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RESEARCH PAPER

Thermotolerant cyclamen with reduced acrolein and methyl vinyl ketone

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Abstract

Reduced levels of trienoic fatty acids (TAs) in chloroplast membranes induce thermotolerance in several plant species, but the underlying mechanisms remain unclear. TA peroxidation in plant cell membranes generates cytotoxic, TA-derived compounds containing α,β-unsaturated carbonyl groups. The relationship between low TA levels and the amounts of cytotoxic TA-derived compounds was examined using thermotolerant transgenic cyclamen (*Cyclamen persicum* Mill.) with low TA contents. Changes in the levels of the cytotoxic TA-derived acrolein (ACR), methyl vinyl ketone (MVK), (E)-2-hexenal, 4-hydroxy-2-nonenal, and malondialdehyde were analysed in the leaf tissues of wild-type (WT) and thermotolerant transgenic cyclamen under heat stress. Levels of ACR and MVK in the WT increased in parallel with the occurrence of heat-induced tissue damage, whereas no such changes were observed in the thermotolerant transgenic lines. Furthermore, exogenous ACR and MVK infiltrated into leaves to concentrations similar to those observed in heat-stressed WT leaves caused similar disease symptoms. These results suggest that thermotolerance in transgenic cyclamen depends on reduced production rates of ACR and MVK under heat stress, due to the low level of TAs in these plants.

Key words: Acrolein, *Cyclamen persicum* Mill., methyl vinyl ketone, thermotolerance, trienoic fatty acids.

Introduction

Global warming may greatly affect plant ecosystems and agricultural production. In addition to droughts and other indirect effects, higher temperatures may have direct negative effects on productivity and crop quality. Active research on responses to global warming is proceeding (Iba, 2002; Sharkey, 2005; Sharkey and Zhang, 2010). One approach focuses on the fact that heat tolerance can be increased by reducing the trienoic fatty acid (TA) content of chloroplast membranes via suppression of the ω-3 fatty acid desaturase (FAD; Murakami *et al.*, 2000; Falcone *et al.*, 2004; Liu *et al.*, 2006; Wang *et al.*, 2006). However, the mechanism by which reduced TA levels lead to increased thermotolerance has not been clarified.

In vivo, TA peroxidation via enzymatic and non-enzymatic pathways generates a wide variety of metabolites (Esterbauer *et al.*, 1991; Rustérucci *et al.*, 1999; Alméras *et al.*, 2003). Some of these metabolites contain α,β-unsaturated carbonyl groups and are strongly cytotoxic (Esterbauer *et al.*, 1991; Vollenweider *et al.*, 2000; Alméras *et al.*, 2003; Mano *et al.*, 2009). It was hypothesized that the reduced TA content may lead to decreases in the production of cytotoxic compounds in thermotolerant transgenic plants. Among the products of TA peroxidation, acrolein (ACR), methyl vinyl ketone (MVK), and 4-hydroxy-2-nonenal (HNE) contribute to significant reductions in photosystem II fluorescence (Alméras *et al.*, 2003). ACR, (E)-2-hexenal,

and HNE inhibit photosynthesis and inactivate multiple enzymes in the Calvin cycle (Mano *et al.*, 2009). Furthermore, ACR and MVK activate defence-related genes that are associated with cell damage. In addition to ACR and MVK, malondialdehyde (MDA) can also act as a powerful activator of defence-related genes, and prolonged exposure to these compounds results in damage to leaf tissues (Alméras *et al.*, 2003).

For these reasons, the potential contribution of ACR, MVK, (E)-2-hexenal, HNE, and MDA to the acquisition of thermotolerance in higher plants was studied. In this study, the induction of thermotolerance in cyclamen (*Cyclamen persicum* Mill.), which develops leaf damage under heat conditions (>38 °C), was demonstrated. This commercially important plant was employed rather than one of the standard experimental models because, due to global warming, cultivars derived from *C. persicum* may be difficult to produce commercially in the future, while wild cyclamen species will face an increased risk of extinction (Yesson and Culham, 2006). Furthermore, the changes in the amounts of ACR, MVK, (E)-2-hexenal, HNE, and MDA in leaf tissues of wild-type (WT) and thermotolerant transgenic cyclamen with low TA content under heat stress were examined.

Materials and methods

Plant materials and growth conditions

The fixed diploid cyclamen (*C. persicum* Mill.) cultivar 'Victoria' was employed as the WT to produce transgenic lines. The tetraploid 'Victoria' cyclamen cultivar was used to extract genomic DNA and identify *C. persicum FAD7* (*CpFAD7*). These lines were obtained from the Kage Shinkoen Nursery (Japan). Commercial cultivars were obtained from Morel Diffusion S.A.S, Goldsmith, Varinova, Schoneveld Twello B.V., and Syngenta Seeds. Seeds were sterilized and sown on half-strength Murashige and Skoog (MS) medium without growth regulators in plant boxes (Magenta vessel GA-7; Sigma-Aldrich, USA), and kept in the dark at 20 °C until germination. Then, seedlings in plant boxes were grown at 20 °C under 16 h light conditions (70 μmol m^{-2} s^{-1}) until utilization. To evaluate drought stress in seedlings and heat stress in mature plants, seedlings were transferred to horticultural soil (ZEN-NOH, Japan Agricultural Cooperatives, Japan) and grown under otherwise identical conditions.

Determination of the genomic CpFAD7 sequence

Genomic DNA was extracted from leaves (200 mg) using a modified cetyltrimethylammonium bromide (CTAB) method. To identify *CpFAD7*, the degenerate primers, iF1 (5′-ACDCAYCAYCARAACCAYGG-3′) and iR2 (5′-CTCCAYKCCTYKCCDCKRTACCA-3′) derived from the sequence of *FAD7* in *Arabidopsis thaliana* were used. The sequence of *CpFAD7* was submitted to the DDBJ/EMBL/GenBank databases (accession number AB250917).

Production of transgenic cyclamen with low TA content by RNAi

To reduce the TA contents in cyclamen plants, RNA interference (RNAi) using an RNAi vector containing parts of the *CpFAD7* sequence was employed. A fragment containing the seventh exon and part of the fourth intron of *CpFAD7* was isolated from genomic DNA using the primers Xba-Ex7S (5′-CCCTCTAGAGAATGGAGTTATTTGCGAGGA-3′; *Xba*I site underlined), and Ex7AS-4thint5′ (5′-GAGTAATAGGCTCACTGCTTCAATTAAGT-3′). The resulting amplified fragment served as a template and was amplified again using Xba-Ex7S and intron-EcoRIAS (5′-TTAGAATTCGGAAAACTAAAATGTTAACAAACTTGTGGATCAATTCAGCAAGGGGATGGAGTAATAGGCTCAC-3′; *Eco*RI site underlined). This *Xba*I–seventh exon–fourth intron–*Eco*RI fragment was digested with *Xba*I and *Eco*RI (TaKaRa, Japan), and ligated into the pGEM-7Zf (+) vector (Promega, USA). Similarly, a *Sac*I–seventh exon antisense–fourth intron–*Eco*RI fragment was amplified and subcloned into pGEM-7Zf (+) using Sac-Ex7S (5′-GGGGAGCTCGAATGGAGTTATTTGCGAGG-3′; *Sac*I site underlined), Ex7AS-4thint3′ (5′-CTGTTTATTCCTCATGCTTCAATTAAGTGA-3′), and EcoRI-intron (5′-TTCCGAATTCTAATGATTTTTTACGTTTTTTCTGTTTATTCCTCAG-3′; *Eco*RI site underlined). Finally, an *Xba*I–seventh exon–fourth intron–seventh exon antisense–*Sac*I fragment was constructed by ligation at the *Eco*RI site, and then inserted into the *Xba*I–*Sac*I site of the binary Ti vector pBI121. For more effective gene expression and antibiotic selection in cyclamen, the *Cauliflower mosaic virus* (CaMV) 35S promoter and the *NPTII* gene in the pBI121 vectors were replaced with the soybean *Cucumber mosiac virus* (CMV) non-coding region promoter and *HPT*, respectively. The resulting RNAi vector was introduced into the *Agrobacterium tumefaciens* strain EHA105 which was then used to transform *C. persicum* diploid 'Victoria' using etiolated petiole explants (Aida *et al.*, 1999). Cyclamen seedlings were grown in the dark to obtain the etiolated petioles for transformation. Sixty days after sowing without subculture, etiolated petioles were cut into 1 cm segments and used as explants to introduce the constructed vector. The explants were incubated in the EHA105 suspension for 30 min, then transferred to solidified MS co-culture medium containing 1.0 mg l^{-1} thidiazuron, 0.1 mg l^{-1} 2,4-dichlorophenoxyacetic acid, and 100 μM acetosyringone. The explants and bacteria were co-cultured in the dark at 20 °C for 7 d, then the explants were transferred to solidified MS selective medium containing 10 mg l^{-1} hygromycin and 10 mg l^{-1} meropenem. The explants were incubated in the dark for 60 d with subculturing every 2 weeks, then the hygromycin-resistant calli were transferred to half-strength MS solidified medium without growth regulators, and incubated at 20 °C under 16 h light (70 μmol m^{-2} s^{-1}). Transgenic plants were transferred to pots with horticultural soil and grown at 20 °C under 16 h light (70 μmol m^{-2} s^{-1}) to the reproductive stage. To investigate thermotolerance, drought tolerance, and levels of TA-derived compounds in the transgenic plants, they were pollinated with pollen from WT plants. From the segregating offspring, individuals with low TA contents as established by gas chromatography were selected, and these plants, named T15 and T31, were used for further analyses.

Northern blot analysis

Total RNA was extracted from leaves, tubers, and roots of cyclamen; 10 μg samples were used for northern blot analysis. The RNA was separated on a denaturing 1% agarose gel and then transferred to a positively charged nylon membrane (Roche Applied Science, Germany) in 20× SSC. To synthesize the *CpFAD7* probe, mRNA was purified from total leaf RNA using the Oligotex™-dT30 mRNA purification kit (TaKaRa, Japan). cDNA was synthesized using the SuperScript™ Choice System for cDNA Synthesis (Invitrogen, USA) with an oligo$(dT)_{12–18}$ primer. The *CpFAD7* cDNA was amplified using the primers 5′-GCCCCTCTCCAGAATCTACC-3′ and 5′-GGGATCTGAGGGAATAGATGG-3′, ligated into the pGEM-T vector (Promega, USA), and then a digoxigenin-labelled *CpFAD7* probe was synthesized using the PCR DIG Probe Synthesis kit (Roche Applied Science, Germany).

Fatty acid analysis

The fatty acid compositions of leaf tissues were analysed by gas chromatography (Kodama *et al.*, 1994). The analyses were

conducted with three replications from three independent plants, using 4.0×4.0 cm leaf sections.

Heat and drought stress treatments

For heat stress treatments, plants on MS medium or in soil were exposed to 38 °C under constant light (70 μmol m^{-2} s^{-1}), according to the method of Murakami *et al.* (2000), with some modifications. For statistical analysis of leaf damage, leaves with a few wilting or browning parts were counted as injured leaves, and seedlings with at least two injured leaves were scored as damaged seedlings according to Zhang *et al.* (2005). Each line was analysed using six individuals and three replications.

For evaluation of drought tolerance, seedlings were grown in soil for 6 months (to the 4–5 leaf stage) and irrigated every 3 d. Irrigation was then withheld for 18 d, after which irrigation was resumed. Drought tolerance was evaluated at 12 d after the beginning of the drought treatment as the ratio of withered leaves to all leaves. Each line was analysed using six individuals and three replications. The plants were photographed at the beginning of the experiment, on the 18th day of drought treatment, and 14 d after resuming irrigation.

Measurement of TA-derived compounds

TA-derived compounds were extracted from leaves, and derivatized (Deighton *et al.*, 1999). Leaves detached from treated seedlings or detached leaves that had been infiltrated with ACR or MVK were analysed. The tissue sample (1 g) was homogenized in liquid nitrogen, and 5 ml of methanol containing 0.005% butylated hydroxytoluene were added. After centrifugation (10 min, 500 *g*, 4 °C), 3 ml of the supernatant were transferred to a new tube containing 5 ml of 350 mg l^{-1} 2,4-dinitrophenylhydrazine (DNPH) in 1 M HCl. The reaction was incubated for 30 min at 20 °C. The mixture was extracted twice with 5 ml of dichloromethane, then dried at room temperature. The solid residue was dissolved in 400 μl of acetonitrile. ACR–DNPH was determined using an liquid chromatography–mass spectrometry (LC-MS) system (Grosjean *et al.*, 1999). The molecular mass of the ACR–DNPH derivative was 234.9, as determined using a standard ACR (Sigma-Aldrich, USA). The amount of ACR in leaf samples was determined by comparison with the standard ACR. MVK–DNPH, (E)-2-hexenal–DNPH, and HNE–DNPH were assayed using reversed-phase high-performance liquid chromatography (HPLC) by measuring the absorbance at 360 nm, while MDA–DNPH was assayed by its absorbance at 307 nm (Korchazhkina *et al.*, 2003). The retention times and amounts of MVK–DNPH, (E)-2-hexenal–DNPH, and HNE–DNPH were established using standard chemicals (Sigma-Aldrich), and those of MDA–DNPH were determined with standard MDA prepared from 1,1,3,3-tetramethoxypropane (Sigma-Aldrich). Each experiment was carried out with three replications using independent plants.

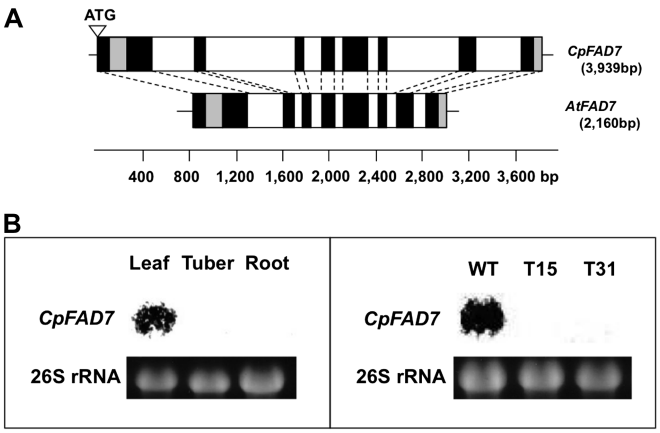
Effects of TA-derived compounds on infiltrated leaves

To investigate whether ACR and MVK in leaves contributed to damage resulting from heat stress, infiltration tests were conducted with ACR and MVK in WT leaves. Detached whole leaves (~4.0×4.0 cm) of WT seedlings (4–5 leaf stage) were floated with their abaxial side on the surface of water (water-infiltrated) or on freshly prepared solutions of 5 ppm ACR or 50 ppm MVK in a sealed container at 20 °C under 16 h light (70 μmol m^{-2} s^{-1}). The treatment periods were 3 d for ACR infiltration tests and 2 d for MVK infiltration tests. The infiltrated samples were washed three times with 1 litre of distilled water and then were analysed for their ACR and MVK contents as described above. Each experiment was carried out with three replications.

Results

Production of transgenic cyclamen with low TA content

The cyclamen gene encoding the plastid-localized CpFAD7 enzyme (accession number AB250917) was identified using the degenerate primers based on the *FAD7* gene sequence from *A. thaliana* (Iba *et al.*, 1993) (Fig. 1A). The sequence of *CpFAD7* included 3939 bp with a 1305 bp open reading frame encoding 435 amino acid residues. Its first exon showed high homology with the transit peptide region of *FAD7* in *A. thaliana*. Northern blot analysis supported the idea that *CpFAD7* encodes the plastid-located ω-3 FAD, since the expression of endogenous *CpFAD7* mRNA was detected in leaves but not in tuber and root tissues which lack chloroplasts (Fig. 1B, left). Two transgenic cyclamen lines, T15 and T31, with decreased TAs were produced using RNAi vectors to inhibit the expression of *CpFAD7*. No endogenous *CpFAD7* mRNA was detectable in leaves of these lines (Fig. 1B, right). The levels of 18:3 fatty acid, the only TA in cyclamen, were dramatically reduced in leaves of the transgenic plants, while 18:2 dienoic fatty acid (DA), showed a corresponding increase (Fig. 1C).



C

Line	Fatty acid (mol%)				
	16:0	18:0	18:1	18:2	18:3
WT	16.7 ± 1.6	2.9 ± 1.0	6.0 ± 1.0	22.5 ± 0.5	52.0 ± 0.4
T15	19.9 ± 1.6	2.5 ± 0.3	4.5 ± 0.2	71.7 ± 1.7	1.4 ± 0.1
T31	19.9 ± 0.8	2.3 ± 0.1	4.1 ± 0.7	70.9 ± 0.5	2.8 ± 0.3

Fig. 1. Production of transgenic lines with low TA contents. (A) Comparison of *CpFAD7* and *AtFAD7* gene structures. White and black boxes represent introns and exons, respectively. The grey boxes indicate regions of low homology between *CpFAD7* and *AtFAD7*. (B) Expression of *CpFAD7* in WT and transgenic lines. Northern blot analysis of *CpFAD7* expression in WT leaves, tubers, and roots (left), and in leaves of WT, T15, and T31 plants (right). The blots were hybridized with a full-length *CpFAD7* cDNA as probe. The denaturing RNA gels were stained with ethidium bromide, and 26S rRNA bands were used as loading controls. (C) Composition of fatty acids in leaves of WT and transgenic lines (T15 and T31). Values are means ±SD (n=3). Plants were grown to seedling stage (4–5 leaves) on half-strength MS medium at 20 °C under 16 h light (70 μmol m^{-2} s^{-1}).

Thermotolerance in transgenic cyclamen with low TA content

Conditions were sought which allowed for a reliable and reproductive evaluation of phenotypic signs of thermotolerance in cyclamen (Supplementary Fig. S1 available at *JXB* online). A treatment of 35 °C for 7 d led to quite variable results in terms of medium desiccation and leaf damage. On the other hand, a temperature of 41 °C resulted in severe browning symptoms on leaves after <2 d. Consequently, a moderately high temperature (38 °C) was applied which avoided significant medium desiccation for 5 d of treatment. At this temperature, some WT seedlings displayed symptoms of heat damage (wilting and browning of leaves) after 2 d, and all WT seedlings showed these symptoms after 5 d. The occurrence of such wilting and browning symptoms was significantly delayed in the two transgenic lines with decreased TA levels, T15 and T31 (Fig. 2A, B; Supplementary Table S1). Moreover, the thermotolerance of 15 commercial cultivars did not differ from that of the WT in spite of the varying TA contents (45.9–59.7 mol%) in these cultivars (Fig. 2B; Supplementary Tables S1, S2). These results demonstrated that the transgenic seedlings were more thermotolerant than the commercial cultivars and the WT. The increased thermotolerance of the T15 line was maintained at the flowering stage (Fig. 2C).

Changes in TA-derived compounds in heat-stressed leaves

TA peroxidation via enzymatic and non-enzymatic pathways generates a wide variety of metabolites that contain toxic α,β-unsaturated carbonyl groups. Among these metabolites, ACR, MVK, (E)-2-hexenal, HNE, and MDA are known to inhibit photosynthesis, inactivate multiple enzymes in the Calvin cycle, and result in damage to leaf tissue following prolonged exposure. Changes in the levels of ACR, MVK, (E)-2-hexenal, HNE, and MDA in leaf tissues of WT and transgenic cyclamen under heat stress were evaluated (Fig. 3A). ACR levels remained similar in WT and T15 leaves for 2 d, but then increased in the WT while remaining low in T15 (Fig. 3A). MVK showed a similar trend, although its concentrations generally were two orders of magnitude higher than those of ACR (Fig. 3A).

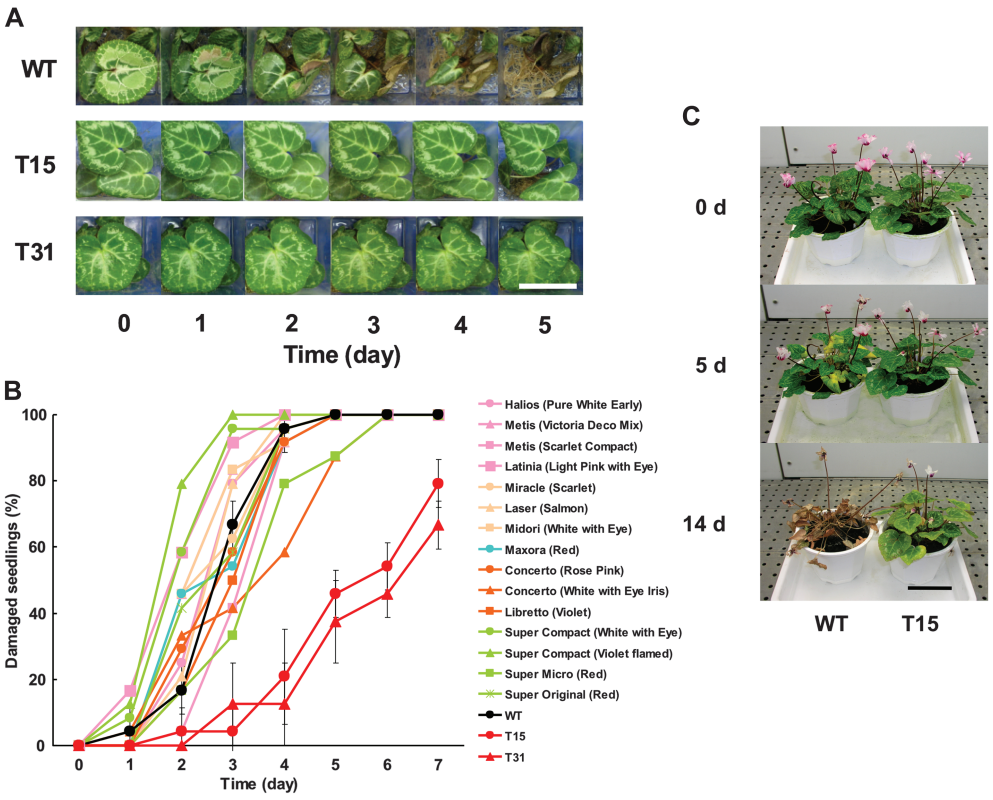


Fig. 2. Thermotolerance of transgenic lines with low TA contents. Plants were grown to the 4–5 leaf stage on half-strength MS medium (A and B) or to the reproductive stage (20–25 leaves) on soil (C) at 20 °C under 16 h light (70 μmol m^{-2} s^{-1}). (A) Development of heat damage symptoms in WT and transgenic seedlings under heat stress treatment (38 °C, constant light). The seedlings were photographed daily for 5 d. The bar indicates 5 cm. (B) Comparison of thermotolerance in seedlings of commercial cultivars, WT, and transgenic lines (pink, lines obtained from Morel Diffusion S.A.S.; pale orange, Goldsmith; blue, Varinova; orange, Syngenta Seeds; pale green, Schoneveld Twello B.V. black, WT; red, transgenic lines). Each line was analysed using six individuals and three replications. Leaves with wilting or browning parts were counted as injured, and seedlings with at least two injured leaves were scored as damaged. Values are means ±SD (n=3). (C) Comparison of thermotolerance in WT and T15 plants at the reproductive stage (20–25 leaves) under heat stress (38 °C, constant light). Scale bar, 10 cm.

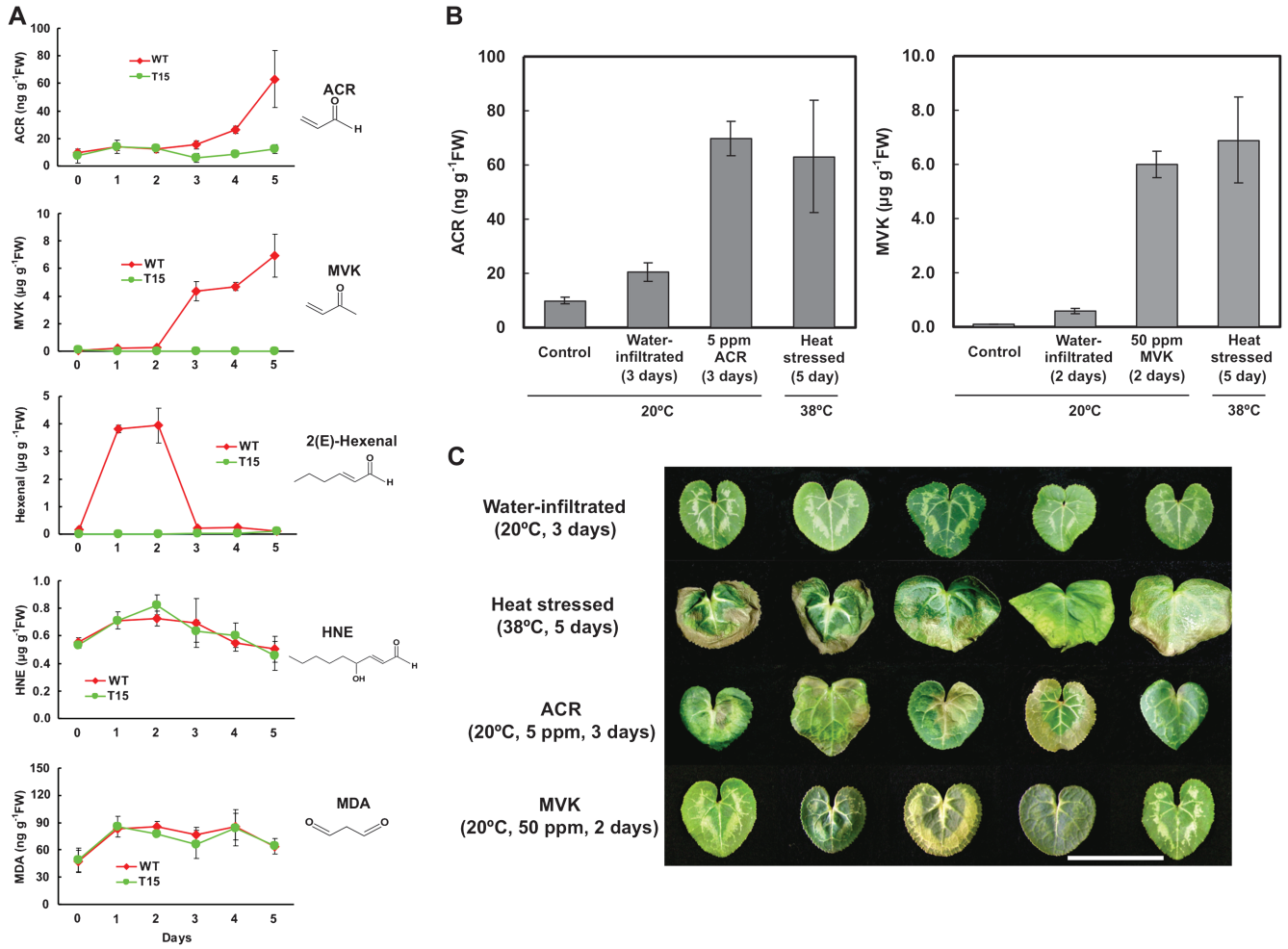


Fig. 3. Changes in the levels of TA-derived compounds during heat treatment, and leaf damage caused by these compounds. Plants were grown to the seedling stage (4–5 leaves) at 20 °C under 16 h light (70 μmol m^{-2} s^{-1}). (A) Changes of candidate cytotoxic compounds in the WT and T15 line under heat stress (38 °C, constant light). ACR, acrolein; MVK, methyl vinyl ketone; HNE, 4-hydroxy-2-nonenal; MDA, malondialdehyde. Values are means ±SD (n=3). (B) Amounts of ACR and MVK in control, water-infiltrated, heat-stressed (38 °C, 5 d, constant light), and ACR-/MVK-infiltrated leaves excised from WT seedlings. The infiltrated leaves were treated with 5 ppm ACR for 3 d (ACR) or 50 ppm MVK for 2 d (MVK) at 20 °C under 16 h light. Values shown are means ±SD (n=3). (C) Damage symptoms displayed by excised WT leaves after heat stress treatment or infiltration with water (water-infiltrated), ACR, or MVK. The treatment conditions were as described for B. Scale bar, 5 cm.

Evidently, the increases in ACR and MVK in the WT seedlings coincided with the development of heat damage in leaves (compare Fig. 2B). In contrast, (E)-2-hexenal levels were dramatically but transiently elevated after 1 d and 2 d in WT but not in T15 seedlings (Fig. 3A). The amounts of HNE and MDA remained fairly stable and similar in WT and T15 seedlings throughout the duration of the experiment (Fig. 3A). It was concluded that ACR and MVK were likely to be involved in the causation of leaf damage during the heat exposure.

Effects of exogenous ACR and MVK on leaves

The above findings suggested that ACR and MVK were involved in heat stress-induced leaf damage. To test this idea, detached WT leaves were infiltrated with ACR and MVK. Under normal growth conditions, the amounts of ACR and MVK in WT leaves (Control) were 9.9 ng g^{-1} FW and 0.1 μg g^{-1} FW, respectively (Fig. 3B). In contrast, WT leaves showed wilting or browning at least over parts of the leaf area under heat stress treatment, and ACR and MVK accumulated to 63.0 ng g^{-1} FW and 6.9 μg g^{-1} FW, respectively (Fig. 3B). Infiltration conditions that resulted in similar levels of ACR and MVK were identified. For ACR, the optimum infiltration conditions were 5 ppm ACR for 3 d, resulting in 69.7 ng g^{-1} FW (Fig. 3B, left). For MVK, the optimum conditions were 50 ppm MVK for 2 d, resulting in 6.0 μg g^{-1} FW (Fig. 3B, right). On the other hand, the amounts of ACR and MVK in water-infiltrated leaves reached 20.5 ng g^{-1} FW after 3 d of treatment, and 0.6 μg g^{-1} FW after 2 d of treatment, respectively (Fig. 3B). Under these conditions, ACR- and MVK-infiltrated leaves developed browning and wilting symptoms that resembled those of heat-stressed leaves, but water-infiltrated leaves did

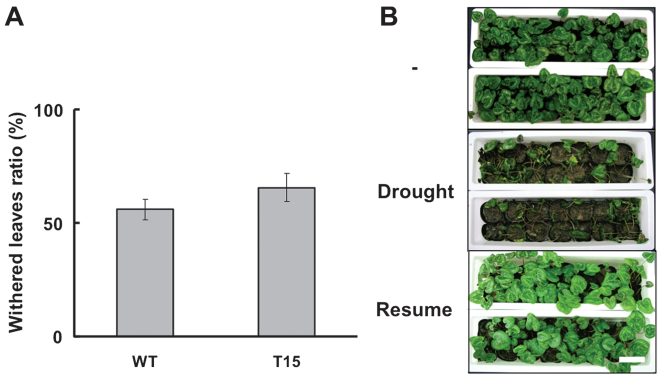


Fig. 4. Evaluation of drought tolerance in the WT and T15 line. After initial growth for 6 months in soil at 20 °C under 16 h light (70 μmol m^{-2} s^{-1}), plants with 4–5 leaves were evaluated for drought tolerance. (A) The statistical evaluation of drought tolerance was conducted after withholding irrigation for 12 d. The numbers of withered leaves were counted and expressed as the percentage of all leaves. The values are means ±SD (n=3). (B) WT (upper container) and T15 (lower container) seedlings initially were irrigated every 3 d for 6 months (–). Irrigation was withheld for 18 d (Drought), and then resumed for 14 d (Resume). Scale bar, 10 cm.

not after 3 d of treatment (Fig. 3C). These results suggested that the enhanced amounts of ACR and MVK in leaves under heat stress could be causing the browning and wilting symptoms.

Drought tolerance in transgenic cyclamen

Decreases in TAs of chloroplast membranes seemed to correlate with reduced drought tolerance in tobacco (Im *et al.*, 2002). To see whether this also is true for cyclamen, irrigation of WT and T15 plants was stopped for 12 d. As a consequence, slightly more than half of all leaves showed signs of withering, but the results for the two lines did not differ significantly (Fig. 4A). Furthermore, when irrigation of the plants was resumed after 18 d of drought stress, both WT and T15 plants recovered similarly (Fig. 4B). These results suggested that transgenic cyclamen plants with decreased TA contents did not suffer from reduced drought tolerance.

Discussion

Thermotolerance of transgenic cyclamen with low TA content

To reveal the relationship between low TA levels and thermotolerance in higher plants, transgenic cyclamen with decreased TA content were produced and the amounts of cytotoxic TA-derived compounds under heat stress were monitored. The suppression of the activity of *CpFAD7* resulted in a reduction of TA from ~50 mol% to <3 mol%, and a corresponding increase of DA from 20 mol% to 70 mol% (Fig. 1). Thus, the DA/TA ratio changed dramatically, from <0.5 in the WT to 25 in T31 and to ~50 in T15. In tobacco, two transgenic lines with decreased TA content in leaf tissue produced by gene silencing of *AtFAD7* showed DA/TA ratios of 1.5 and 2.2, compared with ~0.1 in the WT (Murakami *et al.*, 2000). Similarly, the DA/TA ratio in two transgenic tomato lines with low TA contents that had been generated through antisense *LeFAD7* and the WT were 2.8, 1.2, and 0.5, respectively (Liu *et al.*, 2006). The significantly higher DA/TA ratios that were found in this study in transgenic cyclamen as compared with other species may be due to peculiarities of the lipid biosynthesis pathway in cyclamen and/or a particularly efficient suppression of endogenous *CpFAD7*. In the *A. thaliana fad3fad7-fad8* mutant which lacks plastidial TAs altogether, a reduction of photosynthetic activity and retardation of growth under heat stress have been observed. The growth inhibition was ascribed to a deficiency of TA-derived jasmonates and their derivatives (Routaboul and Browse, 2002; Wallis and Browse, 2002). In contrast, transgenic cyclamen containing 1.4–2.8 mol% of TA may still be able to produce sufficient essential TA derivatives to survive under heat stress and acquire thermotolerance. Furthermore, TA-derived jasmonates contribute to plant responses to abiotic and biotic stresses (McConn *et al.*, 1997; Tuteja and Sopory, 2008) as well as anther development and pollen maturation (McConn and Browse, 1996). In the present study, transgenic cyclamen produced viable pollen and grew normally under appropriate conditions. It would be interesting to determine changes in jasmonate levels in response to biotic and abiotic stresses in transgenic cyclamen with low TA contents.

On the other hand, heat and drought stress responses are closely related in higher plants. In a previous study, decreased TA levels in chloroplast membranes seemed to induce thermotolerance but reduced drought tolerance (Im *et al.*, 2002). In the present study, the performance of transgenic cyclamen in a drought stress test resembled that of the WT.

Changes in TA-derived compounds under heat stress

TA is the substrate for the synthesis of various compounds including fatty acid hydroperoxides, fatty acid ketodienes, 4-hydroxynonenal, smaller aldehydes, and numerous other oxygenated fatty acid derivatives (Esterbauer *et al.*, 1991; Rustérucci *et al.*, 1999; Howe and Schilmiller, 2002; Alméras *et al.*, 2003). These compounds are produced by enzymatic and non-enzymatic oxygenation in diseased, wounded, and stressed plant tissues, and some of these substances appear to be involved in stress responses (Deighton *et al.*, 1999; Imbusch and Mueller, 2000; Vollenweider *et al.*, 2000; Jalloul *et al.*, 2002; Weber *et al.*, 2004). On the other hand, compounds containing α,β-unsaturated carbonyl groups may induce programmed cell death, and may function as genotoxic agents because of their potential to form Michael adducts. Among these compounds, the focus of the present study was on ACR, MVK, (E)-2-hexenal, HNE, and MDA because their cytotoxicity has been characterized in previous studies. Increasing accumulation of ACR and MVK was found in

WT plants from 3 d after the start of the heat treatment, whereas the concentration in T15 plants remained at the basal level. Since these changes correlated with the occurrence of heat stress symptoms, a role for ACR and MVK in the development of heat stress-induced leaf damage was implied. On the other hand, the amount of (E)-2-hexenal increased transiently in WT but not in T15 plants, from day 1 to 3. Moreover, to reveal the possible involvement in thermotolerance of the transiently increasing (E)-2-hexenal, heat stress treatment (38 °C) was applied for 2 d, and leaf damage at day 7 after the end of the treatment was subsequently evaluated. Heat-inducible damage did not appear in the leaves (data not shown). These results did not suggest an involvement of (E)-2-hexenal in the thermotolerance of cyclamen with low TA content. Though (E)-2-hexenal was reported as a damaging factor in plant tissues (Mano *et al.*, 2009), it did not affect leaves in cyclamen under the present experimental conditions. The amounts of HNE in the WT and T15 increased similarly to those of (E)-2-hexenal up to day 2 of the heat treatment, and then returned to basal levels. The similarity of patterns in WT and T15 argued against an involvement of HNE in the thermotolerance observed in T15. HNE is produced mainly from DA in *Capsicum annuum* (Deighton *et al.* 1999), but the biosynthesis pathway for HNE may differ in cyclamen. Increased MDA levels in WT and T15 were maintained from day 1 throughout the duration of the experiment, suggesting that MDA plays no role in T15 thermotolerance. Large amounts of MDA originate from TAs in *A. thaliana*, and changes in the amount and localization of free MDA strongly affected abiotic stress-related gene transcription (Muckenschnabel *et al.*, 2002; Weber *et al.*, 2004). In cyclamen, MDA may be derived from unsaturated fatty acids other than 18:3. Alternatively, the 18:3 remaining in transgenic cyclamen may be sufficient for generating MDA. While it is known that MDA can strongly affect abiotic stress-related gene expression, an involvement of MDA in heat stress effects has not been demonstrated.

Effects of ACR and MVK applied to leaves

ACR and MVK levels were unaffected by heat stress until day 3 in WT and T15 plants. Afterwards, the levels of both substances increased in the WT but not in T15. In leaves infiltrated with exogenous ACR and MVK, distinctive necrotic areas appeared that resembled the damage seen in WT leaves after heat stress for 5 d. The amount of ACR in ACR-infiltrated leaves, the amount of MVK in MVK-infiltrated leaves, and the amounts of ACR and MVK in heat-stressed leaves were comparable. ACR generally causes lesions on leaf surfaces (Haagen-Smit *et al.*, 1952; Darley *et al.*, 1960). Furthermore, ACR and MVK are powerful cytotoxic compounds affecting photosystem II (Alméras *et al.*, 2003; Mano *et al.*, 2009). These studies suggested that a variety of TA-derived compounds, including ACR and MVK, may be generated under heat stress, and that some of these compounds may bring about the heat damage observed. These results may explain why transgenic plants with suppressed *FAD7* expression and reduced TA content show reduced levels of cell death under heat stress. Moreover, an assay for TA-derived cytotoxic compounds such as ACR or MVK in leaves may prove to be useful for the evaluation of thermotolerance in plants. With such a tool it would be possible to produce thermotolerant plants with suppressed expression of plastid-localized FAD, and reduced TA content, by mutagenesis.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Establishment of suitable conditions for the evaluation of thermotolerance in cyclamen.

Table S1. Thermotolerance of cyclamen cultivars, WT, and transgenic lines.

Table S2. Compositions of fatty acids in leaf tissues of various cyclamen cultivars.

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