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THE EFFECTS OF DROUGHT ON THE EXPRESSION OF *TAO1*, *NCED* AND *EIL1* GENES AND ABA CONTENT IN TOMATO WILD-TYPE AND *FLACCA* MUTANT

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Abstract- The effects of drought (partial root-zone drying-PRD and regulated deficit irrigation-RDI) and full irrigation (FI) on the expression of ABA biosynthetic genes (*TAO1* and *NCED*), *EIL1* gene and ABA content in the leaves of tomato wild-type (WT) and *flacca* mutant were investigated. Results confirmed differences in the expression of the investigated genes under the investigated treatments, during treatment duration as well as between investigated WT and *flacca* plants. The most significant differences between WT and *flacca* were found under PRD treatment. The similar expression pattern of all genes in the WT plants could indicate synergistic signaling pathways for ABA and ethylene. In *flacca,* reduced *NCED* and significant *EIL1* expression might reflect the increase in ethylene production, which could influence the ABA signaling and production that occurred under PRD. Drought also induced an increase in ABA content that is most expressed in *flacca* under RDI.

Key words: Tomato, partial root-zone drying, regulated deficit irrigation, *TAO1*, *NCED*, *EIL1*, ABA content.

INTRODUCTION

Investigations of the effects of drought on plants are becoming increasingly important because most of the climate change scenarios suggest an increase in temperature and decrease in precipitation in many areas of the world (IPCC, 2007). This could significantly affect the survival of many plants and productivity of agricultural crops. Currently, irrigation is essential for successful production but many areas are facing water scarcity. Water supplies are also under pressure from other users than agricultural and the saving of water resources is becoming of strategic importance for many countries. Therefore, considerable emphasis in plant research is placed on understanding the effects of drought on plants that is essential for improving agricultural managing practice and breeding for drought-resistant genotypes, as well as for predicting the effects of climate change on natural vegetation (Davies et al., 2011).

In the last decades, one of the options to overcome the effects of drought on plants is the use of deficit irrigation methods: regulated deficit irrigation and partial root-zone drying (FAO, 2002). Both methods are based on an understanding of the physiological responses of plants to water supply and water deficit, especially the perception and transduction of root-to-shoot drought signals (Loveys et al., 2004).

Regulated deficit irrigation (RDI) is a method that irrigates the entire root zone with an amount of water less than the potential evapotranspiration during whole or specific periods of the crop cycle (English and Raja, 1996). The principle of the RDI technique is that plant sensitivity to drought is not

constant during the growing season and that intermittent water deficit during specific periods of ontogenesis may increase water savings and improve yield quality. Under partial root-zone drying (PRD) only half of the root zone is irrigated, while the other half is allowed to dry out. The treatment is then reversed, allowing the previously well-watered side of the root system to dry down while fully irrigating the previously dry side. The frequency of the switch is determined according to soil type, genotypes or other factors such as rainfall and temperature. In most of the published data, the PRD cycle includes 10 to 15 days (Davies et al., 2000). Effects of PRD on plant physiology are different from RDI because wet roots under PRD sustain shoot and fruit turgor that are important for plant growth. The drying roots in PRD produce a sufficient amount of the so-called chemical signals (plant hormones, particularly the abscisic acid, xylem pH, ions) that are transported *via* the xylem from roots to shoot to maintain optimal physiological response to water stress (Dodd et al., 2006). Triggering the reduction of shoot growth and partial stomatal closure under PRD irrigation prevents excessive water loss and also the metabolic inhibition of CO_2 assimilation that otherwise would occur in extensive development of drought stress (Chaves et al., 2002).

Many studies have demonstrated significant and beneficial effects of deficit irrigation techniques on increasing water-use efficiency and saving water for irrigation (Costa et al., 2007; Savic et al., 2009; Stikic et al., 2010). At present, research on the signaling mechanisms of drought stress show significant progress that includes the identification of many genes and their functions (Chaves et al., 2003). Surprisingly, despite the intensive research on RDI or PRD effects on plants, little work has been done regarding the genetic background of these methods, although it could significantly contribute to the understanding and exploitation of the beneficial effects of these methods.

The aim of this paper was to assess the expression of *TAO1*, *NCED* and *EIL1* genes and ABA content in the leaves of tomato wild type and *flacca* under drought treatments of RDI and PRD. The investigated genes are involved in the biosynthesis and signaling pathways of abscisic acid (ABA) and ethylene as key hormones in the reactions of plants to drought. The biosynthetic pathway of ABA is well established (Xiong and Zhu, 2003). ABA is synthesized from zeaxanthin, a C40 carotenoid, whose conversion to xanthoxin (the C15 intermediate) is catalyzed by several enzymes, including zeaxanthin epoxidase, neoxanthin synthase and 9-cisepoxycarotenoiddioxygenase (NCED). Xanthoxin is then converted to ABA via the oxidation of xanthoxin to abscisic aldehyde and finally, to ABA. The last step in the conversion of abscisic aldehyde to ABA is catalyzed by *Arabidopsis* aldehyde oxidase 3 (AAO3). Numerous biochemical and genetic evidence have confirmed that NCED is the key regulatory step in ABA biosynthesis (Iuchi et al., 2001). Since *NCED* was first isolated from the maize vp14 mutant, the *NCED* gene has been cloned and characterized in various plant species, including tomato. The investigated *TAO1* gene belongs to a multigene AO family (Ori et al., 1997). There are four *AAO* genes in *Arabidopsis* and one of the known *Arabidopsis* AO genes, *AAO3*, was found to encode an abscisic aldehyde oxidase (AAO). AAO in conjunction with the sulfurylated MoCo form catalyzes the conversion of ABA-aldehyde to ABA (Seo et al., 2000).

The tomato mutant *flacca,* which has been chosen for investigation, is defective in the last step of ABA biosynthesis when abscisic aldehyde is oxidized to form ABA by aldehyde oxidase enzyme (Taylor et al., 1988). As a consequence, the endogenous level of ABA in *flacca* tissues is significantly lower than in wild type (Sagi et al., 1999). There is now substantial evidence showing that ethylene production was greater in ABA-deficient mutants of tomato, and these tomato mutants exhibited the morphological characteristics of excess ethylene, such as leaf epinasty and adventitious rooting (Tal et al., 1979). The results of Sharp et al. (2000) have revealed that an important role of endogenous ABA is to limit ethylene production and that this reduction is required for the maintenance of plant growth.

There is also increasing evidence that the ABA and ethylene signaling pathways have a close interplay in plant growth, development, and stress response (Cheng et al., 2009). The ethylene signal is transmitted via a pathway that includes a transcriptional cascade, and EIN3 has been identified as a critical component within this cascade (Guo and Ecker, 2004). The essential step in the ethylene response pathway is the transduction of ethylene signal into the nucleus to regulate gene expression via the stabilizing of two transcription factors: ethylene-insensitive 3 (EIN3) and ethylene insensitive 3-like1 (EIL1). A product of the *EIL1* gene is the transcription factor in the ethylene signal transduction which may also have an effect on the activity of other genes (Solano et al., 1988). Therefore, in our investigations of gene expression in tomato plants exposed to different stress conditions under PRD and RDI treatments, we also included *EIL1*.

MATERIAL AND METHODS

Tomato plants (*Lycopersicon esculentum* Mill., cv. Ailsa Craig) and *flacca* mutant were raised from seed and transplanted into 20 L pots (one plant per pot) filled with commercial compost (Klasmann Potground H) in a growth chamber (photoperiod was 14h; light intensity at plant level 250 $\mu molm^{-2}s^{-1}$, temperature 26/17°C and relative humidity 60%). For PRD experiments, the pots were specially designed in such a way that they were separated with plastic sheets into two equally sized compartments. Washed roots of the seedlings were divided into approximate halves and repotted into these two hydraulically separated compartments. The compartments were classified as PRD-L (left side) and PRD-R (right side). After transplantation, all the plants were irrigated daily to full-pot holding capacity, volumetric soil water content (θ) of 36%. The θ of both compartments of each pot was measured daily using TDR probes (time domain reflectometer, TRASE, Soil Moisture Equipment Corp., USA).

Ten days after transplantation, the plants were subjected to the following irrigation treatments: 1) full irrigation (FI), in which the whole root system was irrigated daily at 9:00 h to a θ of 36%; 2) deficit irrigation (DI), in which water was evenly applied to the whole root system to reach 15-20% θ of; 3) partial root drying (PRD), where one half of the root was irrigated to reach θ of 36% while the other half was allowed to dry, and the irrigation was shifted when θ of the dry side had decreased to 15-20%.

Leaf samples for analyses of both WT and *flacca* plants' *TAO1*, *NCED* and *EIL1* genes were collected at the beginning and during the experimental period. In total, there were 6 sampling periods including the beginning of treatments (starting point). Samples of leaves under PRD were taken after every PRD shifting period one hour before irrigating (in total 5 sampling). At the same time, samples were collected from FI and RDI treatments.

Leaves for the analyses of genes were harvested from the same third trusses of a single plant. To preserve the genetic material, leaves were immediately transferred to liquid nitrogen and then stored in a refrigerator at a temperature of -80°C. Samples were grounded in liquid nitrogen, and 70 mg of homogenized material was used for the isolation of RNA. Isolation was done by using an RNAeasy Plant Mini Kit (Quiagen, Germany) according to the manufacturer's instructions. Determination of the RNA concentration and quality was done spectrophotometrically by measuring absorbance at 260 nm, and by electrophoretic separation on 1% agarose gels stained with ethidium bromide. For synthesis of cDNA, the First Strand cDNA Synthesis Kit (Fermentas, Lithuania) was used according to the manufacturer's instructions. Gene-specific primers were designed by using the program Primer3 (Metabion International AG, Deutschland). Primer sequences used were: TAO1 (F- CCATAAGAGCAGCACGTG, R- CTTTGGAGAGTCCGAGCA); NCED (F- CTGAAATGATCCGTGGAG, R- CTGCTTCTTCCCAAGCAT) and EIL1 (F- CAGCACTGTGATCCTCCTCA, R- ATCACCGCTGTTAGGACACC). Expression of these genes was monitored by using RT-PCR (Real Time PCR SYBR-Green technique, RT-PCR 7500, Applied Biosystems, UK). Complementary DNA and ampli-

fied products of RT-PCR were checked by electrophoresis on agarose gel.

The samples for ABA measurements were taken at the end of the experimental period. Leaves for analyses were immediately frozen in liquid N and stored at -80°C. Prior to extraction, the samples were ground to a fine powder in pre-chilled steel cylinders by mixer mill. ABA extraction was done using water as a solvent without further purification. Cross-reaction of the antibody with other compounds in the extract was avoided by briefly boiling the plant tissue in water before extraction in a Thermomixer comfort (Eppendorf) at 4°C in a dark room during the night. Measurement of ABA content in the leaves of the investigated plants was done by the ELISA method using a MAC 252 monoclonal antibody for ABA (Quarrie et al., 1988) according to Asch (2000). Plate contents (Nunc: F96 Maxisorp immuno plate) were read at 405 nm by ELISA reader (Sunrise, Tecan).

The statistical analyses for ABA content was done by Student's t test (Sigma Plot 6.0 for Windows - SPW 6.0, Jandel Scientific, Erckhart, Germany), with significance level less than 0.05.

RESULTS AND DISCUSSION

Changes in the soil water content during the FI, RDI and PRD treatments in the course of the experimental period are shown in Fig. 1. Soil water content values (θ) for both wild-type (WT) and *flacca* mutant in FI treatment were kept close to the field capacity (36%), in RDI below 18%, while θ in PRD depended on whether a side was irrigated or not (Fig. 1A and B). The pattern of alternating soil drying and re-watering of the PRD treatment was similar in both tomato genotypes. Differences between investigated genotypes were expressed on a temporal basis under PRD, especially after the third shift when the wet/dry cycle was more rapid in WT (8 days) compared to *flacca* (12 days). In total, 6 shiftings of the dry/wet side were done in the WT plants under PRD treatment, while in *flacca* the number was smaller (5).

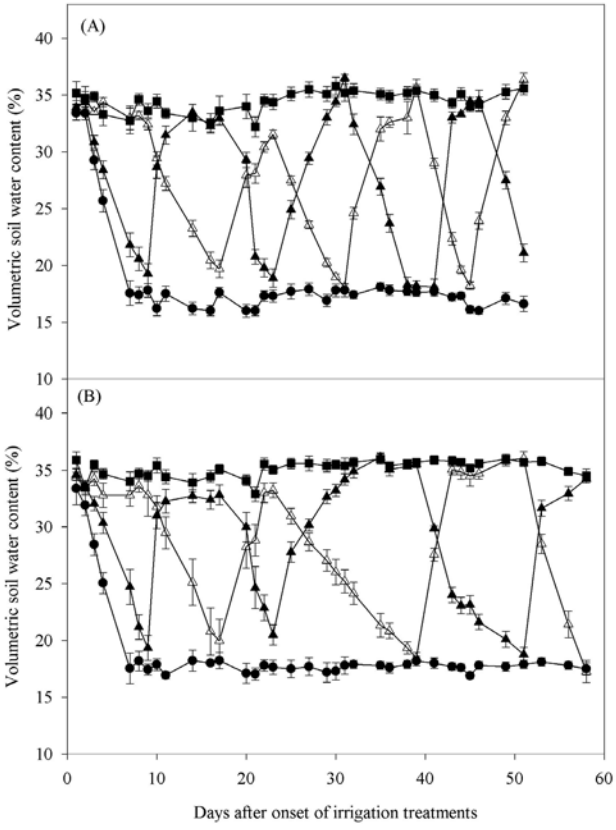

Fig. 1. Changes in volumetric soil water content (%) for full irrigation (FI, ▪), partial root-zone drying (PRD-L, ▲ and PRD-R, Δ) and regulated deficit irrigation (RDI, •) treatments of Ailsa Craig plants (A) and *flacca* mutants (B).

The soil water content data and longer period of drying and re-watering in *flacca* compared to WT suggested that the extraction of water by the *flacca* roots under PRD was smaller than that of the WT plants. The higher water uptake may be the result of the increase in root growth, biomass or root hydraulic conductivity. Our previous results of root biomass showed that the fresh weight of WT roots was significantly bigger in WT plants (22.8 g per plant) compared to *flacca* (9.59 g per plant). Very recently, Hu et al. (2011) demonstrated that PRD plants can compensate for water stress in a drying site by taking up water from the hydrated parts of the root zone where water is available. This might reflect plant acclimation to water distribution in the soil.

Results of the ABA content in the leaves of wild-type and *flacca* plants under FI, PRD and RDI treatments are presented in Table 1. These results show that the mean ABA content in *flacca* in all experimental treatments was significantly lower compared to the WT plants (by about 70%). The results of Sagi et al. (1999) also confirmed the reduced capacity of *flacca* to produce ABA compared to WT. ABA results for both WT and *flacca* also showed differences between treatments and sampling period. In WT plants, PRD had the greatest effect on ABA accumulation (*ca*.1.3 fold), while in *flacca* RDI had (*ca* 2.3 fold). PRD treatment had a similar effect on ABA increase in both genotypes (*ca* 1.3 fold).

Table 1. The content of ABA (ng g FW^{-1}) in the leaves of Ailsa Craig and *flacca* under FI, PRD and RDI treatments. Results are mean values ± S.E.

Genotype	FI	PRD	RDI
Ailsa Craig	338.90 ± 16	435.18 ± 42	376.25 ± 18
flacca	70.76 ± 6	91.98 ± 8	161.04 ± 25

The ABA detected in the leaves may have originated in the roots and been transported via the xylem. It might be the product of a novel biosynthesis in the leaves or the results of recirculation between roots and shoots (Davies et al., 2000). In the majority of PRD experiments, there is a general agreement that xylem ABA should be used as a key chemical signaling molecule. However, our PRD experiments were not designed to measure xylem ABA because of the difficulty in isolating the necessary amounts of xylem sap for analyses from *flacca* leaves. Nevertheless, the results for ABA content increase in drought stress conditions (especially RDI) indicated that we could consider the leaf ABA content as a signal of drought and a reflection of the soil water content available to the plants. Although we did not measure xylem ABA or root ABA, the similar increase of the leaf ABA content in both genotypes under PRD conditions indicates that the *flacca* mutation did not affect ABA transport from the root to the shoot as was shown by Sagi et al. (1993). The discrepancy between our results and Sagi's could be explained by the different degree of stress of the investigated tomato mutants.

Many changes in gene expression occur in response to water deficit and elevation of ABA content (Qin et al., 2011). In our study, water deficit was imposed to determine whether genes known to be included in ABA biosynthesis and the ethylene signal transduction pathway respond to a stress induced by PRD or RDI. In addition to gene expression, the research also included the temporal pattern of gene expression during the investigated treatments.

Our results confirmed the differences in expression of the investigated *TAO1, NCED* and *EIL1* genes under FI, PRD and RDI treatments, during treatment duration as well as between the investigated WT and *flacca* plants.

Results for the *TAO1* gene in WT plants (Fig. 2A) showed a similar temporal pattern under FI and PRD treatments, with maximal expression in the third (PRD) and fourth sampling periods (FI). These sampling periods corresponded to the second and third shifting periods under PRD. The expression of this gene under RDI during almost all investigated periods, especially during the last sampling period (fifth shifting), was smaller compared to the expression under FI or PRD. Our results also confirmed that *TAO1* was expressed in *flacca* leaves after all treatments, but to a lesser extent than in WT (Fig. 2B). Comparison between the investigated treatments showed that the expression of this gene was greater under FI and PRD than under RDI. The temporal pattern of change also differed between treatments, and maximal *TAO1* expression in *flacca* leaves was found under FI after the fourth and fifth shifting periods, while under PRD and RDI this was earlier (during the second shifting period).

The expression of *TAO1* in the leaves of both WT and *flacca* plants confirmed the previously demonstrated tissue-specificity of this gene. Minn et al. (2000) showed that in the leaves of tomato mutants (*flacca* and *syt*) and WT the gene *TAO1* was expressed mainly in vegetative tissues, *TAO2* in both vegeta-

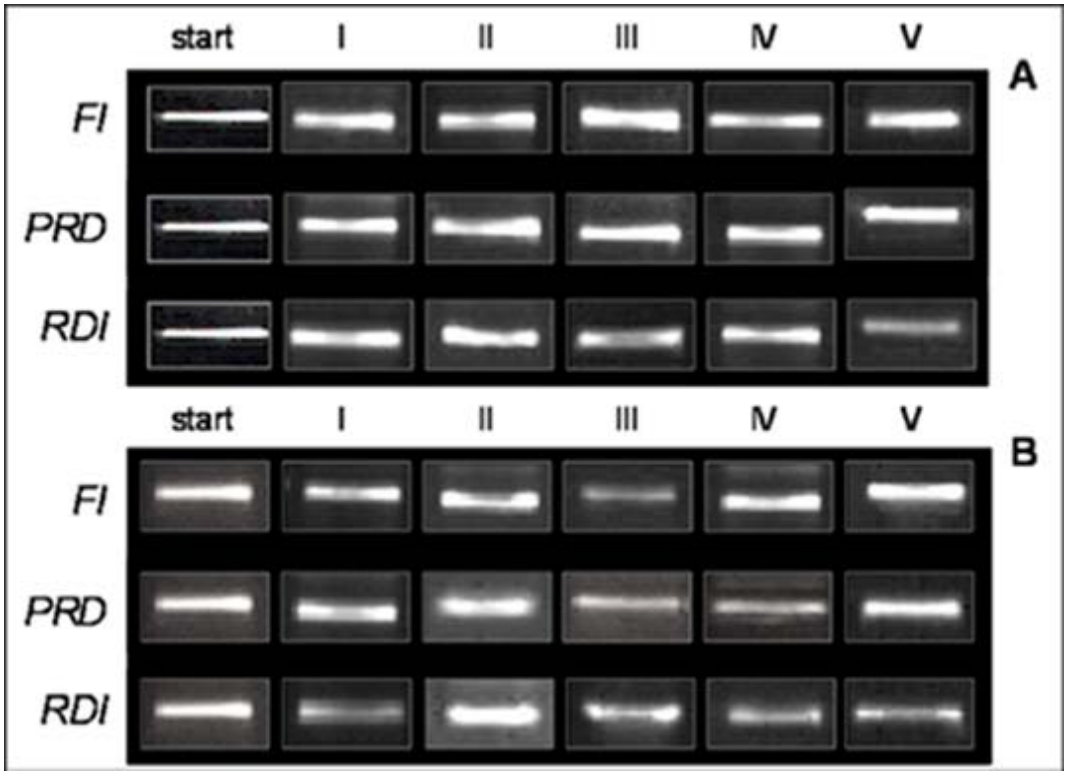

Fig.e 2. Expression patterns of the *TAO1* gene in the leaves of Ailsa Craig (A) and *flacca* (B) under FI, PRD and RDI during six sampling periods.

tive and reproductive tissues, while the expression of *TAO3* was not detectable by Northern hybridization. Within the AO family, the results of Minn et al. (2000) for tomato and similarly, Seo et al. (2000) for *Arabidopsis*, demonstrated the key role of *TAO3* and *AAO3* for ABA biosynthesis. Since the *flacca* mutant showed both expression of the *TAO1* gene and accumulation of ABA in the leaves (Tab. 1), our results indicated that another gene from the TAO family and not *TAO1* (probably *TAO3*) was responsible for encoding enzyme abscisic aldehyde oxidase in *flacca*.

Our study also included *NCED* as another biosynthetic gene (Fig. 3A and B). Similarly to the expression of *TAO1* gene for all treatments, the expression of *NCED* was more upregulated in WT than in *flacca*. Comparison between the investigated treatments showed that the expression of this gene in WT plants was greater under PRD than under FI (Fig. 3A). The maximal expression of *NCED* was during the second shifting period under both PRD and RDI. Later the *NCED* expression was downregulated, especially under RDI. At the end of the experiment (fifth shift) the smallest expression of this gene was found in the leaves of RDI plants.

It is well known that environmental stresses (especially drought and salt stress) activate ABA biosynthesis. Drought stress was also shown to induce the expression of *NCED* in many plants, including tomato (Burbidge et al., 1999). The absence of stress in WT plants under FI could explain the relatively small increase of *NCED* expression during the experimental period compared to PRD or RDI treatments (especially in the first and second shifting periods). At the same time, the expression of *TAO1* gene in FI plants indicated that TAO1 is responsible for ABA biosynthesis and the final content in the leaves under this treatment (Table 1).

The temporal expression of *NCED* in *flacca* under the investigated treatments is presented in Fig. 3B.

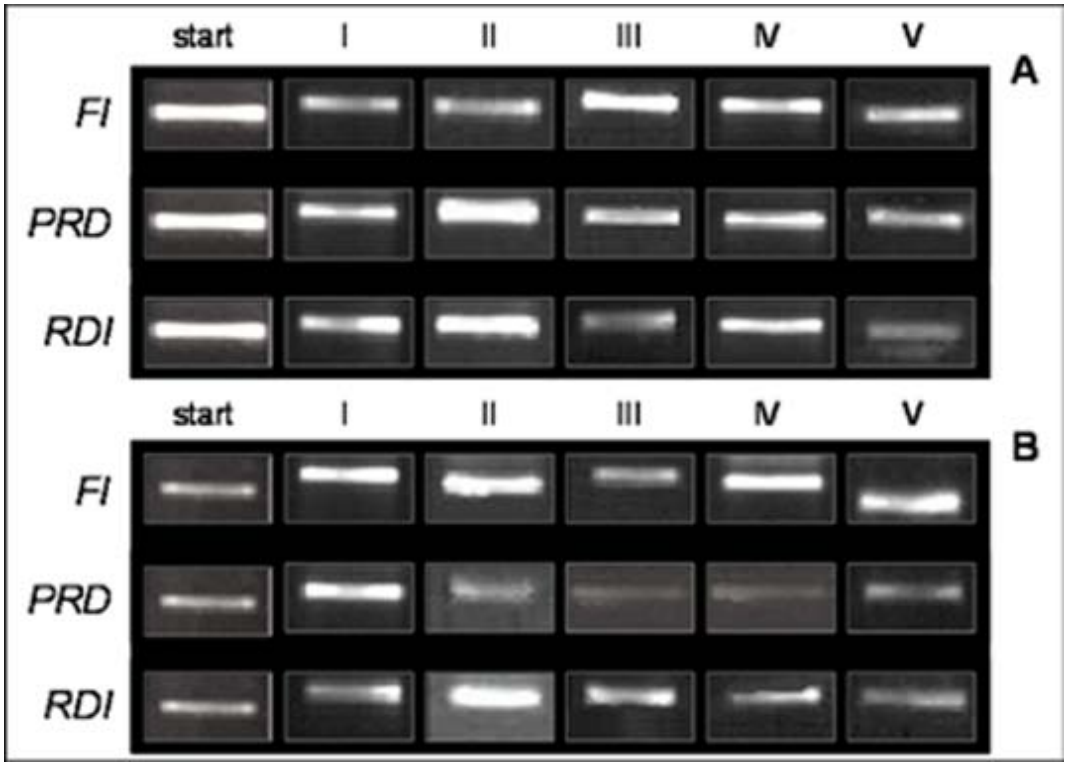

Fig. 3. Expression patterns of the *NCED* gene in the leaves of Ailsa Craig (A) and *flacca* (B) under FI, PRD and RDI during six sampling periods.

Results demonstrated that the expression of *NCED* was more upregulated by FI and RDI than by PRD. Under the PRD treatment, the maximal expression was during the first shift and later the expression was significantly reduced. The maximal expression of *NCED* was under FI and RDI, during the second shift. High expression of both genes (*TAO1* during almost all investigated period and *NCED* during the first shift) was noticed in *flacca* under FI and this increase could be responsible for the ABA content in the leaves of these plants (Table 1).

The results of Thompson et al. (2000) showed a very rapid increase in NCED transcript in tomato leaves. However, our results did not reveal a rapid increase in gene expression (our experiments were designed to examine the long-term adaptive gene response) because during almost all treatments, even under RDI, the upregulation of *NCED* began after the second shifting periods. Discrepancy between these results and Thompson's could be explained by differences in the degree and duration of drought stress or differences in type of drought (PRD and RDI). Thompson's experiment was performed with the detached leaf test and the degree of stress was higher short-term than under our *in vivo* experimental conditions.

One possible mechanism to modulate developmental or tissue-specific responses to ABA could also be through the regulation of ABA biosynthesis and/or response by ethylene. Ethylene is another hormone that regulates a plant's responses to water deficits by acting to control the vegetative growth of plants in drying soils (Sharp et al., 2000; Chaves et al., 2003). It is accepted that ABA is the inhibitor of shoot growth (Trewavas and Jones, 1991), and thus, it is paradoxical that many results have confirmed that ABA mutants are shorter and have a smaller leaves than the corresponding wild types. Our results also showed that *flacca* plants were about 30% smaller than WT (data not shown). One of the explanations

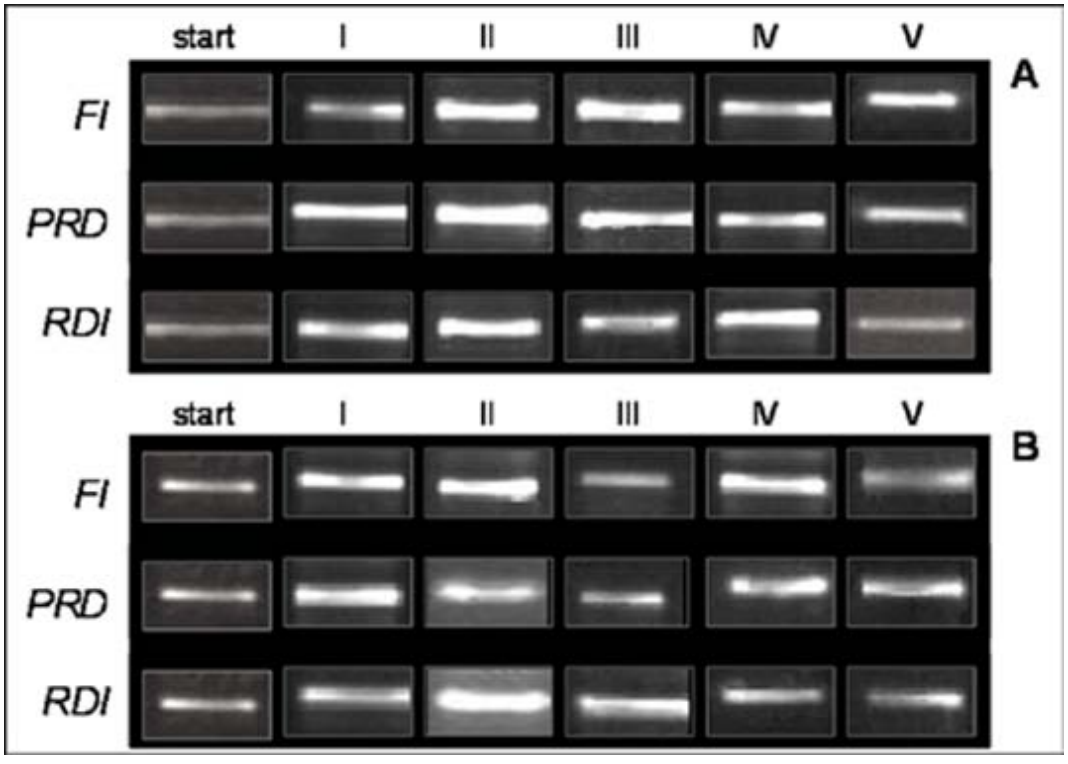

Fig. 4. Expression patterns of the *EIL1* gene in the leaves of Ailsa Craig (A) and *flacca* (B) under FI, PRD and RDI during six sampling periods.

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for smaller shoot growth of ABA mutants is their higher ethylene production compared to WT plants (Neill et al., 1986). According to Sharp et al. (2000), the role of ABA is to maintain growth by limiting the production of ethylene.

To address the question of involvement of ethylene in the reaction of WT and *flacca* to FI, PRD and RDI treatments, we investigated the expression of *EIL1* (Figs. 4A and B). The expression pattern of *EIL1*, similarly to other genes, differed depending on both treatments and temporal pattern. In WT plants under FI, the maximal expression was noticed in the period corresponded to the third shifting (Fig. 4A), while under PRD and RDI this maximum was earlier (second shift). In *flacca* plants, the maximal expression in all treatments was after the second shifting period (Fig. 4B). Differences also occurred during the last two sampling periods. Under RDI treatments during the last sampling period, the expression of *EIL1* in leaves was downregulated compared to the other periods and this was especially expressed in WT plants. The end of the sampling period corresponds to the phase of fruit ripening which is known to be controlled by ethylene (Yang, 1987).

The results of Rosado et al. (2006) demonstrated that the *flacca* mutant produced ethylene at a significantly higher concentration than Ailsa Craig. In our experiment we did not measure the ethylene concentration, but the genetic analysis of Tieman et al. (2001) showed that EIL1 is a positive regulator of ethylene responses (including leaf epinasty, flower abscission and senescence, fruit ripening). Temporal expression of *EIL1* in the last phases of our experiments (fifth and sixth) also coincides with the rapidity of development and fruit ripening of the investigated plants. Our previous results (data not shown) demonstrated that plant development and fruit ripening of both WT and *flacca* were faster under RDI treatment compared to FI and PRD.

Although an increase in PRD-induced ABA content was similar in both WT and *flacca* (*ca* 1.3 fold) leaves, PRD treatment had a different impact on the expression of the investigated ABA biosynthetic genes. The most significant increase of expression was found during the second shift for *NCED* in WT, while in *flacca* the maximal overexpression was for *TAO1*. These results indicate that *NCED* and *TAO1* might be responsible for ABA biosynthesis in WT and *flacca*, respectively. The similar expression pattern of *TAO1*, *NCED* and *EIL1* (maximum during the second shift was followed by downexpression) in WT plants under PRD could possibly indicate synergistic signaling pathways for ABA and ethylene. However, such a pattern was not found in *flacca* under PRD. Reduced *NCED* and significant *EIL1* expression (especially after the second shift) probably reflect the increase in ethylene production which could further influence ABA signaling and biosynthesis.

Under RDI treatment, the pattern of all genes in WT and *flacca* leaves was similar and once again it raises the possibility of a synergistic effect of ABA and ethylene signaling pathways. The very significant increase of ABA content in *flacca* (*ca* 2.3 fold) is probably a result of the very high expression of *TAO1* and *NCED* genes, which is also followed by significant *EIL1* expression.

In conclusion, our results provide novel insights into the understanding of tomato response to drought induced by PRD and RDI. Future investigations should analyze more genes (by microarray) that underlie the ABA and ethylene biosynthetic and/or signaling pathways.

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