A Temperature-sensitive Mechanism That Regulates Post-translational Stability of a Plastidial ω-3 Fatty Acid Desaturase (FAD8) in Arabidopsis Leaf Tissues*∆

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Trienoic fatty acids (TAs) are the major constituents in plant membrane lipids. In Arabidopsis, two plastidial isozymes of ω-3 fatty acid desaturase, FAD7 and FAD8, are the major contributors for TA production in leaf tissues. Despite a high degree of structural relatedness, activities of these two isozymes are regulated differently in response to temperature. Elevated temperatures lead to decreases in leaf TA level due to temperature sensitivity of FAD8 activity. A series of FAD7-FAD8 chimeric genes, each encoding a functional plastidial ω-3 desaturase, were introduced into the Arabidopsis fad7fad8 double mutant. Constructs with or without a c-Myc epitope tag were tested. Functionality of each chimeric gene in response to temperature was assayed by Northern and Western analyses and by examining the fatty acid composition. All transformants harboring a chimeric gene containing the FAD8-derived C-terminal coding region (44 amino acids) showed a marked decrease in TA level when exposed to high temperature, similarly as transgenic lines complemented with the native form of FAD8. The reduction of TA level was accompanied by a decrease in the amount of ω-3 desaturase protein but not necessarily by a decrease in its transcript level. Analysis of the decay of c-Myc-tagged products after inhibiting protein synthesis revealed that the FAD8-derived C-terminal region acts in an autoregulatory fashion to destabilize the protein at high temperature. This suggests that the regulation of post-translational stability of FAD8 provides an important regulatory mechanism for modifying its activity in response to temperature, mediating a decrease in TA level at elevated temperatures.

Membrane lipids of plant cells are characterized by a high content of polyunsaturated fatty acids. Typically, dienoic and trienoic fatty acids (DAs and TAs, respectively), qualify as at least 70% of total fatty acids in leaf and root lipids (1). The abundance of TAs relative to DAs changes in accordance with environmental conditions. In particular, increasing and decreasing in TA levels have been observed in a variety of plant species exposed to low and high temperatures, respectively (2–4). TAs are formed from DAs in the endoplasmic reticulum (ER) and in plastids by ω-3 fatty acid desaturases (5). In Arabidopsis, FAD3 encodes an ER-localized ω-3 desaturase (6), whereas FAD7 and FAD8 encode plastidial isozymes of this enzyme (7, 8).

The physiological relevance of the responses to temperature has been demonstrated in transgenic plants with modified leaf TA contents. For example, FAD7-deficient tobacco plants showed reduced leaf TA levels but performed better regarding growth and photosynthesis at high temperature (9). Thus, regulation of plastidial ω-3 desaturase activity is likely associated with the adaptation of plants to elevated temperatures.

The production of TAs in root tissues depends predominantly on the activity of ER-localized FAD3 desaturase (10). According to previous studies in Arabidopsis and wheat, it is likely that temperature-regulated post-transcriptional mechanisms are involved in FAD3 expression (11, 12). In wheat root tips, the enhanced accumulation of TAs upon exposure to lower temperatures was accompanied by an increase in the amount of FAD3 protein, which was not preceded by a concurrent increase in its transcript level (12).

In contrast to the metabolism of TAs in the non-photosynthetic root tissues, the plastidial FAD7 and FAD8 desaturases are the major contributors for TA production in leaf tissues (13). FAD8 was originally identified as a temperature-sensitive plastidial isozyme by phenotypic analysis of the fad7 mutant (14), in which FAD8 is the only functional ω-3 desaturase in plastids. Reduction in leaf TA level caused by the fad7 mutation is less pronounced at lower temperatures, implying that FAD8 activity is induced by low temperature. Consistent with this observation, the steady-state level of FAD8 transcripts increases in response to temperature drops within the physiological range, whereas FAD7 transcript accumulation is almost unresponsive to changing temperature (8). It has therefore been postulated that temperature-dependent changes in leaf TA level are mostly due to transcriptional regulation of FAD8 expression.

Despite this convincing explanation, there has been no direct evidence showing that the transcriptional regulation of FAD8 expression is actually the decisive event controlling the temperature sensitivity of FAD8 activity. A major hindrance preventing thorough investigation into the regulation of FAD8 expression has been the lack of an effective way to quantify the amount and activity of ω-3 desaturase. To overcome these difficulties, a series of FAD7-FAD8 chimeric genes, each encoding a functional plastidial ω-3 desaturase, either with or without a c-Myc epitope tag, was introduced into the Arabidopsis fad7fad8 double mutant. The functionality of each chimeric...
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In response to temperature was analyzed from the transcriptional to post-translational levels. Here we describe a novel post-translational mechanism in which the C-terminal region of FAD8 desaturase acts in an autoregulatory fashion to destabilize the protein at high temperature, leading to a reduction of the concentration of this enzyme in the absence of a concurrent decrease in its transcript level.

EXPERIMENTAL PROCEDURES

Plant Materials and Growth Conditions—All lines of Arabidopsis thaliana described here were derived from the Columbia wild type. The mutant lines fad7fad8 (Ref. 13, stock number C58036) and fad7 (Ref. 14, stock number CS8036) are available from the Arabidopsis Biological Resource Center at Ohio State University. The mutant fad7 line was isolated from the progeny of a cross between the mutant genotypes and wild-type Columbia plants using restriction enzyme polymorphisms specific to the fad7 (15) and fad8 (8) alleles. All transgenic lines were confirmed to be homozygous for the transgenes after two rounds of backcrossing. Unless otherwise described, plants were routinely grown at 22 °C under continuous white light on solid Murashige-Skoog (MS) medium (16) containing 1% (w/v) sucrose and 0.5% (w/v) gellan gum (Kanto Chemical, Tokyo, Japan) and harvested on day 18 for subsequent analyses.

Construction of Chimeric Genes—All cloning techniques used in this study were essentially as described in Ref. 17. The 5.9-kb F8 fragment, corresponding to nucleotides 40510–46390 of the P1 clone MOP10 (GenBank accession number AB005241) and including the endogenous 3′-untranslated sequences of the FAD7 gene, was amplified by PCR from genomic DNA of wild-type Columbia plants. The 7.0-kb F7 fragment, corresponding to nucleotides 34315–41363 of the BAC clone F11B9 (GenBankTM accession number AC073935) and including the endogenous FAD7 gene, was amplified by PCR from the same genomic DNA. All chimeric genes shown in Fig. 1 were constructed based on these two fragments according to exon-intron structures described in the GenBankTM records. The FAD8-derived region encoding the C-terminal 52 amino acids of C6 was replaced in C6alt with a synthetic oligonucleotide 5′-TGA GAG GCT AAA AAC TAC GTC GGA CGT TGC CGA TTA CAT TTA CTC GGA AGT CTG ATA AAA AGT AAA CAA GAT CAT TTC GTG AGC GAC ACA GAG GTT GTC TAC TAT GAA GCA GAT CCA AAA CTC AAT GGA CAA ACA ACA-3′ (the substituted bases are underlined). To construct the chimeric genes for c-Myc-tagged desaturases, a duplex oligonucleotide 5′-GCC GGA CAA AAG TGT ATT TCT GAA GAA GAT TTG GCT AGC-3′ (the area encoding the 9E10 epitope of c-Myc protein (18) is underlined) was inserted immediately at the 5′-end of the conserved AGA codon (Arg) at position 87 of the FAD7 protein and position 80 of the FAD8 protein (Fig. 3A). Recombination between the F8 and F7 fragments and insertion of the oligonucleotides at positions achieved by the combined PCR procedure described in Ref. 19. All PCR products were sequenced to confirm the absence of PCR errors. Sequences of all chimeric genes used in this study are available on request.

Transformation of Arabidopsis Plants—Each chimeric gene shown in Fig. 1 was unidirectionally ligated to the EcoRI/HindIII site of the binary vector pBI121 (Clontech, Palo Alto, CA) along with appropriate linker oligonucleotides. Agrobacterium-mediated transformation of Arabidopsis plants was performed by the vacuum infiltration procedure (20). Positive transformants were selected for kanamycin resistance as described previously (21).

Fatty Acid Analysis—The fatty acid composition of total leaf lipids was determined by gas chromatography as described previously (22). For temperature-shift experiments, transgenic and fad mutant plants were shifted to 27 °C on day 13, and young rosette leaves (5 mm in length) that had developed after the temperature shift were harvested on day 18 for fatty acid analysis. Control plants were maintained at 22 °C until analysis on day 18. At least six independent measurements were performed for each line (X) to determine the mean (FA[X]), and (DA[X]), and S.D. of leaf TA and DA levels at each temperature (X). The TA level was calculated as the sum of 18:3 (ω-6-linoleic acid) and 16:3 (hexadecatrienoic acid) levels, whereas the DA level was calculated as the sum of 18:2 (ω-6-linoleic acid) and 16:2 (hexadecadienoic acid) levels. Values of these parameters are available on-line as Supplemental Material. Changes in leaf TA and DA levels, upon temperature upshift from 22 to 27 °C (ΔTA[X]12–27 and ΔDA[X]12–27), were calculated as follows, and S.D. was calculated with the law of propagation of errors (23).

\[ \Delta \text{TA}[X]_{12-27} = \text{TA}[X]_{27} - \text{TA}[X]_{12} \] (Eq. 1)

\[ \Delta \text{DA}[X]_{12-27} = \text{DA}[X]_{27} - \text{DA}[X]_{12} \] (Eq. 2)

Values of TA, DA, TA, and DA (X and t are usually omitted for simplicity) in different transgenic and mutant lines are summarized in Table I.

Northern Blot Analysis—Total RNA was extracted from leaf tissues as described in Ref. 24. Twenty micrograms of RNA was separated on a 1% (w/v) denaturing agarose-formaldehyde gel. Blotting and hybridization were performed based on the method of Amano (25) as described previously (15). The 3′-untranslated sequences of FAD7 and FAD8, corresponding to nucleotides 39282–39458 of F11B9 and 44952–45088 of MOP10, respectively, were used as hybridization probes (Fig. 1). Plants were shifted to 27 °C on day 17 and harvested on day 18 (after 24 h) for Northern blot analysis. Control plants were maintained at 22 °C until harvest on day 18.

Western Blot Analysis—Total leaf proteins were prepared by the EZ extraction procedure as described in Ref. 26. Protein concentration was determined by the method of Lowry et al. (27) as modified in Ref. 28. Forty micrograms of proteins were separated on a 10% (w/v) SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Amersham Biosciences). Antibodies used in this study included anti-c-Myc rabbit polyclonal antibody A14 (1.500 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-rabbit goat polyclonal antibody conjugated with horseradish peroxidase (1:30,000 dilution) (Stressgen Biotechnologies, Victoria, Canada). Immunoreactive proteins were visualized by the ECL Plus reagents according to the manufacturer’s instructions (Amersham Biosciences). After chemiluminescence detection, total proteins were visualized by staining the blots with Amido Black.

Cycloheximide Treatment—Plants were transferred onto a nylon mesh (50 μm), layered onto solid MS medium on day 7, and allowed to grow at 22 °C until cycloheximide (CHX) treatment. The mesh prevented the growing roots from penetrating into the solid medium. On day 14, plants were transferred to liquid MS medium containing 200 μg/ml CHX in 0.2% (v/v) ethanol. The selected concentration of CHX has been shown to inhibit more than 92% of [35S]Met incorporation (29), and, under our experimental conditions, plants became extensively chlorotic 3 days after starting the treatment. After incubation at either 22 or 27 °C for the times indicated in Fig. 5, total leaf proteins were extracted and used for Western blot analysis as described above.

RESULTS

Novel Assay System for Temperature-sensitive FAD8 Gene Expression Based on Functional Complementation of the fad7fad8 Double Mutant—To develop an experimental system for assaying temperature-sensitive FAD8 expression, genetic complementation of the fad7fad8 double mutant was conducted using functional genomic clones of FAD7 and FAD8. It was expected that expression of FAD8 in this genetic background would result in acquisition of an fad7 mutant-like phenotype, which is discernible by its fatty acid composition. For this analysis, DNA fragments of the two genes covering the entire transcribed region associated with the 5′- and 3′-flanking regions (usually containing intronic promoters and terminators, respectively) were recovered from the wild-type genome (Fig. 1, F8 and F7). These fragments were then transferred into the fad7fad8 double mutant. Transgenic fad7fad8 lines harboring exogenous copies of FAD7 and FAD8, respectively, are designated as F7 and F8 lines.

To identify the temperature-sensitive FAD8 expression, plants of several fad mutant and the transgenic lines were grown at two temperatures, 22 and 27 °C, prior to preparation of fatty acid samples. Within this temperature range, the leaf TA level changes drastically in the fad7 mutant, but only slightly in wild-type plants (14). The fad8 mutant has been...
shown to be almost indistinguishable from the wild type on the basis of fatty acid composition (13). Under our experimental conditions, a temperature upshift from 22 to 27 °C caused a 12.1 ± 1.3% (mean ± S.D.) decrease in leaf TA level in the fad7 mutant (Table I, ΔTA), but only 3.1 ± 0.8% in wild-type plants. In the fad8 mutant, the decrease in leaf TA level resulting from the same treatment was 5.5 ± 0.7% (Table I, ΔTA), an amount significantly lower than the value obtained in the fad7 mutant (p < 0.01).

In most of the F7 and F8 lines, the overall levels of TAs in leaf tissues were higher than in the fad8 and fad7 mutants, respectively (Fig. 3C). Despite such overexpression phenotypes, changes in leaf TA level in response to temperature were similar in all of the independent transgenic lines harboring the same construct. As examples for the low levels of endogenous FAD7 and FAD8 expression, we show the analyses of a single transgenic line of each construct (e.g. Fig. 3C, closed circles). In the representative F7 and F8 lines, the 5 °C-upshift in temperature resulted in decreases of leaf TA levels by 5.8 ± 1.2% and 15.1 ± 1.6%, respectively (Table I, ΔTA). These values differ significantly from each other (p < 0.01), whereas they are comparable to the values obtained in the fad8 and fad7 mutants, respectively. This result suggests the presence of regulatory sequences required for temperature-sensitive expression within the F8 fragment that are not present in the F7 fragment.

C-terminal Coding Region of FAD8 Gene Is Essential for Temperature-sensitive FAD8 Desaturase Activity—To identify the regulatory sequences required for the temperature-sensitive FAD8 expression, a series of FAD7-FAD8 chimeric genes, each encoding a functional plastidial ω-3 desaturase, were constructed based on structural relatedness between the two genes (Fig. 1, C1–C6). As described above for the F7 and F8 transgenes, each construct was introduced into the fad7fad8 double mutant, and the level of complementation in response to temperature was assayed by examining the fatty acid composition. All transformants showed a temperature response similar to that observed in either the F7 or F8 line, and none of the transformants exhibited an exceptional phenotype (Table I and Fig. 2).

To display clearly the type of temperature response that was acquired by expression of a given chimeric gene, net changes in leaf TA level attributable to transgene expression were calculated (Table I and Fig. 2A, ΔΔTA). These values were obtained by subtracting the effects of FAD3 activity inherent to the fad7fad8 genetic background (see “Experimental Procedures”). Because the flux of both TAs and DAs from the plastidial to the
ER membrane is very low (30), it is unlikely that this baseline desaturase activity in the ER is largely affected by the activity of plastidial \( \omega-3 \) desaturase derived from transient expression. We estimated that, upon the 5 °C-upshift in temperature, FAD8 expression in the cis-regulatory mutant plastidial desaturase activity in the endoplasmic reticulum (ER) is largely affected by the activity of the FAD7-FAD8 gene. Accordingly, only the transcripts derived from transgene expression to decreases (or increases) of leaf TA level (\( \Delta TA \)) was accompanied by a corresponding increase to increases (or decreases) of leaf DA level (\( \Delta DA \)). This consistency of the data is highly supportive of the above conclusion and suggests that TA as well as DA levels provide a measure for the degree of temperature sensitivity of expression of the chimeric genes.

**C-terminal Region of FAD8 Desaturase Rather Than Its Encoding Nucleotide Sequence Is Essential for Temperature-sensitive Activity**—To evaluate whether the temperature-sensitive FAD8 activity, regulated via the C-terminal coding region, is associated with transcriptional regulation of FAD8 expression, steady-state transcript levels of several FAD7-FAD8 chimeric genes were quantified. In the fad7fad8 double mutant, aberrant transcripts from the mutated FAD7 and FAD8 loci are rapidly degraded via nonsense-mediated decay processes (15). Accordingly, only the transcripts derived from transgene expression are detectable by FAD7 or FAD8 gene-specific probes (Fig. 1) in the transgenic fad7fad8 lines. As shown by Northern blot analysis in Fig. 4B, steady-state levels of F8 and F7 transcripts in response to temperature were comparable to those of FAD8 and FAD7 transcripts, respectively, detected in wild-type plants. However, inconsistent with the F8-type expression of C2 and C6 as confirmed by the fatty acid analysis (Table I and Fig. 2), transcript accumulation of these chimeric genes was almost unresponsive to temperature (Fig. 4B). Similar inconsistency was also detected in the expression of C4 construct (data not shown). We concluded that the transcriptional regulation of FAD8 expression is not necessarily the decisive event controlling the temperature sensitivity of FAD8 activity.

To examine further the possibility that the C-terminal coding region of FAD8 acts in cis to affect transcription, a variant of C6 was constructed, containing a nucleotide substitution within the FAD8-derived C-terminal coding region (Fig. 1, C6alt). Throughout this region of the FAD8 sequence, 21 silent mutations were introduced to mimic the corresponding sequence of FAD7 (see “Experimental Procedures”). Despite such an extensive modification, this C6alt construct retained F8-type expression to an extent comparable to the unmodified C6 construct. The net change in leaf TA level attributable to C6alt expression upon the 5 °C-upshift in temperature was estimated to be −6.8 ± 1.8% (Table I and Fig. 2A, \( \Delta TA \)). This result alone is not sufficiently conclusive; but when considered in combination with the results of Northern blot analysis (Fig. 4B), it suggests that the C-terminal region of FAD8 protein, rather than its encoding nucleotide sequence, is essential for modulating the desaturase activity in response to temperature, possibly at the post-translational level.

### Table 1

**Summary of fatty acid analysis**

| Genotype       | \( \Delta TA \) | \( \Delta DA \) |
|----------------|-----------------|-----------------|
| fad7           | −12.1 ± 1.3     | +9.2 ± 1.3      |
| fad8           | −5.5 ± 0.7      | +1.7 ± 0.7      |
| fad7fad8       | −6.0 ± 1.1      | +3.7 ± 1.1      |
| +F8            | −15.1 ± 1.6     | +11.8 ± 1.3     |
| +F7            | −5.8 ± 1.2      | +2.7 ± 0.9      |
| +C1            | −5.4 ± 1.0      | +2.4 ± 1.0      |
| +C2            | −12.6 ± 1.3     | +7.3 ± 1.1      |
| +C3            | −15.3 ± 1.4     | +12.5 ± 1.0     |
| +C4            | −5.6 ± 1.0      | +1.9 ± 1.2      |
| +C5            | −14.0 ± 1.2     | +8.0 ± 1.6      |
| +C6            | −13.3 ± 1.5     | +10.3 ± 1.5     |
| +C6alt         | −12.8 ± 1.5     | +10.8 ± 1.3     |

\( ^a \) Net changes in leaf TA and DA levels attributable to endogenous FAD8 expression.

\( ^b \) Net changes in leaf TA and DA levels attributable to endogenous FAD7 expression.
sulted in a complete loss of enzymatic activity (data not shown). To incorporate an epitope tag without affecting the overall structure and functional properties of the desaturase, a short peptide that was immunoreactive with anti-c-Myc antibodies was appended to the N-terminal potentially extramembranous domain of the mature desaturase protein (Fig. 3A). Based on an analysis using the ChloroP program (31), both FAD7 and FAD8 were predicted to contain N-terminal pre-sequences for plastid targeting. The estimated lengths of the transit peptides were 81 and 42 amino acids for FAD7 and FAD8, respectively (Fig. 3A). To reduce the risk of dissociation of the epitope tag after processing the preproteins, the c-Myc sequence was inserted behind the putative cleavage site of FAD7 and the corresponding site of FAD8 (Figs. 1 and 3A). Variants encoding c-Myc-tagged desaturases (myc-FADs) were constructed for four of the transgenes shown in Fig. 1, F7, F8, C2, and C6. These modified constructs were designated as F7myc, F8myc, C2myc, and C6myc, respectively.

Fig. 3B shows Western blot detection of myc-FADs expressed in leaves of transgenic lines. Immunoreactive proteins of ~45 kDa were detected only in the transgenic lines, suggesting that these proteins represent myc-FADs derived from transgene expression. In the F8myc and C2myc lines, myc-FADs migrated as a single band with a mobility corresponding to 44.9 kDa. This molecular mass was in good agreement with the theoretical mass of mature myc-FAD8 cleaved at the position predicted by the ChloroP program (46.8 kDa, see Fig. 3A). In contrast, myc-FADs in the F7myc and C6myc lines were detected as doublet bands of 47.2 and 45.8 kDa, the former being the major subtype. Both subtypes of myc-FAD7 (expressed in the F7myc line) migrated slower than myc-FAD8, contrary to what might have been expected from the smaller theoretical mass of mature myc-FAD7 (43.6 kDa, see Fig. 3A).

Fig. 3C shows phenotypic complementation of the fad7fad8 double mutant by functional expression of myc-FADs. The leaf TA levels in all of the transgenic lines expressing myc-FADs were substantially higher than the level in the parental fad7fad8 double mutant, typically exceeding 40% at 22 °C. Between each pair of transgenic fad7fad8 lines expressing either myc-FADs (e.g. the F8myc line) or their non-tagged counterparts (e.g. the F8 line), no appreciable difference was noted in terms of leaf TA levels. Similar data were collected for all temperatures tested, including 27 °C (data not shown). These results suggest that myc-FADs retained specific activities comparable to those of their non-tagged counterparts. We conclude that the expression of myc-FADs, as monitored by Western blot and fatty acid analyses, provides critical information regarding overall expression patterns of endogenous FAD7 and FAD8 proteins.

C-terminal Region of FAD8 Desaturase Is Essential for Destabilization at High Temperature—To verify regulatory roles of the C-terminal region on the accumulation of FAD8 protein, effects of temperature on steady-state levels of myc-FADs derived from the four different transgenes were tested. After initial growth at 22 °C, plants expressing the myc-FADs were subjected to three different temperature-shift protocols. As shown by Western blot analysis in Fig. 4A, the amounts of both subtypes of myc-FAD7 accumulated in the F7myc line were nearly unaffected by the temperature shift. In contrast, the amounts of myc-FADs in the other three transgenic lines, including both subtypes in the C6myc line, decreased remarkably at 27 °C. This decrease was reversible, recovering to control levels after reincubation at 22 °C. Similar results were obtained also with the wild-type genetic background (data not shown). These results are consistent with the results shown in Fig. 2, suggesting that the temperature-mediated modulation of FAD8 activity is due to a change in the amount of FAD8 protein, rather than a change in its specific activity.

To dissect the mechanisms restricting the accumulation of FAD8 protein at high temperature, the time course of the decay of myc-FADs after treatment with cycloheximide (CHX), an inhibitor of protein synthesis, was examined at different temperatures. As shown in Fig. 5, the amount of myc-FAD7 did not change during the course of CHX treatment at both 22 and 27 °C. On the other hand, the products of F8myc and C6myc were somewhat more susceptible to CHX at 22 °C. The decay of
these myc-FADs was further accelerated at 27 °C; after 4 h, the amounts of the proteins were half of those detected at 22 °C. These results suggest that the C-terminal region of FAD8 desaturase acts in an autoregulatory fashion to destabilize the protein at high temperature, causing a reduction in the amount of active enzyme (Fig. 4A) in the absence of a concurrent decrease in its transcript level (Fig. 4B).

**DISCUSSION**

In this study, we developed an experimental system allowing the identification of post-transcriptional mechanisms that regulate the activities of FAD7 and FAD8 desaturases in response to temperature. Based on structural relatedness, a series of FAD7/FAD8 chimeric genes were constructed that encoded a functional plastidial ω-3 desaturase, either with or without a c-Myc epitope tag (Fig. 1). Introduction of these chimeric genes into the Arabidopsis fad7fad8 double mutant resulted in accumulation of their transcripts and proteins (Figs. 3B and 4) followed by enhanced production of TAs in leaf tissues (Fig. 3C), which appeared to correlate with the ω-3 desaturase activity of the gene products. Although each chimeric gene exhibited either temperature-sensitive or -insensitive expression, an inconsistency was noted between changes in the amounts of transcripts and proteins (Fig. 4, compare A and B). Upon examination by Northern blot analysis, it appeared that transcript levels of chimeric genes reflected the origin of the 5′-flanking region, generally containing the transcriptional promoter (Fig. 4B). In contrast, as evidenced by Western blot and fatty acid analyses, changes in the amounts of translational products (Fig. 4A) and their metabolite TAs (Table I and Fig. 2) were regulated via the C-terminal coding region. Such inconsistency was at least in part due to post-translational destabilization of the ω-3 desaturase protein at high temperature, which was triggered only if the desaturase contained the FAD8-derived C-terminal region (Fig. 5). These findings suggest that, although the temperature-sensitive expression of FAD8 desaturase is regulated at both the transcriptional and post-translational levels, the response of its enzymatic activity to temperature changes within the physiological range depends mainly on post-translational mechanisms.

Although it is possible to define the C-terminal 44 amino acids of FAD8 desaturase as the temperature-sensitive regulatory region, the mechanism of its action on the stability of ω-3...
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Fig. 4. C-terminal coding region of FAD8 is sufficient to suppress the accumulation of plastidial \(\omega\)-3 desaturase protein at high temperature. After initial growth at 22 °C for 16 days, the indicated transgenic and wild-type (WT) plants were subjected to different temperature-shift protocols as follows: 22 °C (control), kept at 22 °C for 2 days, 22–27 °C, kept at 22 °C for 1 day and shifted to 27 °C for 1 day; 22–27–22, shifted to 27 °C for 1 day and reincubated at 22 °C for 1 day. A, total leaf proteins (40 \(\mu\)g) from each treatment were subjected to Western blot analysis using anti-c-Myc antibody (Anti c-Myc). The same blots were stained with Amido Black to verify equal loading and transfer of proteins (Total). Minor subtypes of myc-FADs in F7myc and C6myc lines (Fig. 3B) are not visible in this figure but showed a temperature response similar to that of major subtypes. RBCL, large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. B, total RNA (20 \(\mu\)g) from each treatment was subjected to Northern blot analysis using duplicated DNA probes specific to \(3'-\)untranslated sequences of FAD8 and FAD7 (Fig. 1). Equal loading of RNA was confirmed by ethidium bromide staining (data not shown). UTR, untranslated region.

A

B

Temperature shift (°C)

22

22–27

22–27–22

22

22–27

22–27–22

F8myc

F7myc

C2myc

C6myc

Anti c-Myc

Total (RBCL)

FAD8

FAD7

probe (3’UTR)

RNA

mRNA

Unlike the \(\Delta9\) stearoyl-acyl carrier protein desaturase localized in the soluble stromal fraction (37), the regulation of plastidial acyl-lipid desaturases, including \(\omega\)-3 desaturases, has not been thoroughly investigated at the post-transcriptional level (38). This is primarily due to difficulties in isolating active forms of such membrane-bound enzymes (39) and in obtaining monospecific antibodies against them (12). A heterologous expression system using yeast as the host has proven advantageous in dissecting biochemical properties (substrate specificity and regioselectivity) (40) and post-transcriptional regulation (41) of ER-localized \(\omega\)-3 desaturases. A heterotrophic host, however, is not suited for the analysis of plastidial isozymes, because they require electron transfer chains specific to plastidial membranes (38, 42). Neither are photosynthetic unicellular organisms, such as cyanobacteria, the free-living ancestors of plastids (43), ideal heterologous hosts for the analysis of plastidial \(\omega\)-3 desaturases from higher plants. Unlike yeast cells, they typically contain high levels of polyunsaturated fatty acids and several endogenous fatty acid desaturases acting on TAs and/or DAs (44), which would make it difficult to estimate the net activities of exogenous \(\omega\)-3 desaturases from the fatty acid composition. The first attempt to immunodetect the \(\omega\)-3 desaturase protein by means of an epitope-tagging strategy has been performed
with an ER-localized desaturase expressed in the yeast system (41). Again, it is highly difficult to apply this strategy to plastidial isozymes, because they contain N-terminal presequences for plastid targeting. Moreover, the introduction of C-terminal epitope tags into the desaturase results in a complete loss of enzymatic activity. Nevertheless, we succeeded in complementing the fad7fad8 double mutant through functional expression of c-Myc-tagged FAD7 and FAD8 desaturases (Fig. 3). With a set of transgenic plants expressing the different epitope-tagged variant of the three ω-3 desaturase isozymes now available, it will be feasible to investigate further their differential regulation from the transcriptional to post-translational levels. As is evident from Fig. 3B, functional structures of the two plastidial isozymes appear to be different. To establish whether these structural differences are related to their responsiveness to temperature and other physiological properties, we are planning to study the assembly and intramolecular modification (by both proteolysis and covalent modification) of the isozymes, using affinity-purified epitope-tagged desaturases. A deeper understanding of the mechanisms underlying the regulation of ω-3 desaturase activity will help to clarify the adaptive strategies of plants in elevated temperatures.

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