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Xanthoxin Metabolism in Cell-free Preparations from Wild Type and Wilty Mutants of Tomato[1]

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ABSTRACT

Extracts prepared from the turgid and water-stressed leaves of wild-type tomato (*Lycopersicon esculentum* Mill cv Ailsa Craig) and the wilty mutants *sitiens*, *notabilis*, and *flacca* were tested for their ability to metabolize xanthoxin to ABA. Extracts from wild type and *notabilis* converted xanthoxin at similar rates, while extracts from *sitiens* and *flacca* showed little or no activity. We also observed no activity when extracts of *sitiens* and *flacca* were mixed. Similar results were obtained when ABA aldehyde was used as a substrate, in that extracts from wild type and *notabilis* were equally active, but extracts from *flacca* and *sitiens* showed little activity. None of the tomato extracts showed significant activity with xanthoxin acid, xanthoxin alcohol, or ABA-1′,4′-*Trans*-diol as substrates. Extracts from bean leaves (*Phaseolus vulgaris* L. cv Blue Lake) were similar to the wild-type tomato extracts in their ability to convert the various substrates to ABA, although excised bean leaves did convert ABA-1′,4′-*trans*-diol and xanthoxin alcohol to ABA when these substances were taken up through the petiole. These results are consistent with a role for xanthoxin as a normal intermediate on the ABA biosynthetic pathway, and they suggest that ABA aldehyde is the final ABA precursor.

Although the details of the ABA biosynthetic pathway have not yet been delineated, several lines of evidence suggest that the pathway includes the oxidative cleavage of a xanthophyll to a C-15 compound that is metabolized to ABA. Mutants lacking xanthophylls, or in which xanthophyll biosynthesis is blocked by inhibitors, show a greatly reduced ability to accumulate ABA (11–13). ABA produced by water-stressed plants in the presence of $^{18}O_2$ has one ^{18}O atom incorporated into the carboxyl group (2, 7). This result is consistent with the dioxygenase cleavage of a xanthophyll to a C-15 aldehyde followed by oxidation of the aldehyde to the carboxyl of ABA by a dehydrogenase so that the second oxygen is supplied by water (3). One candidate for the C-15 aldehyde is the naturally occurring compound Xan[2] (II) (20). Xan was produced *in vitro* from violaxanthin by lipoxygenase and UV light (5, 21), was converted to ABA when fed in radioactive form to tomatoes and beans (22), and was converted to ABA by extracts from the leaves of several plants (19). Despite these results, the role of Xan as an ABA precursor has remained questionable for several reasons. It is present at very low levels in plants and does not increase significantly in water-stressed plants even when ABA levels rise 50-fold (4, 16). Xan was not observed to be labeled significantly when plants were stressed in the presence of $^{18}O_2$ or ^2H_2O, suggesting that its synthesis is not concurrent with that of ABA that is labeled under those conditions (3, 14, 16).

Recently, Parry *et al.* (16) reported that the wilty tomato mutants *flc* and *sit*, which contain carotenoids but are unable to accumulate ABA when their leaves are water stressed, converted ^{13}C-Xan to ABA very poorly. The wild-type tomato and a third wilty mutant, *not*, readily metabolized Xan to ABA (16). These results are consistent with a precursor role for Xan, suggesting that the failure of *flc* and *sit* to accumulate ABA is due to their inability to convert Xan to ABA. We reported that extracts from the leaves of several plants convert Xan to ABA at rates which appear to be sufficient to account for the formation of ABA in water-stressed leaves (19). The work reported here was undertaken to determine how cell-free extracts from the various tomato mutants compare in their abilities to metabolize Xan and related compounds to ABA.

MATERIALS AND METHODS

Plant Material. Seeds of *Lycopersicon esculentum* Mill. cv Ailsa Craig wild type, *not*, *flc*, and *sit* were a gift from Dr. Roger Horgan, University College of Wales, UK. The seeds were germinated in flats in a 1:1 (v/v) vermiculite-soil mixture in a greenhouse at about 21°C. The seedlings were transferred to 20-cm pots containing the same mixture when they were about 5 weeks old. The plants were grown in the greenhouse under natural light and were watered twice daily. Detached leaves were stressed by allowing them to lose 12% of their fresh weight in a growth chamber. The leaves were then wrapped in aluminum foil and kept at ambient temperature for various periods of time. Plants of *Phaseolus vulgaris* L., cv Blue Lake, were grown in flats containing the soil-vermiculite mixture in the greenhouse. Fully expanded primary leaves from 2 to 4 week old plants were used for experiments. The detached bean leaves were stressed as described for tomatoes.

Preparation of Enzyme Extracts. Tomato leaves were ground with a mortar and pestle in 0.2 M KPi, pH 7.5, containing 7.5 mM DTT (3 mL/g leaf material). The extract was passed through 4 layers of cheesecloth and was centrifuged at 13,000 *g* for 20 min at 4°C, and the supernatant used for enzyme assays. Bean leaves were homogenized with a mortar and pestle in 50 mM KPi, pH 7.5 (3 mL/g leaf material). The extract was passed through four layers of cheesecloth and centrifuged at 13,000*g* for 20 min at 4°C. The supernatant was precipitated with acetone as previously described (19). The acetone precipitate was extracted with 50 mM KPi, pH 7.5, and was centrifuged at 13,000*g* for 20 min, and the supernatant was used for enzyme assays.

Enzyme Assays. The assay tubes contained 0.4 to 0.5 mL 0.2 M KPi, 0.25 to 0.4 mL enzyme extract, 1 μmol NADP, and

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[2] Abbreviations: Xan, xanthoxin; Xan acid, Xanthoxin acid; Xan alc, xanthoxin alcohol; ABA-*t*-diol; ABA 1′,4′-*trans*-diol; *t*-ABA alc, 2-*trans*-ABA alcohol; *t*-Xan alc, 2-*trans*-xanthoxin alcohol; ABA alc, ABA alcohol; ABA ald, ABA aldehyde; *flc*, *flacca*; *sit*, *sitiens*; *not*, *notabilis*.

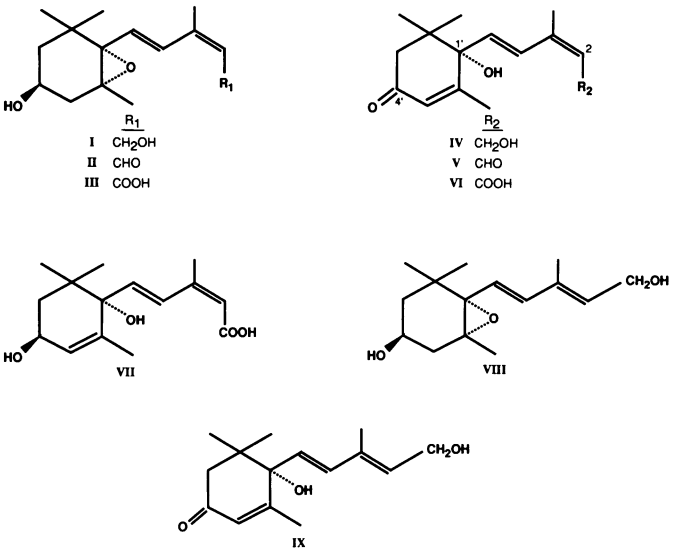

FIG. 1. Structures of compounds discussed in text. I, Xan alc; II, Xan, III, Xan acid; IV, ABA alc; V, ABA ald: VI, ABA; VII, ABA-*t*-diol; VIII, *t*-Xan alc; LX, *t*-ABA alc.

various amounts of substrate in a total volume of 0.85 mL. The tubes were incubated at 28°C for 1 h, and the reaction was stopped by the addition of 90 μL concentrated HCl. [^{3}H-]ABA (12,000 dpm, specific activity 10 Ci mmol^{-1}) (24) was added to the solution, which was then chilled and centrifuged. The purification and estimation of ABA has been described previously (19).

Preparation of Xan, ABA Ald, ABA-*t*-Diol, Xan Acid, and Xan Alc. Xan was prepared by zinc permanganate oxidation of violaxanthin as described by Taylor and Burden (21) with some modifications (19).

ABA ald and its 2-*trans* isomer were prepared by the chromium trioxide/pyridine oxidation of Xan (21). The products were initially purified by HPLC on a 5 μm ODS column. Elution was with a linear gradient of 50 to 100% methanol over 15 min at a flow rate of 0.8 mL min^{-1}. The isomers were further purified by HPLC on a 3 μm silica column (4.6 × 150 mm). Elution was isocratic with hexane containing 5% isopropanol at 1 mL min^{-1}.

The Xan acids were prepared from Xan according to (21). The isomers of the methyl esters were separated by HPLC on a 3 μm silica column (4.6 × 150 mm) isocratically with hexane containing 5% isopropanol at 1 mL min^{-1}. After hyrolysis, the acids were further purified by HPLC on an ODS column (4.6 × 150 mm). Elution was with a linear gradient of 50 to 100% methanol containing 0.1 N acetic acid in 15 min at 0.8 mL min^{-1}.

The 1′,4′-diols of ABA were prepared from ABA according to Hirai *et al.* (6). The *cis*- and *trans*-diols were separated by HPLC on an ODS column (4.6 × 250 mm). Elution was with a linear gradient of out 40 to 100% methanol over 15 min at 0.8 mL min^{-1}.

The Xan alc were prepared by $NaBH_4$ reduction of Xan (21). The alcohols were initially purified by HPLC on a 5 μm ODS column (4.6 × 150 mm) using a linear gradient of 40 to 100% methanol over 15 min at 0.8 mL min^{-1}. The two isomeric alcohols were separated by HPLC on a 3 μm silica column (4.6 × 150 mm) isocratically with hexane containing 5% isopropanol at 1 mL min^{-1}.

GC-MS. The identities of all of the compounds prepared were confirmed by GC-MS. Methyl esters (18) of ABA and ABA-*t*-diol were analyzed by GC-MS with a Finnegan 4000 GC/MS/DS system. The GC was performed on a SPB-1 fused silica capillary column (30 m × 0.25 mm i.d.) with a film thickness of 0.25 mm (Supelco, Bellefonte, PA). The carrier gas was helium at 1 mL min^{-1}. After a 2-min hold at 50°C, the oven temperature was raised by 10°C min^{-1}. Mass spectrometry was by electron impact at 70 eV. For all of the other compounds, the conditions were the same except that the column was 8 m long.

RESULTS

When crude extracts from the leaves of wild-type tomatoes were incubated with Xan, no detectable ABA was produced. Neither dialysis nor acetone precipitation produced active fractions as they do with bean leaves (19). Consequently, EDTA, DTT, BSA, and phenylmethylsulfonyl fluoride were added to the homogenizing and incubation media. In the presence of these compounds, Xan was converted to ABA. Of these, only DTT appears to be necessary for activity (Table I). With 10 mM DTT in both the homogenizing and assay media, a second compound was produced in addition to ABA. This compound was identified as *t*-ABA, and it accumulated to a level twice that of ABA. The formation of *t*-ABA was shown to be due to the rapid nonenzymic conversion of xan to *t*-xan by DTT. Since DTT appeared to be necessary for activity, we modified our procedure to reduce the isomerization of Xan. When DTT was added to the homogenization medium at 7.5 mM, but left out of the assay medium, near maximal enzymic activity was observed with relatively little production of *t*-ABA. In the case of bean leaf extracts, activity is greatly enhanced after dialysis or acetone precipitation. The tomato extracts lost all activity, however, after either dialysis or acetone precipitation even when DTT was included in all solutions. As is the case with beans, the crude tomato leaf extracts do not require the addition of NADP.

When leaves from wild type and the three mutants were water stressed, only the wild type accumulated ABA (Table II). When extracts from either stressed or unstressed leaves of wild type, *flc*, *sit*, and *not* were incubated with Xan, wild type and *not* produced similar amounts of ABA, but *flc* and *sit* produced little or no ABA (Table III). When extracts from *flc* and *sit* were mixed, there was still no observable activity.

We were interested to determine if other compounds related to Xan or ABA would be good substrates for conversion to ABA

Table I. *Effect of DTT, EDTA, BSA, and PMSF on Xan Oxidizing Activity by Extracts of Wild-Type Tomato*

Assay contained 1 μg Xan, 1.25 mM NADP, extract equivalent to 100 mg fresh weight leaf tissue; incubation 1 h at 28°C. EDTA, BSA, DTT, and PMSF in homogenization and incubation media at 0.25 mM, 0.5 mg mL^{-1}, 10 mM, and 1 mM, respectively.

Media	ABA	*t*-ABA
	ng	
Complete	151	250
−EDTA	83	222
−DTT	ND[a]	ND
−PMSF	85	171
−BSA	151	298

[a] ND = not detected.

Table II. *ABA Levels in Turgid and Stressed Leaves of Wild Type and Wilty Tomato Mutants*

Leaves harvested 4 h after 12% fresh weight loss.

Leaves	Turgid	Stressed
	ng ABA/g fresh wt	
Wild type	223	644
not	231	196
flc	103	98
sit	61	62

Table III. *Xan Oxidizing Activity in Extracts from Turgid and Water-Stressed Leaves of Wild-Type and Mutant Tomatoes*

Assay contains 500 ng Xan, 1.2 mM NADP, extract equivalent to 100 mg fresh weight tissue; homogenization medium contains 7.5 mM DTT; incubation 1 h at 28°C.

Extract	Turgid	Stressed
	ng ABA	
Wild type	166	307
not	191	155
sit	0	8
flc	6	3
sit + *flc*	10	ND[a]

[a] Not detected.

Table IV. *Conversion of Xan, Xan Acid, ABA Ald and ABA-t-Diol to ABA by Extracts from Turgid Wild-Type and Mutant Tomato Leaves*

Assay contains 1 μg substrate; incubation for 2 h. Other conditions as in Table III.

Extract	Substrate			
	Xan	Xan acid	ABA ald	ABA-*t*-diol
	ng ABA			
Wild type	241	24	245	16
not	190	17	461	15
sit	2	3	ND[a]	10
flc	ND	15	ND	14

[a] Not detected.

Table V. *Conversion of Xan, ABA Ald, t-ABA Ald, ABA-t-diol, and Xan Acid to ABA by Bean Leaf Extracts*

Assay contains 500 ng substrate, acetone-precipitated leaf extracts equivalent to 80 mg tissue fresh weight, 1.2 mM NADP. Incubation 1 h at 28°C.

Substrate	ABA	*t*-ABA
	ng	
Xan	135	ND[a]
ABA ald	153	30
t-ABA ald	27	164
ABA-*t*-diol	5	ND
Xan acid	19	ND

[a] Not detected.

Table VI. *Conversion of Xan and Xan Alc to ABA and t-ABA by Extracts of Wild-Type Tomato Leaves*

Assay contains 1 μg Xan or Xan alc. Other conditions as in Table III.

Compound	ABA	*t*-ABA
Xan	568	ND[a]
Xan alc	20	ND
t-Xan alc	11	46
Xan + Xan alc	598	ND

[a] Not detected.

under the same conditions. Xan acid (III) has the same ring structure as Xan, but the sidechain carbonyl is oxidized to a carboxyl. Xan acid was converted to ABA at low rates when fed to tomatoes (9) and accumulated at low levels when bean and tomato plants were incubated with ^{14}C-Xan (22). We observed little or no conversion of Xan acid to ABA by either tomato or bean leaf extracts under our standard conditions (Tables IV and V). We also measured the conversion of Xan alc (I) to ABA. Cell-free extracts from tomato showed very little activity with this compound (Table VI). Bean leaf extracts showed little activity with Xan alc, but they did convert *t*-Xan alc (VIII) to *t*-ABA at an appreciable rate (Table VIII). Conversely, intact bean leaves converted Xan alc to ABA at good rates, but conversion of *t*-Xan alc to either ABA or *t*-ABA was considerably slower (Table VII).

Table VII. *Conversion of Xan, Xan Alc, and t-Xan Alc to ABA and t-ABA by Intact Bean Leaves*

Compounds fed through the petiole for 135 min.

Compound Fed	Uptake	ABA	*t*-ABA
		ng/g fresh wt	
Control		54	ND[a]
Xan	1880	537	ND
Xan alc	1650	708	35
t-Xan alc	2430	151	187

[a] Not detected.

Table VIII. *Conversion of Xan Alc and t-Xan Ac to ABA and t-ABA by Bean Leaf Extracts*

Conditions as in Table V except that 2 μg substrate used.

Substrate	Products	
	ABA	*t*-ABA
	ng	
Xan	1385	ND
Xan alc	112	120
t-Xan alc	20	406

ABA-*t*-diol (VII) differs from ABA in that the 4′-keto group is reduced to a hydroxyl. This compound has been isolated from plants fed ABA (1, 10, 15). Parry *et al.* (16) reported that tomato plants fed the diol converted it to ABA at about the same rate as they converted Xan but that there was no difference in rates between the tomato mutants. When the various tomato extracts were incubated with ABA-*t*-diol, we observed little or no formation of ABA by any of them (Table IV). We observed similar results when bean leaf extracts were incubated with the diol (Table V). The diol was converted to ABA, but at a slower rate than Xan, when fed to intact bean leaves through their petioles (data not shown).

ABA ald (V) has not been reported in plants to our knowledge, nor has it apparently been observed as a metabolite when ABA has been fed to intact tissues. With Xan as substrate, ABA ald(V) would be a precursor to ABA, if the Xan ring transformations precede the oxidation of the side-chain carbonyl. Since Xan acid was not a substrate, this suggested to us that the ring transformations do precede the side-chain oxidation. As shown in Table VI, ABA ald was converted to ABA by the extracts from wild-type and *not* leaves at rates which are similar to the conversion of Xan. Extracts from either *flc* or *sit* leaves, however, showed little or no activity. Bean leaf extracts also converted ABA ald to ABA at rates similar to those for Xan, while *t*-ABA ald was converted predominantly to *t*-ABA at rates comparable to that for the conversion of ABA ald to ABA (Table V).

DISCUSSION

The results obtained for the conversion of Xan to ABA by the extracts from the wild type and the three tomato mutants are similar to the ^{13}C-Xan feeding studies reported by Parry *et al.* (16). *Not* and wild type have similar rates of conversion, while extracts from either turgid or stressed leaves of *sit* and *flc* show little activity. As previously noted, these results suggest that the inability of *not* to accumulate ABA is due to a metabolic block prior to the cleavage of a xanthophyll, while *sit* and *flc* are blocked in the conversion of a precursor C-15 compound to

ABA (16). Taylor (23) has also suggested similar metabolic blocks for these mutants on the basis of other evidence.

The results obtained with Xan acid and ABA-*t*-diol suggest that neither are intermediates in the cell-free conversion of Xan to ABA under the conditions used in our assays. In the case of beans, we observed that ABA-*t*-diol was converted to ABA when fed to detached leaves, but the cell-free extracts did not convert the diol to ABA. In tomato, the conversion of the diol by the cell-free extracts was very low and did not appear to depend on the cultivar used. In contrast, Parry *et al.* (16) fed ABA-*t*-diol to these tomatoes and found the conversion to ABA to be similar to that produced when Xan was fed, except that neither *sit* nor *flc* was impaired in its oxidative ability.

Unlike the other two compounds, ABA ald was converted to ABA by cell-free extracts from beans, the wild-type tomato, and *not* at rates comparable to that for Xan. Extracts from *flc* and *sit*, however, did not convert ABA ald to ABA at significant rates. Parry *et al.* (16) noted that when Xan is converted to ABA the final ABA precursor could be Xan acid, ABA-*t*-diol, or ABA ald (16). Our results suggest that ABA ald is the penultimate compound in the conversion of Xan to ABA.

Recently, Linforth *et al.* (8) reported that stressed leaves of *sit* and *flc*, unlike *not* and the wild type, accumulate *t*-ABA alc (IX). They suggested that this compound is on the ABA biosynthetic pathway and is normally converted to ABA via ABA ald. The required isomerization would occur either at the level of *t*-ABA alc or *t*-ABA ald. They suggested that the mutations in *flc* and *sit* block the normal metabolism of *t*-ABA alc. Another possibility is that ABA aldehyde is reduced to ABA alcohol in the mutants, since it cannot be oxidized to ABA. As Linforth *et al.* observed, it is not possible for both Xan and ABA alc to be on the normal metabolic pathway to ABA. They speculated that the conversion of exogenous Xan to ABA may be due either to the similarity of Xan to ABA ald or to the nonspecific conversion of Xan to ABA ald. The fact that Xan, but not Xan acid, is converted to ABA suggests to us that the required changes in the ring are specific.

If *t*-ABA alc is an ABA intermediate, and not the product of an abnormal metabolism, another explanation is possible for both our results and those of Linforth *et al.* (8) If the normal cleavage of violaxanthin produces *t*-Xan alc (VIII) rather than Xan, both exogenous Xan and endogenous *t*-Xan alc could be converted to ABA via ABA ald (Fig. 2). The facile conversion of Xan to ABA would be because it resembles Xan alc. Our results suggest that such an explanation is unlikely. Neither the bean nor tomato extracts readily converted *t*-Xan alc to ABA. Since the assays were optimized for the conversion of Xan to ABA, the low conversion of *t*-Xan alc may have been due to suboptimal assay conditions. When *t*-Xan alc was fed to detached leaves, however, it was converted mostly to *t*-ABA at low rates compared with the conversion of Xan alc to ABA. These results suggest that *t*-Xan alc is not an ABA precursor. They do not, however, preclude Xan alc as a possible ABA precursor.

The mutations in *sit* and *flc* have been mapped to different chromosomes (17), which suggests that they affect different polypeptides. Our observation that a mixture of extracts from the two mutants was not able to convert Xan to ABA makes it unlikely that the lesions are in two different enzymes acting independently of one another. The inability of either *flc* or *sit* to oxidize ABA ald to ABA is consistent with the failure of the two extracts to be complementary, although it remains to be determined how mutations in two different proteins affect the same enzymic activity. We did not isolate any products when Xan was incubated with the mutants, so we do not know whether the initial steps in the reaction are also blocked by either one of the mutants. These mutants do not accumulate Xan when their leaves are stressed (16), so further metabolism must occur if Xan is a normal intermediate on the ABA biosynthetic pathway. The results reported by Linforth *et al.* (8) suggest that *t*-ABA alc may be such a metabolite.

The results reported in this paper are consistent with the assumption that Xan is a normal intermediate on the ABA biosynthetic pathway. Tomato mutants that do not accumulate ABA are unable to convert Xan to ABA. Our results still do not provide definitive evidence that Xan is a normal intermediate on the ABA biosynthetic pathway, however, since the lesion(s) affect the metabolism of an ABA precursor that can be derived from Xan, and not the metabolism of Xan directly.

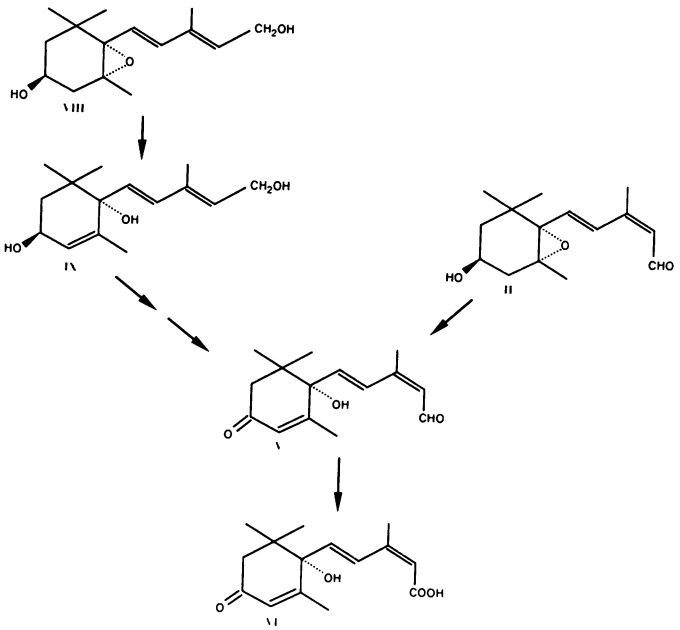

FIG. 2. Pathways to ABA (VI) from Xan (II) and *t*-Xan alc (VIII).

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