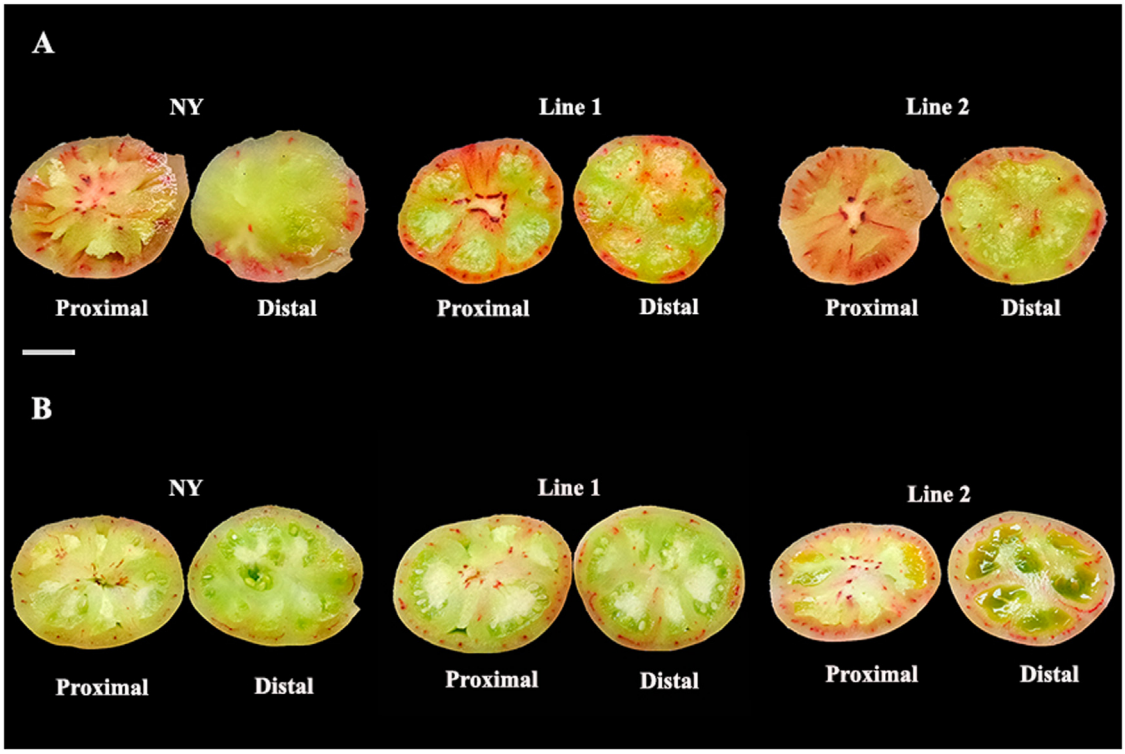

Fig. 4. Functional xylem vessels in the proximal and distal tissues of tomato fruit harvested at–A - 15 DAP and B - 30 DAP from wild type 'New Yorker' (NY) and ALC::NCED transgenic tomato lines 1, 2. Xylem vessels correspond to the red dots. Wild type and ALC::NCED transgenic plants were sprayed weekly with 2% ethanol, starting at 40 days after transplanting. Bar–1 cm.

reduction in fruit susceptibility to BER, when *NCED* gene expression was stimulated by ethanol treatment, compared to wild type plants. Similar ABA responses on decreasing BER incidence have been reported in other studies with external ABA spray treatments (De Freitas et al. 2014, Riboldi et al. 2018b).

4.1. Xylem functionality regulating BER incidence

Abscisic acid has been reported to affect xylem vessels functionality during fruit growth and development (De Freitas et al. 2014). Treating tomato plants with ABA resulted in higher number of functional xylem vessels at later stages of fruit growth and development, favoring higher xylemic water and Ca^{2+} uptake into the fruit. Indeed, our study has also shown that triggering *NCED* expression, and possibly higher ABA biosynthesis in *ALC::NCED* transformed plants, resulted in a higher number of functional xylem vessels at earlier stage (15 DAP) and later stages of growth and development (30 DAP). The higher number of functional xylems in *ALC::NCED* fruit resulted in higher fruit Ca^{2+} uptake and translocation into distal tissues, reducing fruit susceptibility to BER, as suggested in previous studies (Balate et al. 2018, Riboldi et al. 2018b).

Some studies have shown that ABA plays an important role on increasing primary and secondary vascular bundle development (Popko et al. 2010, Campbell et al. 2018, Bloch et al. 2019). Ramachandran et al. (2021) showed that xylem vessel differentiation is increased through protoxylem in response to *VASCULAR-RELATED*

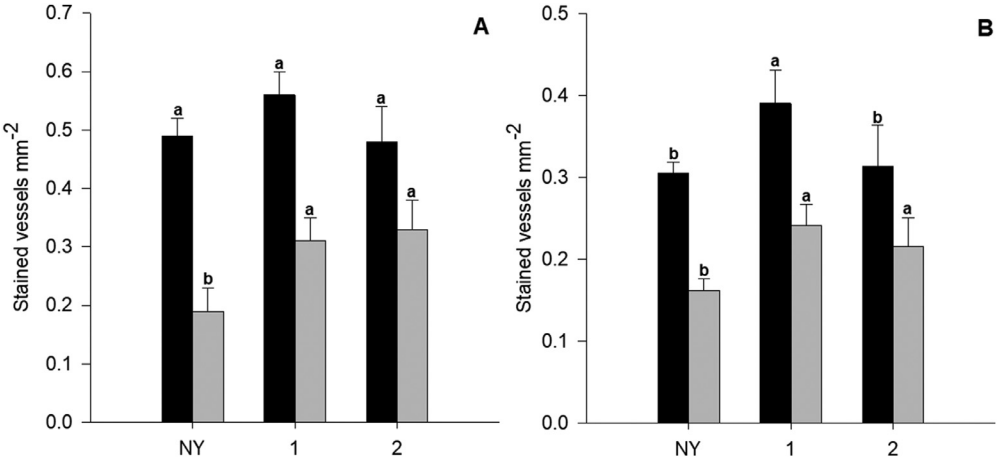

Fig. 5. A - Number of functional xylem vessels in tomato fruit harvested at 15 DAP and B - number of functional xylem vessels in tomato fruit harvested at 30 DAP from wild type 'New Yorker' (NY) and ALC::NCED transgenic tomato lines 1, 2. Wild type and ALC::NCED transgenic plants were weekly sprayed with 2% ethanol, starting at 40 days after transplanting. Black bars correspond to fruit proximal part and grey bars correspond to fruit distal side. Averages followed by different letters (n=6) are statistically different according to Tukey's test (5%), within the same fruit side, proximal and distal. Data shown mean ± standard deviation.

Table 1
Fruit number (FN), fruit fresh (FW) and dry weight (DW), fruit diameter (FD) and fruit length (FL) of wild type 'New Yorker' and ALC::NCED transgenic tomato lines 1, 2. Wild type and ALC::NCED transgenic plants were weekly sprayed with 2% ethanol, starting at 40 days after transplanting. Fruit were harvested at the red-ripe maturity stage.

Lines	Fruit Number	Fresh Weight (g)	Dry weight (g)	Fruit Diameter (mm)	Fruit Length (mm)
NY	15.8±1.2 a	128.1±11.1 a	9.4±1.0 a	65.0±3.1 a	48.2±3.1 a
1	11.3±2.7 b	37.1±2.0 b	2.85±0.1 b	45.3±1.5 b	39.1±1.4 b
2	10±0.6 bc	25.7±2.1 c	2.29±0.3 bc	37.8±1.7 c	33.7±1.1 c

*Averages followed by the same letter (n=6) are statistically different according to Tukey test (5%). Data shown mean ± standard deviation.

NAC DOMAIN (*VND*) transcription factors expression that are activated by ABA treatment in *Arabidopsis thaliana*. Molecular and genetic analyses revealed that the two ABA-mediated xylem developmental changes are regulated by distinct members of this transcription factor family, with *VND2* and *VND3* promoting differentiation of metaxylem cells, while *VND7* promotes the conversion of metaxylem into protoxylem (Ramachandran et al. 2021).

Moreover, higher *NCED* expression in transgenic lines also resulted in different plant and fruit growth and development, compared to the wild type plants. Transgenic plants and fruit grew slower than wild types. In addition, transgenic lines had fewer flowers and more abortion that resulted in lower number of fruit per plant, compared to wild type plants.

The increase in *NCED* expression and ABA biosynthesis have also been suggested to result in higher expression of genes coding for *DELLA* proteins that inhibit plant responses to gibberellins. The ABA inhibition of gibberellin responses could explain the smaller fruit observed in the transgenic tomato lines, considering that gibberellins play an important role on triggering fruit growth (Burbidge et al. 1999, Harrison et al. 2011, Carrera et al. 2012). Indeed, mutant genotypes with repressed *DELLA* expression, such as PROCERA, have bigger plant phenotypes that produce larger fruit and leaves (Carrera et al., 2012). In addition, previous studies have shown that gibberellins increase fruit susceptibility to BER (De Freitas et al. 2017, Gaion et al. 2019). Therefore, triggering *NCED* expression in *ALC::NCED* transformed genotypes possibly decreasing BER incidence by inhibiting plant and/or fruit responses to gibberellins.

4.2. Plant water use efficiency

In our study, triggering *NCED* expression in *ALC::NCED* transgenic plants resulted in a reduction of plant water loss, possibly due to reduced stomatal conductance, which is one of the main plant responses to ABA (Danquah et al., 2014). ABA plays an important role in the plant response to drought by regulating stomatal conductance (Saradadevi et al. 2017) and root hydraulic conductivity (Olaetxea et al. 2015). When applied exogenously, ABA causes rapid stomatal closure and reduces water loss through transpiration (Qin and Zeevaart 1999). However, CO_2 diffusion into leaves becomes limiting to photosynthesis due to stomatal closure (Sorrentino et al. 2016), and this also explains the smaller fruit growth, partially caused by *DELLA* repression, discussed above.

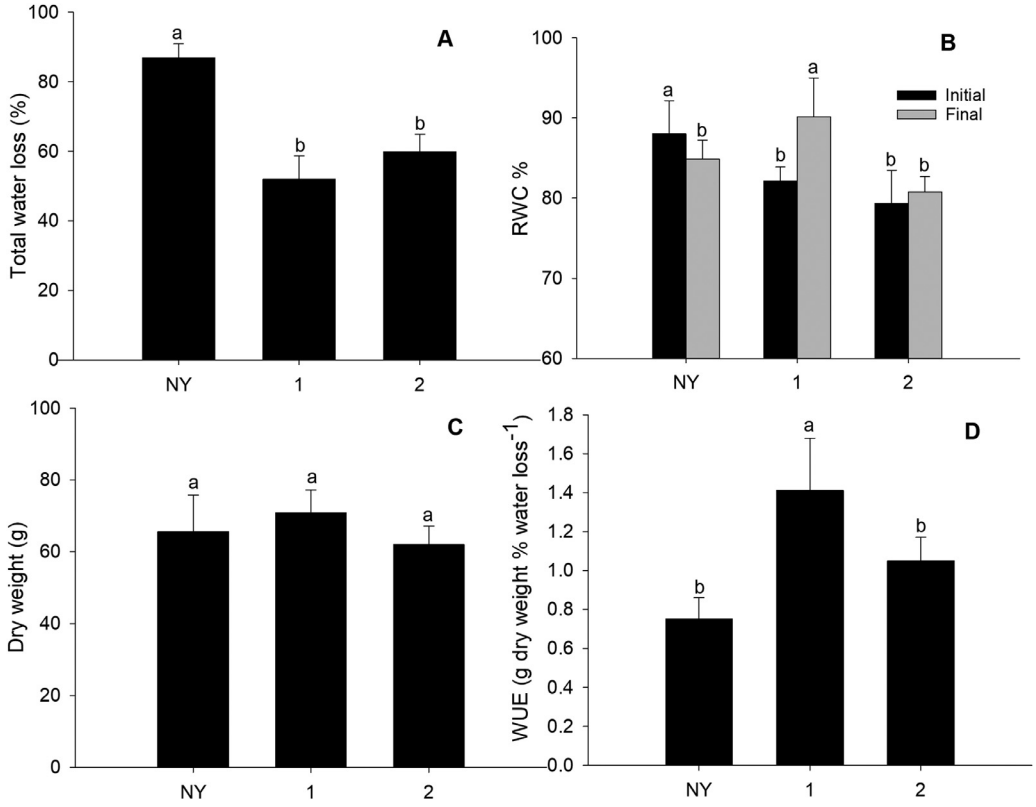

Fig. 6. Percentage of total water loss (A), relative water content (B), plant dry weight (C), and plant water use efficiency (WUE) (D) of wild type 'New Yorker' (NY) and ALC::NCED transgenic tomato lines 1, 2. Wild type and ALC::NCED transgenic plants were weekly sprayed with 2% ethanol, starting at 40 days after transplanting. Analyses were accomplished every day for a total of ten days. Results were normalized, using the first evaluation as a reference for fully hydrated plants and substrate water capacity. Water loss was monitored when plants reached full leaf coverage. Averages followed by different letters (n=6) are statistically different according to Tukey's test (5%). Data shown mean ± standard deviation.

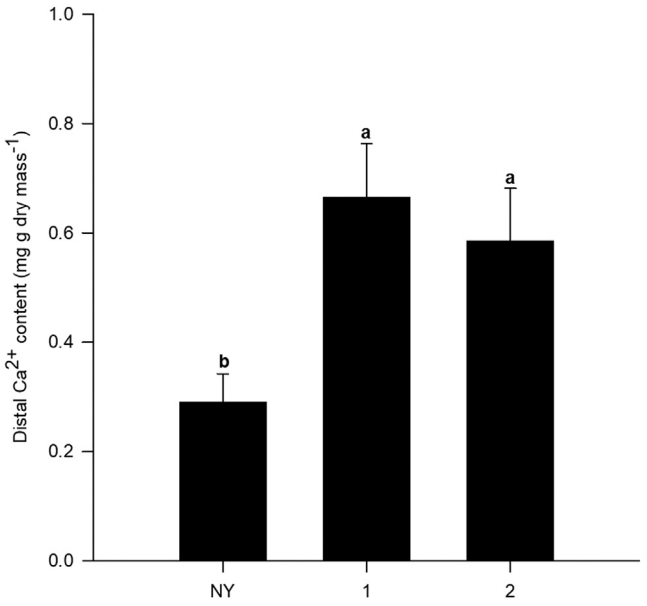

Fig. 7. Ca2+ content in distal portion of wild type 'New Yorker' (NY) and ALC::NCED transgenic tomato lines 1, 2. Wild type and ALC::NCED transgenic plants were weekly sprayed with 2% ethanol, starting at 40 days after transplanting. Fruit were harvested at 15 DAP. Averages followed by different letters (n=6) are statistically different according to Tukey's test (5%). Data shown mean ± standard deviation.

Besides the fact that ABA can increase WUE by changing how plants can use water to produce assimilated carbon, a new study shows that ABA overexpressing tomato lines can also increase xylem embolism under water stress conditions, in response to high ABA levels in plant tissue (Lamarque et al. 2020). However, the causes for the increase vascular embolism are still unclear. Thus, increases in WUE must be seek and analyzed case by case.

Our results show that treating *ALC::NCED* transgenic lines with ethanol might have resulted in higher ABA biosynthesis in these plants, which was not observed in the wild type plants. In addition, triggering *NCED* expression in *ALC::NCED* transgenic plants also increased leaf chlorophyll and carotenoid contents (Barickman et al. 2014), as well as leaf and fruit antioxidant capacity.

Recent studies also show that ABA can play an important role on regulating antioxidant defenses during water stress conditions (Du et al. 2013, Shu et al. 2016, Tao et al. 2020). According to Jiang and Zhang (2002), the accumulation of ABA induced by water stress causes an increase in the levels of ROS, triggering the antioxidant defense system in plants that leads to an increase in the levels of superoxide and hydrogen peroxide radicals, which was followed by an increase in the activity of superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase.

Table 2
Leaf chlorophyll a (Chl A), chlorophyll b (Chl B) and carotenoids (CAR) contents in wild type 'New Yorker' and ALC::NCED transgenic tomato lines 1, 2. Wild type and ALC::NCED transgenic plants were sprayed weekly with 2% ethanol, starting at 40 DAT. Leaves were harvested after ethanol treatment.

Lines	Chl A (mg g^{-1})	Chl B (mg g^{-1})	CAR (mg g^{-1})
NY	0.96 ± 0.21 b	0.32 ± 0.06 b	33.2 ± 0.71 b
1	1.25 ± 0.1 a	0.4 ± 0.03 a	42.5 ± 0.32 a
2	1.41 ± 0.22 a	0.43 ± 0.05 a	44.6 ±0.7 a

*Averages followed by the same letter (n=6) are statistically different according to Tukey test (5%). Data shown mean ± standard deviation.

In our study, the antioxidant capacity was higher in leaf and fruit tissues of *ALC::NCED* transgenic plants treated with ethanol, compared to the wild type. Proteomic and transcriptomic analyses performed in several plant species subjected to different stress combinations have highlighted the importance of the antioxidant defense machinery to avoid cell death and plant tissue damage (Huang et al. 2019, Dumanović et al. 2021). Plants with higher antioxidant capacity and/or lower ROS levels have been shown to have higher tolerance to stress conditions (Suzuki et al. 2014, Rached et al. 2018). Indeed, our results show that higher antioxidant capacity was highly correlated with lower fruit susceptibility to BER in *ALC::NCED* transgenic plants. Calcium is a nutrient that acts as structural compound, binding to pectins and improving the structure and strength of the cell wall (De Freitas et al. 2014), but it is also an essential nutrient, involved in the proper membrane stability and functionality (Melcrová et al. 2016). The symptoms of BER have been suggested to develop in response to membrane damage caused by low apoplastic soluble calcium content and/or oxidative stresses (De Freitas et al. 2017, Riboldi et al. 2019), which also corroborate with our results showing that higher fruit antioxidant capacity due to higher *NCED* expression inhibited BER symptoms development possibly by inhibiting membrane damage caused by high ROS levels.

4.3. *Practical application of* ALC::NCED *system to improve water use efficiency and reduce fruit susceptibility to BER*

Blossom-end rot is a physiological disorder believed to be regulated by the interaction between the genotype and environmental conditions. Under stress, such as drought, heat, salinity, and others, each genotype must have the capacity to trigger morphological, biochemical, physiological, and molecular changes that will maintain proper cell and plant tissue metabolism, avoiding cell death and tissue damage.

According to our study, triggering *NCED* expression in *ALC::NCED* transgenic plants resulted in higher ABA biosynthesis that is supported by the observed lower plant water loss, leading to morphological changes such as lower fruit number, size, weight, and higher functional xylem vessels in the fruit, as well as biochemical changes such as increased chlorophyll and carotenoids contents and antioxidant capacity in leaves and fruit, and physiological changes such as reduced leaf transpiration and improved plant WUE, and possibly molecular changes related to the communication among ABA and other plant hormones that resulted in tomato plant with better performance under stress, using less water to produce fruit with less BER.

The possibility to control plant ABA biosynthesis, using the *ALC::NCED* approach, could enable the maintenance of higher plant ABA levels, promoting a reduction of cultivation costs in regions where irrigation water is limited, as well as turning well irrigated region in more efficient crop production sites. Besides that, this approach could help studies in which ABA is necessary only during certain parts of plant growth and development, in order to avoid negative effects of higher ABA levels, like short size, smaller fruits, floral abortion or even during a specific timing, when it is necessary to maintain the stomata close. In addition, the crosstalk between plant hormones could also be studied since ABA production can be easily manipulated.

Therefore, this technique represents a powerful tool to improve plant WUE and allow for Ca^{2+} to reach the distal end of the fruit, decreasing BER incidence and reducing fruit losses. In addition, more studies must also be conducted, to check if ABA could lead to a higher xylem density and maintenance in fruit tissues, if the VND transcription factor family were highly expressed, and finally if genes related to GA-repressed growth were expressed in response to ABA treatment.

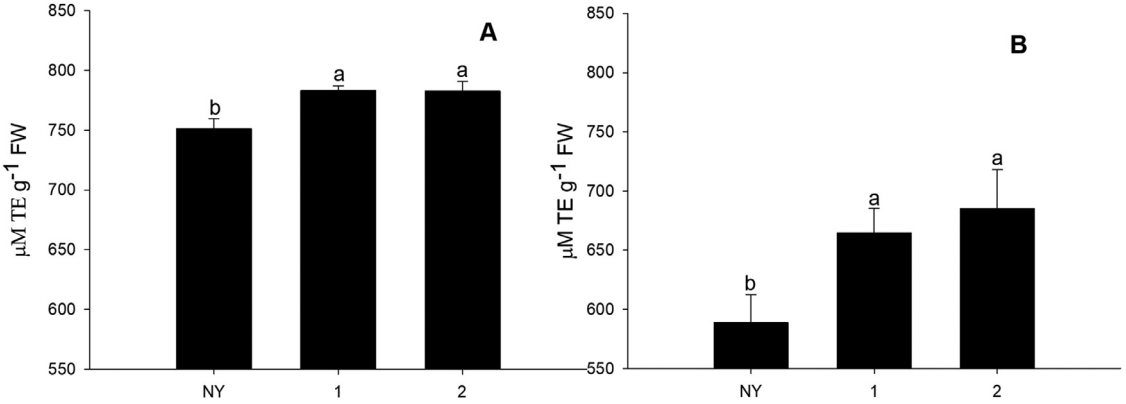

Fig. 8. Antioxidant capacity of fruit (A) and leaf (B) of wild type 'New Yorker' (NY) and ALC::NCED transgenic tomato lines 1, 2. Wild type and ALC::NCED transgenic plants were weekly sprayed with 2% ethanol, starting at 40 days after transplanting. Fruit and leaf were harvested at 15 DAP. Averages followed by different letters (n=6) are statistically different according to Tukey's test (5%). Data shown mean ± standard deviation.

5. Conclusion

Triggering *NCED* expression in *ALC::NCED* genotypes, maintained higher number of fruit functional xylem vessels and Ca^{2+} uptake throughout growth and development, reducing BER incidence.

Author contributions

All authors contributed to the study conception and design. Project administration, formal analysis, data curation and writing - original draft were performed by Lucas Baiochi Riboldi. Ayla Norris assisted in formal analysis, resources and writing - review& editing. Sérgio Tonetto de Freitas and Cai-Zhong Jiang contributed to funding acquisition, conceptualization, resources and writing - review & editing. The first draft of the manuscript was written by Lucas Baiochi Riboldi, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

Authors have no conflicts of interest to declare that are relevant to this article content.

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Further Reading

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