Auxin-Induced Ethylene Triggers Abscisic Acid Biosynthesis and Growth Inhibition[1]

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The growth-inhibiting effects of indole-3-acetic acid (IAA) at high concentration and the synthetic auxins 7-chloro-3-methyl-8-quinolinecarboxylic acid (quinmerac), 2-methoxy-3,6-dichlorobenzoic acid (dicamba), 4-amino-3,6,6-trichloropicolinic acid (picloram), and naphthalene acetic acid, were investigated in cleavers (*Galium aparine*). When plants were root treated with 0.5 mM IAA, shoot epinasty and inhibition of root and shoot growth developed during 24 h. Concomitantly, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase activity, and ACC and ethylene production were transiently stimulated in the shoot tissue within 2 h, followed by increases in immunoreactive (+)-abscisic acid (ABA) and its precursor xanthoxal (xanthoxin) after 5 h. After 24 h of treatment, levels of xanthoxal and ABA were elevated up to 2- and 24-fold, relative to control, respectively. In plants treated with IAA, 7-chloro-3-methyl-8-quinolinecarboxylic acid, naphthalene acetic acid, 2-methoxy-3,6-dichlorobenzoic acid, and 4-amino-3,6,6-trichloropicolinic acid, levels of ethylene, ACC, and ABA increased in close correlation with inhibition of shoot growth. Aminoethoxyvinyl-glycine and cobalt ions, which inhibit ethylene synthesis, decreased ABA accumulation and growth inhibition, whereas the ethylene-releasing ethephon promoted ABA levels and growth inhibition. In accordance, tomato mutants defective in ethylene perception (*never ripe*) did not produce the xanthoxal and ABA increases and growth inhibition induced by auxins in wild-type plants. This suggests that auxin-stimulated ethylene triggers ABA accumulation and the consequent growth inhibition. Reduced catabolism most probably did not contribute to ABA increase, as indicated by immunoanalyses of ABA degradation and conjugation products in shoot tissue and by pulse experiments with [^{3}H]-ABA in cell suspensions of *G. aparine*. In contrast, studies using inhibitors of ABA biosynthesis (fluridone, naproxen, and tungstate), ABA-deficient tomato mutants (*notabilis*, *flacca*, and *sitiens*), and quantification of xanthophylls indicate that ABA biosynthesis is influenced, probably through stimulated cleavage of xanthophylls to xanthoxal in shoot tissue.

Plant growth and development is regulated by hormones, of which auxins are one of the classical five types, together with ethylene, gibberellins, (+)-abscisic acid (ABA), and cytokinins (Kende and Zeevaart, 1997; Taiz and Zeiger, 1998). In cooperation with the other hormones, auxins, with indole-3-acetic acid (IAA) as the principal auxin in higher plants, stimulate fundamental processes such as cell elongation and division (Kende and Zeevaart, 1997). However, with increasing concentrations at the cellular sites of action, IAA induces a variety of growth abnormalities, including epinasty and inhibition of shoot (decreased internode elongation and leaf area) and root growth (Sterling and Hall, 1997). This effect provides the basis for the use of synthetic mimics of auxins as herbicides and bioregulators in agriculture (Sterling and Hall, 1997; Grossmann, 1998). Stimulation of ethylene biosynthesis is a common response when auxins are applied to sensitive species (Abeles et al., 1992; Sterling and Hall, 1997; Grossmann, 1998) or overproduced in transgenic plants (Klee and Lanahan, 1995). Auxins have been shown to induce de novo synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase through increased expression of specific ACC synthase genes or post-transcriptional regulation (Kende and Zeevaart, 1997; Grossmann, 1998; Taiz and Zeiger, 1998; Wei et al., 2000).

Conclusive evidence has recently been presented that the stimulation of ethylene biosynthesis through induction of ACC synthase activity plays the crucial role in auxin-induced growth inhibition (Grossmann and Schmülling, 1995; Sterling and Hall, 1997; Grossmann, 1998; Wei et al., 2000). In sensitive grasses, overproduction of cyanide, which is formed at physiologically damaging concentrations as a coproduct of ethylene during the oxidation of ACC, is implicated in phytotoxic growth inhibition (Grossmann, 1998; Grossmann and Kwiatkowski, 2000). However, the early, growth-retarding effects of auxins in sensitive dicots are not caused by this phytotoxic agent (Grossmann, 1998). As first shown for the action of the auxin herbicide 7-chloro-3-methyl-8-quinolinecarboxylic acid (quinmerac) in cleavers (*Galium aparine*), the stimulation of ethylene biosynthesis was accompanied by a subsequent increase of ABA (Scheltrup and Grossmann, 1995; Grossmann, 1998; Grossmann and Scheltrup, 1998). This increase was initially detectable at 5 h after root treatment. Massive accumulations of ABA in root and even more in shoot tissue proceeded and reached 70 times the level in controls after 48 h (Scheltrup and Grossmann, 1995). Experiments with isolated plant organs revealed that quinmerac-induced increases in ethylene
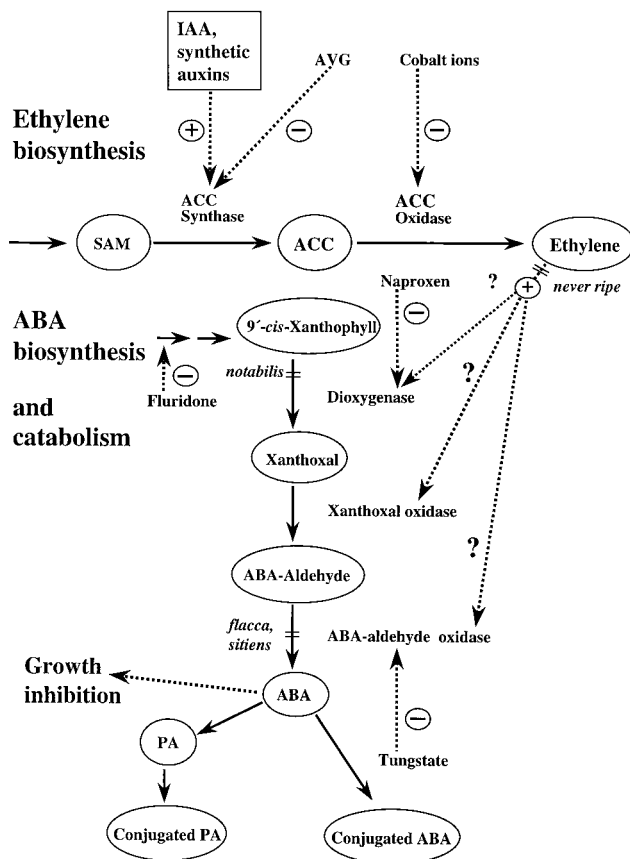
and ABA occur exclusively in the shoot tissue (Grossmann and Scheltrup, 1995). From there, ABA appears to be translocated within the plant. In accordance with its known physiological function in the plant (Taiz and Zeiger, 1998), quinmerac-induced ABA closely correlates with stomatal closure and consequent reductions in transpiration, CO_2 assimilation, and shoot and root growth (Grossmann and Scheltrup, 1995; Scheltrup and Grossmann, 1995; Grossmann et al., 1996). Similar correlations have been found for other auxin herbicides and IAA at high concentration in a variety of dicot species (Grossmann et al., 1996). In Gramineae species, auxin herbicides induced only slight accumulations of ABA, which were not enough to cause reductions in CO_2 assimilation and shoot growth (Grossmann, 1998). Therefore, although phenomena such as leaf and stem epinasty could be attributed to auxin-stimulated ethylene or auxin activity alone (Abeles et al., 1992; Sterling and Hall, 1997; Grossmann 1998), it was hypothesized that auxin-induced ethylene synthesis is connected with ABA accumulation and growth inhibition in sensitive dicots (Fig. 1).

Figure 1. Proposed model illustrating the mechanism of action of IAA at high concentration and synthetic auxins in relation to induced ethylene synthesis and triggered ABA accumulation and consequent growth inhibition. Target sites of inhibitors (dotted arrows) and mutants used in experiments are indicated. SAM, S-Adenosyl-Met.

The present study was undertaken to test the causal relationships of the latter hypothesis and to determine if it applies to other synthetic auxins and particularly to IAA. We have focused our investigations on growth and biochemical changes in the shoot of *G. aparine* plants, because this dicot weed responds very sensitive to auxins and the shoot tissue was found to be the principal site of biochemical auxin action (Grossmann and Scheltrup, 1995, 1998; Scheltrup and Grossmann, 1995). Following root treatment, the time course of IAA effects on ACC synthase activity, ACC and ethylene production, and immunoreactive levels of ABA and its precursor xanthoxal were determined. Causality was established by molecular dissection of the target pathways using inhibitors and tomato mutants defective in hormone synthesis or signaling (Fig. 1). In addition we have characterized the target site of auxin-induced increases in ABA by studying ABA catabolism and changes in biosynthetic precursors (Fig. 1).

RESULTS AND DISCUSSION

Auxin-Induced Ethylene Synthesis, ABA Accumulation, and Growth Inhibition

After hydroponic treatment of *G. aparine* plants at the third whorl stage with 0.5 mM IAA, epinastic symptoms on stems and leaves developed within 4 h and subsequently inhibition of root and shoot growth commenced during the next 20 h. The plants were stunted, internode elongation and leaf area were reduced, and shoot fresh weight remained 17% below those of control plants at 24 h after treatment. During this time, the water content, expressed by the fresh-to-dry weight ratio, and the osmotic potential in the shoot tissue were not changed (data not shown). These phenomena correspond to those elicited by quinmerac or other synthetic auxins in *G. aparine* (Scheltrup and Grossmann, 1995; Grossmann et al., 1996). Within 2 h of IAA exposure, ACC synthase activity, ACC, and ethylene production were transiently stimulated in the shoot tissue (Fig. 2). The increase in ACC synthase was initially detectable as early as 1 h after treatment and peaked at 3 h with an activity 11-fold higher than the basal level. The time course revealed that ACC and ethylene production increased to maximum values of 8- and 6-fold, relative to the control, respectively, after 5 h (Fig. 2). Levels of ACC synthase activity, ACC, and ethylene then declined. Compared with the stimulation of ethylene synthesis over time, immunoreactive levels of ABA and isomers of its precursor xanthoxal began to increase more slowly and, significantly, with a lag phase of 5 h in the shoot tissue (Fig. 2). Immunoreactive xanthoxal levels represent the total of cis- and trans-xanthoxal, because of the selected HPLC conditions and the given cross-reactivities of the antibody for both isomers of xanthoxal in the determination. During 24 h of IAA treatment, xanthoxal and
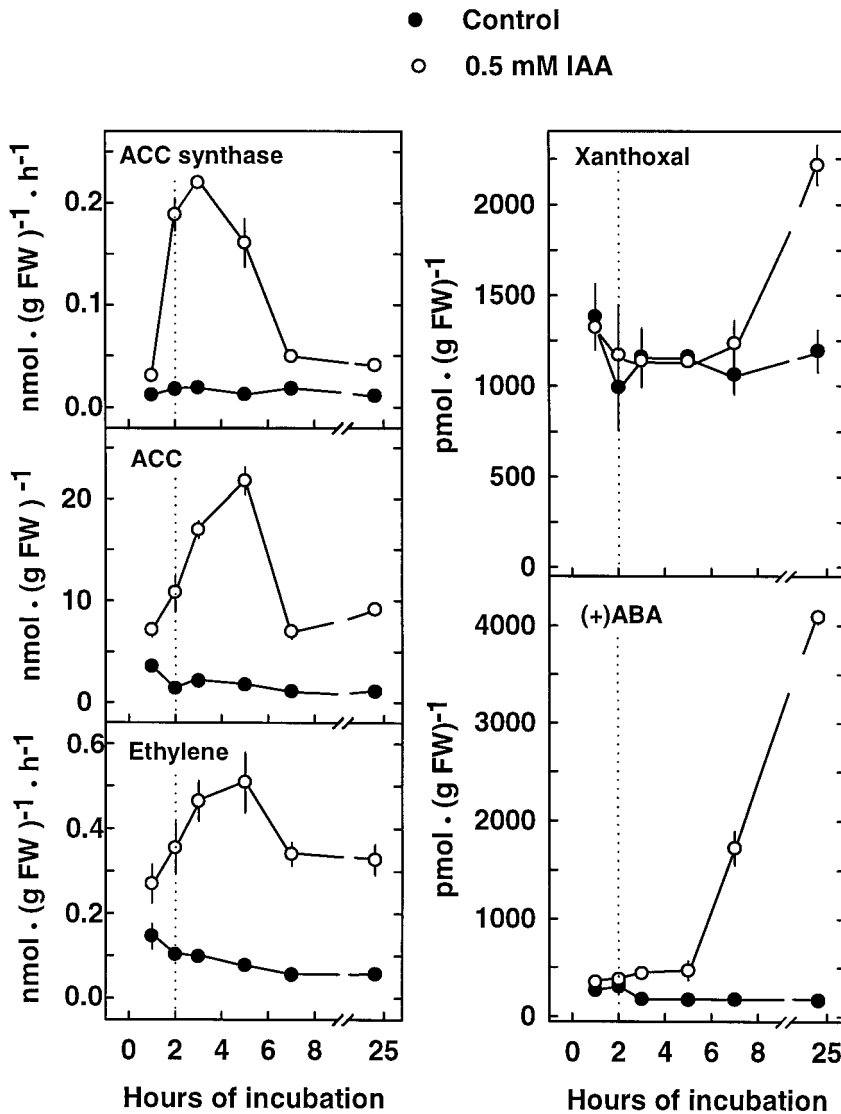
Figure 2. Time course of the effect of IAA on ACC synthase activity, ethylene formation, and levels of ACC, and immunoreactive xanthoxal and ABA in shoot tissue of root-treated *G. aparine* plants at the third whorl stage in hydroponics. Vertical bars represent SE of the mean.

ABA levels were elevated up to 2- and 24-fold, relative to the control, respectively. The increase in xanthoxal and ABA levels in auxin-treated *G. aparine* shoot tissue was also confirmed by liquid chromatography-mass spectroscopy (LC-MS) or gas chromatography (GC)-MS analyses, respectively. The determined amounts of immunoreactive xanthoxal in *G. aparine* are in the range of those reported previously, e.g. for tomato (Yamamoto and Oritani, 1997) or sunflower seedlings (Feyerabend and Weiler, 1988b).

As suggested for the auxin herbicide quinmerac, stimulation of ethylene synthesis in susceptible dicots induces ABA accumulation, which leads to the inhibition of shoot growth (Scheltrup and Grossmann, 1995; Grossmann et al., 1996; Grossmann and Scheltrup, 1998). Comparing the effects of IAA and synthetic auxins from different chemical classes, such as quinmerac, α-naphthalene acetic acid (NAA), dicamba, and 4-amino-3,5,6-trichloropicolinic acid (piclo-

ram) on shoots of root-treated *G. aparine* plants, levels of ethylene, ACC, and ABA increased in close correlation with the inhibition of growth and, as shown for IAA, according to the applied concentration (Figs. 3 and 4). Auxin-induced ethylene production was decreased by the application of amino-ethoxyvinyl-Gly (AVG), an inhibitor of ACC synthase (Abeles et al., 1992; Fig. 1), and cobalt ions, which inhibit the conversion of ACC to ethylene by ACC oxidase (Abeles et al., 1992; Fig. 1). Concomitantly, ABA accumulation and growth inhibition were reduced by both inhibitors (Figs. 3 and 4). Although AVG blocked the production of ACC and ethylene (Fig. 3), cobalt ions substantially decreased ethylene formation, whereas endogenous ACC accumulated (Fig. 4). This suggests that auxin-induced ethylene and not ACC is the trigger for ABA accumulation and the consequent growth inhibition (Fig. 1). In addition, exposure of *G. aparine* and tomato plants to ethylene-releasing ethephon stimulated ABA levels and reduced shoot
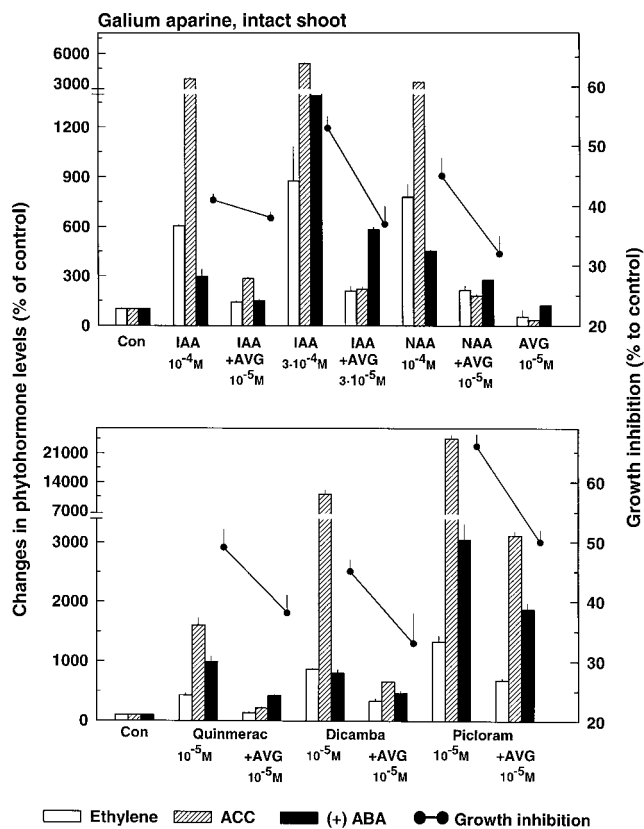
Figure 3. Effects of IAA and synthetic auxins, AVG and combinations of the compounds on shoot growth of *G. aparine* (measured by reduction in fresh weight), and ethylene formation and ACC and immunoreactive ABA contents in shoots. *G. aparine* plants at the fourth whorl stage were root treated with compounds and combinations in hydroponics (three plants per vessel) and ethylene formation and ACC and ABA levels were measured after 24 h of treatment. For determination of changes in shoot fresh weight, plants were incubated for 72 h. Vertical bars represent SE of the mean (*n* = 4). Control values ± SE (100%) were for ethylene formation 0.068 ± 0.005 nmol g^{-1} fresh weight h^{-1}, ACC 1.88 ± 0.10 nmol g^{-1} fresh weight, ABA 83 ± 4 pmol g^{-1} fresh weight, and shoot fresh weight 2.1 ± 0.1 g shoot $^{-1}$.

growth, though, to a significantly lower extent than auxin (Table I).

Further support for a causal relationship of ethylene and ABA in auxin action was obtained in experiments with the tomato mutant *never ripe*, which is impaired in ethylene perception (Wilkinson et al., 1995; Fig. 1). The receptor proteins of this mutant are not able to bind ethylene efficiently. In shoots of mutant plants root treated with IAA, quinmerac, NAA, picloram, or 2-methoxy-3,6-dichlorobenzoic acid (dicamba) for 24 h, accumulation of xanthoxal was completely prevented, compared with wild-type plants (Fig. 5). Moreover, xanthoxal levels decreased substantially below those of controls. Tomato mutants also resisted the ABA increase induced by auxins in wild-type plants (Fig. 5). However, ABA increase was not completely abolished. It is evident that mutant plants that are blocked in the response to

ethylene are no longer able to increase xanthoxal production in the shoot tissue after auxin treatment. Xanthoxal levels even decreased whereas ABA accumulated, which could be explained by a higher metabolic conversion of xanthoxal to ABA in the *never ripe* mutant treated with auxins. In addition, mutant plants could compensate for the growth inhibition induced by IAA in wild-type plants (Table II).

Effects on ABA Metabolism

To elucidate in more detail the mechanism implicated in auxin-induced ABA accumulation in shoots of *G. aparine* plants, our investigations first approached the question of an altered ABA catabolism. The known pathways include conjugation to form abscisic acid glucose ester (ABAGE) or oxidation yielding phaseic acid (PA) and dihydro-PA (Sembdner et al., 1994; Kende and Zeevaart, 1997; Cutler and
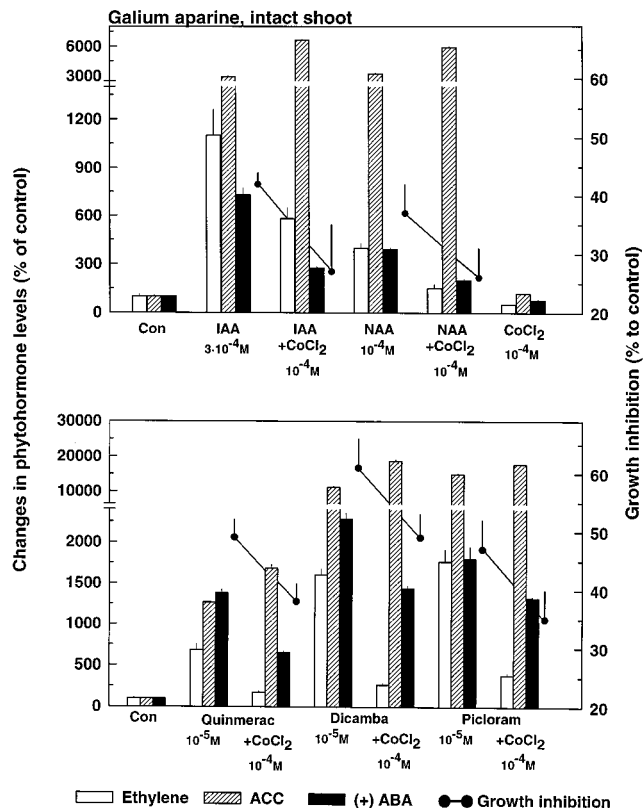
Figure 4. Effects of IAA and synthetic auxins, cobalt chloride, and combinations of the compounds on shoot growth of *G. aparine* (measured by reduction in fresh weight) and ethylene formation and ACC and immunoreactive ABA contents in shoots. *G. aparine* plants at the fourth whorl stage were root treated with compounds and combinations in hydroponics, and ethylene formation and ACC and ABA levels were measured after 24 h of treatment. For determination of changes in shoot fresh weight, plants were incubated for 72 h. Vertical bars represent SE of the mean. Control values ± SE (100%) were for ethylene formation 0.059 ± 0.002 nmol g^{-1} fresh weight h^{-1}, ACC 1.49 ± 0.05 nmol g^{-1} fresh weight, ABA 119 ± 3 pmol g^{-1} fresh weight, and shoot fresh weight 1.5 ± 0.1 g shoot $^{-1}$.

Table I. *Effect of ethephon on ABA contents and shoot growth in G. aparine and tomato*

G. aparine and wild-type tomato plants (cv Ailsa Craig) at the second whorl/leaf stage were root-treated with 0.5 mM ethephon hydroponically and immunoreactive ABA levels were measured in shoot tissue after 24 h. For determination of changes in shoot fresh wt., plants were incubated for 75 h (tomato) and 96 h (*G. aparine*). Data are means ± SE of four replicates statistically analyzed using a *t* test.

Treatment	ABA	Shoot
	pmol g^{-1} fresh wt	*g shoot^{-1} fresh wt*
G. aparine		
Control	142 ± 8	0.71 ± 0.02
Ethephon	420 ± 16[a]	0.56 ± 0.05[a]
Tomato		
Control	522 ± 13	2.69 ± 0.20
Ethephon	856 ± 14[a]	2.20 ± 0.26[a]
[a] Significantly different to control at $P < 0.05$.		

Krochko, 1999; Fig. 1). The concept of the reversible conjugation of plant hormones suggests that under conditions of physiological changes, hormone conjugates can be a source for free hormones (Sembdner et al., 1994). Young plants of *G. aparine* were root treated with IAA or quinmerac for 24 and 48 h (Fig. 6). Dependent on the auxin concentration, increased levels of xanthoxal and ABA were accompanied by a rise in the concentrations of conjugated ABA, PA, and conjugated PA in the shoot tissue (Fig. 6). After exposure to IAA (0.5 mM) or quinmerac (0.01 mM) for 48 h, conjugated ABA levels increased 49- and 131-fold, relative to control, respectively (Fig. 6). The chemical nature of the alkaline hydrolyzable ABA conjugates in *G. aparine* shoots was not analyzed, though it is likely that ABAGE is included, because ABAGE has been found a major conjugate in many plant species (Sembdner et al., 1994). Concomitantly, increases in endogenous ABA amounted to 7- and 59-fold by IAA and quinmerac, respectively (Fig. 6). Quinmerac-elicited ABA accumulation proceeded during the incubation period of 48 h (Fig. 6). In contrast, when plants were treated with IAA, ABA levels peaked at 24 h and then declined, whereas maximum levels of xanthoxal were maintained in the shoot tissue during incubation (Fig. 6). In this case, the conversion of xanthoxal to ABA appears to be less active than ABA catabolism. Furthermore, IAA is known to be subjected to rapid inactivation through conjugation and to degradation by multiple pathways in the plant (Sembdner et al., 1994; Taiz and Zeiger, 1998). In contrast, synthetic auxins, such as quinmerac, are long lasting and more effective than IAA because they are stable and not inactivated by the plant as rapidly as the endogenous phytohormone (Sterling and Hall, 1997; Grossmann and Scheltrup, 1998). Thus rapid inactivation of the absorbed IAA could explain the temporary nature of the stimulation of ethylene synthesis (Fig. 2) and the consequent transience of the increase of ABA in *G. aparine*

shoot tissue (Fig. 6). In a simultaneous manner, free ABA was reduced through conjugation and oxidation (Fig. 6). After the application of IAA (0.5 mM) and quinmerac (0.01 mM) for 48 h, PA levels increased approximately 12-fold and conjugated PA levels 9- and 16-fold, relative to control, respectively (Fig. 6). Conjugation seems to be similar or more effective than oxidative degradation in reducing free ABA in *G. aparine*. At 0.01 mM quinmerac applied for 48 h, levels of conjugated ABA increased from 50 to 6,524 pmol per g shoot fresh weight, whereas levels of PA and conjugated PA were elevated from a total of 176 to 2,347 pmol (Fig. 6). However, in this calculation the formation of dihydro-PA and its conjugates were not considered. To summarize, the results presented here render it highly unlikely that auxin-induced ABA accumulation in *G. aparine* is generated through an increased release of ABA from conjugates or a reduced oxidative degradation. The latter conclusion was further supported in pulse experiments with *G. aparine* cell suspensions, which were fed for 2 h with [³H] ABA before incubation in the presence of 0.03 mM quinmerac. After 4 and 21 h, quinmerac-
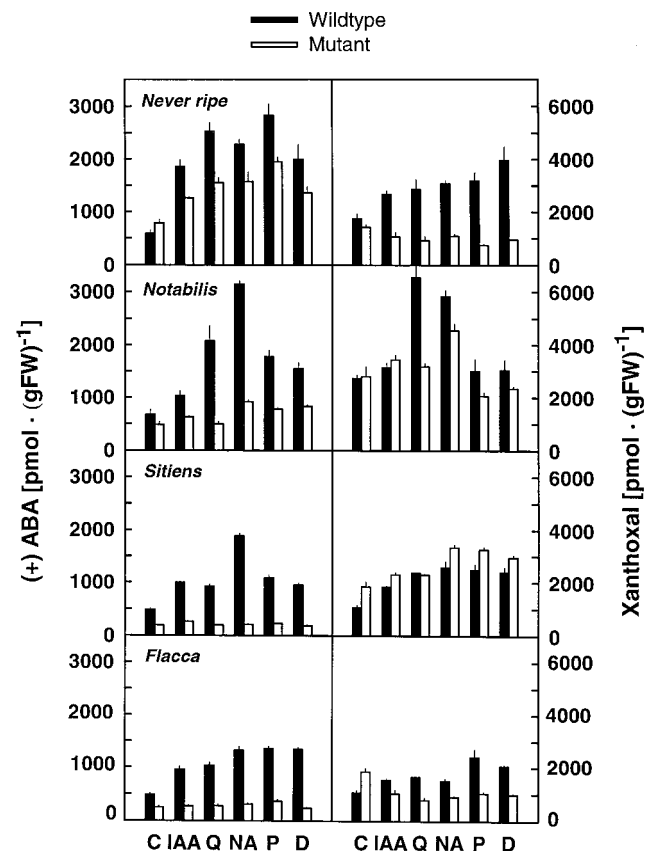
Figure 5. Influence of IAA, quinmerac (Q), NAA (NA), picloram (P), and dicamba (D) on immunoreactive xanthoxal and ABA contents in shoot tissue of the tomato mutants *never ripe, notabilis, sitiens,* and *flacca* and the corresponding wild types. Plants at about the third leaf stage were root treated with compounds at 0.01 mM in hydroponics for 24 h. Data are means ± SE compiled from an experimental series.

Table II. *Effect of IAA on shoot growth of wild-types, never ripe, and flacca tomato plants*

Plants at the third leaf stage were root-treated with compounds in hydroponics. After 6 d of IAA treatments, shoot fresh wt. and plant height were measured as growth parameters. Data are means of 15 plants (five hydroponic vessels), compiled from an experimental series. Means with a common letter are not significantly different ($P = 0.01$; Duncan's multiple range test). In parenthesis, values in percent of control.

| Tomato Plants	Plant Height	Shoot Fresh Wt
	cm	*g shoot^{-1}*
Wild type (Ailsa Craig)		
Control	15.5 ± 0.2 (100)a	6.69 ± 0.22 (100)a
IAA, 10 μM	13.0 ± 0.1 (84)b	3.02 ± 0.12 (45)b
IAA, 50 μM	11.2 ± 0.1 (72)c	1.94 ± 0.10 (29)c
Never ripe mutant		
Control	13.0 ± 0.3 (100)a	5.14 ± 0.24 (100)a
IAA, 10 μM	12.7 ± 0.4 (97)a	4.56 ± 0.24 (89)a
IAA, 50 μM	10.7 ± 0.3 (82)b	2.64 ± 0.19 (51)b
Wild type (Rheinlands Ruhm)		
Control	20.6 ± 0.4 (100)a	5.19 ± 0.11 (100)a
IAA, 50 μM	17.7 ± 0.3 (86)b	3.93 ± 0.08 (76)b
IAA, 100 μM	15.2 ± 0.7 (74)c	2.81 ± 0.20 (54)c
Flacca mutant		
Control	18.2 ± 0.1 (100)a	2.89 ± 0.07 (100)a
IAA, 50 μM	17.1 ± 0.4 (94)a	2.67 ± 0.06 (93)a
IAA, 100 μM	14.5 ± 0.4 (80)b	2.20 ± 0.16 (76)b

treated cells contained the same levels of ABA and PA as found in controls (Table III). Therefore, it is hypothesized that auxin-stimulated ethylene triggers ABA accumulation, mainly from de novo synthesis (Fig. 1).

Effects on ABA Biosynthesis

In higher plants, ABA is produced by the oxidative cleavage of the polyene chain of a C_{40} epoxy-xanthophyll precursor, such as 9'-cis-neoxanthin, which is formed from violaxanthin in carotenoid biosynthesis (for review, see Kende and Zeevaart, 1997; Koornneef et al., 1998; Cutler and Krochko, 1999; Fig. 1). To evaluate whether the synthesis of xanthophylls is influenced by auxins, the contents of neoxanthin, violaxanthin, antheraxanthin, and β-carotene were determined in *G. aparine* shoots after root treatment of plants with 0.01 mM quinmerac for 48 h (Table IV). In contrast to the levels of xanthoxal and ABA, those of violaxanthin, neoxanthin, antheraxanthin, and β-carotene were found to be nearly identical in treated shoot tissue versus control (Table IV). This could indicate that the formation of xanthoxal in the oxidative cleavage reaction of an epoxy-carotenoid, or the following two-step oxidation of xanthoxal to ABA (Sindhu and Walton, 1988; Koornneef et al., 1998; Cutler and Krochko, 1999) is indirectly stimulated by auxins (Fig. 1).

To obtain more information on the target site of ethylene action, inhibitor studies in *G. aparine* plants

and analyses of tomato mutants blocked in ABA biosynthesis were carried out (Fig. 1). Among the inhibitors used, fluridone is known to affect phytoene desaturase in the carotenogenic pathway (Kowalczyk-Schröder and Sandmann, 1992). Naproxen is a putative inhibitor of epoxy-carotenoid cleavage to give xanthoxal (Lee and Milborrow, 1997). Tungstate was shown to affect the formation of ABA from ABA-aldehyde by impairing ABA-aldehyde oxidase (Lee and Milborrow, 1997), an enzyme requiring a molyb-
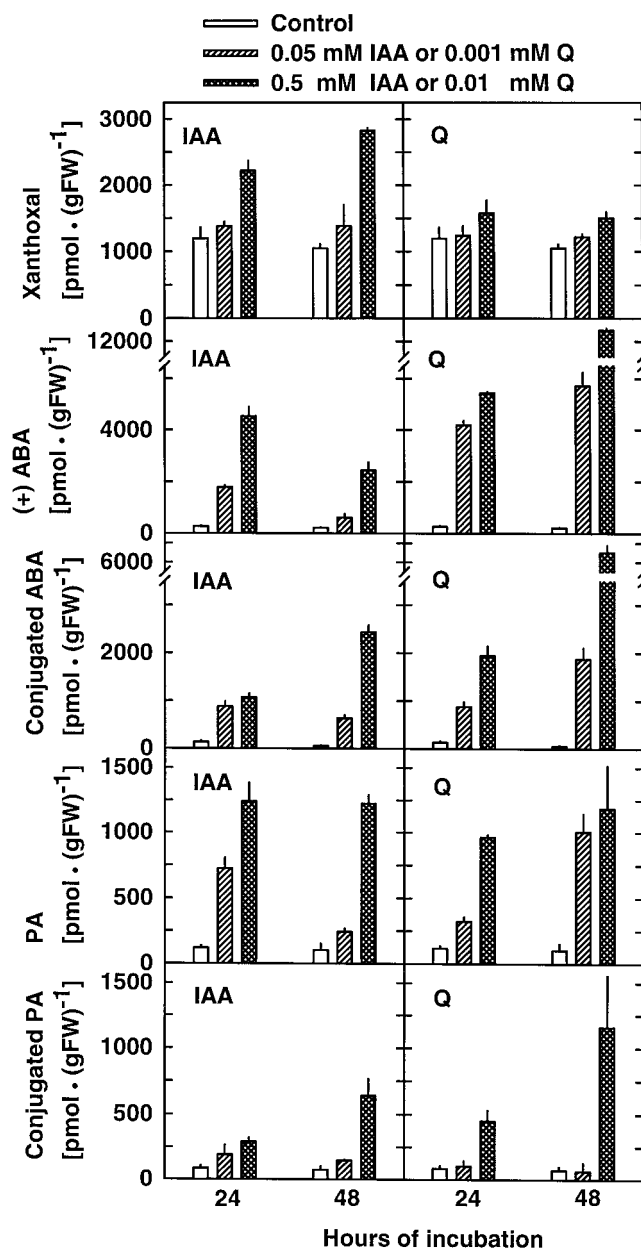
Figure 6. Influence of IAA and quinmerac (Q) on the contents of immunoreactive xanthoxal, ABA, conjugated ABA, PA, and conjugated PA in shoot tissue of *G. aparine* plants at the third whorl stage, which were root treated in hydroponics for the indicated times. Vertical bars represent SE of the mean.

Table III. *Catabolism of ABA in G. aparine cell suspensions after treatment with quinmerac*

Four-day-old cell suspensions were fed for 2 h with 0.01 mM [³H]-(±)-ABA and then were incubated in the presence of 0.03 mM quinmerac for the times indicated. After HPLC-radiocounting of cell extracts, the fractions containing ABA and PA were quantified by immunoassay. Data are means ± SE of five replicates statistically analyzed using a *t* test. Means with NS are not significantly different to control at $P < 0.01$.

Treatment	Time after Treatment	ABA	PA
	h	*nmol g⁻¹ fresh wt.*	
Control	0	65.6 ± 4.0	0.4 ± 0.1
Control	4	49.4 ± 2.4	1.7 ± 0.2
Quinmerac	4	51.1 ± 6.4 NS	1.6 ± 0.1 NS
Control	21	1.5 ± 0.2	2.0 ± 0.1
Quinmerac	21	1.4 ± 0.1 NS	1.7 ± 0.3 NS

denum cofactor (Marin and Marion-Poll, 1997). After root treatment of *G. aparine* plants for 24 h, the effects of quinmerac in combination with ABA inhibitors on the xanthoxal and ABA contents in shoots were investigated (Fig. 7). Quinmerac alone stimulated the levels of xanthoxal and ABA 2- and 26-fold, relative to control, respectively (Fig. 7). A similar increase in xanthoxal levels of 2- to 3-fold was found after exposure of *G. aparine* plants to IAA (Figs. 2, 6, and 7). The smaller extent of xanthoxal increase compared with ABA accumulation could be due to the rapid turnover of xanthoxal to ABA (Sindhu and Walton, 1988; Yamamoto and Oritani, 1997). The enzymes for these steps are constitutively expressed in a variety of plants (Sindhu and Walton, 1988; Parry et al., 1990). This argues in favor of the stimulation of the oxidative cleavage reaction leading to xanthoxal as the target site of auxin-induced ethylene. In accordance, fluridone and naproxen were able to inhibit quinmerac-induced increases in xanthoxal and ABA levels (Fig. 7). On the other hand, tungstate prevented ABA accumulation, whereas xanthoxal levels only gradually dropped (Fig. 7). However, although tungstate is an inhibitor of ABA-aldehyde oxidase, it did not cause a further rise in the xanthoxal levels of *G. aparine* shoot tissue. Feedback inhibition of the formation of xanthoxal from epoxy-carotenoids may have occurred. In an alternate manner and more likely, ABA-aldehyde and/or trans-ABA-alcohol and metabolites (Taylor et al., 1988; Rock et al., 1991) accumulated instead of xanthoxal.

In a further set of experiments, the influence of IAA, quinmerac, NAA, picloram, and dicamba on ABA and xanthoxal levels of ABA-deficient tomato mutant and corresponding wild-type plants was studied (Fig. 5). Although *notabilis* has a defect in the oxidative cleavage reaction of epoxy-carotenoid (Parry et al., 1992; Burbidge et al., 1999; Fig. 1), the mutants *sitiens* and *flacca* are blocked at the final step of ABA biosynthesis, the conversion of ABA-aldehyde to ABA (Taylor et al., 1988; Marin and Marion-Poll, 1997; Fig. 1). In accordance, in the shoot tissue of the mutants ABA levels were reduced whereas xanthoxal levels were similar (*notabilis*) or higher (*sitiens* and *flacca*) than those of wild-type plants (Fig. 5). Concomitantly, in the mutants ACC levels were found approximately 2-fold higher than those of wild-type plants (data not shown). Since ABA-deficient tomato mutants are known to overproduce ethylene (Tal and Nevo, 1973), ethylene-induced synthesis of xanthoxal could explain increased levels found in the *sitiens* and *flacca* mutant (Fig. 5). After root treatment with auxins for 24 h, xanthoxal and ABA levels increased in shoot tissue of wild-type plants (Fig. 5). In contrast, *notabilis* mutants could resist auxin effects on xanthoxal and particularly ABA levels (Fig. 5). In a similar manner, auxin-induced ABA accumulation was completely inhibited in *sitiens* and *flacca* (Fig. 5). Concomitantly, *flacca* mutants could compensate for auxin-induced growth inhibition in wild-type plants (Table II; Grossmann et al., 1996). However, in the shoot tissue of *sitiens*, auxin-stimulated xanthoxal levels were similar to those of wild-type plants, whereas in *flacca*, levels declined (Fig. 5). Similar to the studies using tungstate, it can be logically postulated that in the *sitiens* and *flacca* mutant of tomato, auxin-induced xanthoxal is further metabolized to ABA-aldehyde and trans-ABA-alcohol, as is found in both mutants when subjected to water stress (Taylor et al., 1988; Rock et al., 1991). It appears that this conversion of xanthoxal does not proceed with the same efficiency in both mutants. It is possible that in the *flacca* mutant auxin-induced xanthoxal is more effectively metabolized to ABA-aldehyde and/or ABA-alcohol than in the *sitiens* mutant. Overall, the main target site of auxin-induced ethylene appears to be the oxidative cleavage of 9'-cis-xanthophylls in ABA biosynthesis. This first committed reaction in ABA biosynthesis has been suggested to be the most likely regulatory step

Table IV. *Effect of quinmerac on the contents of xanthophylls, xanthoxal, and ABA in G. aparine shoots*

Plants at the third whorl stage were root-treated with 0.01 mM quinmerac hydroponically. After 48 h of incubation, pigments, xanthoxal, and ABA were extracted from shoot tissue and quantified (based on shoot dry wt.) as described in "Materials and Methods." The data shown are means ± SE of four replicates statistically analyzed using a *t* test. Means with NS are not significantly different to control at $P < 0.01$.

Treatment	β-Carotene	Anthera-Xanthin	Violaxanthin	Neoxanthin	Xanthoxal	ABA
			nmol g⁻¹ dry wt			
Control	886 ± 35	61 ± 13	683 ± 25	496 ± 31	10.5 ± 0.5	2.2 ± 0.2
Quinmerac	822 ± 36 NS	77 ± 4 NS	663 ± 45 NS	493 ± 35 NS	15.5 ± 1.0	127.2 ± 1.3

Figure 7. Effects of quinmerac (Q; 0.01 mM), tungstate (Tu; 0.5 mM), naproxen (Na; 1 mM) (A) and fluridone (Fl; 0.01 mM) and combination of the compounds (B) on immunoreactive xanthoxal and ABA contents in shoot tissue of *G. aparine* plants at the third whorl stage, which were root treated in hydroponics for 24 h. Data are means ± SE compiled from an experimental series.
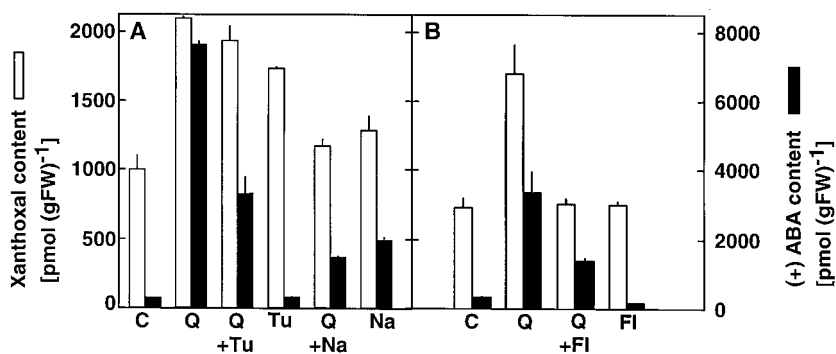
in the pathway (Kende and Zeevaart, 1997; Cutler and Krochko, 1999). Genes that encode 9'-cis-epoxycarotenoid dioxygenase have recently been identified in various species (Tan et al., 1997; Neill et al., 1998; Burbidge et al., 1999; Qin and Zeevaart, 1999). Their expression was up-regulated by water stress.

CONCLUSION

The results presented here demonstrate, to our knowledge for the first time, that the inhibitory effects of IAA at high concentration and of synthetic auxins on plant growth derive from hormonal interaction between ethylene and ABA (Fig. 1). Although stimulation of ethylene production following auxin treatment was described by Zimmerman and Wilcoxon as early as 1935, knowledge of interrelations between auxin and ABA remains limited. In studies with excised stem sections of aspen with lateral buds, Eliasson (1975) observed that high endogenous auxin levels coincided with high ABA levels. Furthermore, ethylene and ethephon treatment have been shown to increase ABA levels in many plant tissues (Abeles et al., 1992). Our experiments suggest that in *G. aparine* shoot tissue auxin stimulation of ethylene, via induction of ACC synthase, triggers an increase of endogenous ABA, which causally leads to growth inhibition (Fig. 1). This effect is common to IAA and synthetic auxins of different chemical classes in a variety of dicot plant species (Grossmann et al., 1996). As shown previously, ABA affects biomass production by reducing stomatal aperture and consequently CO_2 assimilation (Scheltrup and Grossmann, 1995). Direct inhibitory effects of ABA on cell division and expansion have also been reported (Taiz and Zeiger, 1998). In consequence, ABA, together with ethylene, appears to function as a further hormonal second messenger of auxin in the signaling pathway leading to growth inhibition (Fig. 1). Investigations using inhibitors of ABA biosynthesis and ABA-deficient tomato mutants lend further support to the hypothesis that auxin-induced ethylene increases ABA, mainly through a stimulation of the cleavage reaction of epoxy-carotenoids to xanthoxal (Fig. 1). To test this hypothesis, future studies should

explore the situation at the transcript and enzymatic level of epoxy-carotenoid conversion to xanthoxal, probably catalyzed by a dioxygenase, which is up-regulated at the transcript level by stress (Tan et al., 1997; Burbidge et al., 1999; Qin and Zeevaart, 1999; Fig. 1). In addition, it would be interesting to reappraise the morphological and physiological effects observed in transgenic, auxin-overproducing plants containing genes that encode enzymes of IAA biosynthesis (Klee and Lanahan, 1995), against the background of auxin-induced increases in endogenous ABA levels.

MATERIALS AND METHODS

Chemicals

The following compounds were used: 1H-1-methyl-3-(3-trifluormethyl-phenyl)-5-phenylpyridin-4-on (fluridone), IAA, and the synthetic auxins dicamba, picloram, quinmerac, and NAA from Riedel-de Haen (Seelze, Germany) and BASF AG, Ludwigshafen, Germany. ACC, anti-rabbit whole serum developed in goat, cobalt chloride, [-]-sodium 6-methoxy-α-methyl-2-naphthaleneacetate (naproxen), sodium tungstate, 2-chloroethyl-phosphonic acid (ethephon), and (+)- and (±)-ABA were obtained from Calbiochem (Bad Soden, Germany) or from Sigma (Munich). 4-(2-Aminoethyl) phenylsulfonylfluoride hydrochloride (Pefabloc SC), AVG, N-[2-hydroxyethyl]piperazine-N'-3-propanesulfonic acid (EPPS), and Tris-buffered saline were purchased from Fluka (Neu-Ulm, Germany) and Merck (Darmstadt, Germany). Xanthoxal and PA were gifts from Prof. Michael Böttger and Prof. Karl Dörffling (University of Hamburg, Germany), respectively.

Cultivation of Plants in Hydroponics

Seeds of *Galium aparine* were stratified for 4 to 5 weeks at 4°C to 6°C under moist conditions. They were then germinated in soil substrate in the greenhouse (10°C–25°C). Seedlings at the first whorl stage (15 d after sowing) were transferred to vermiculite substrate moistened with one-half concentrated Linsmaier and Skoog (1964) nutrient solution and raised to the third or fourth whorl stage (light/dark: 16/8 h at 24°C/19°C, 250 μmol m^{-2} s^{-1} photon irradiance, 400 to 700 nm; fluorescent lamps, radium

HRLV). Seedlings of the ABA-deficient mutants of tomato Mill. *flacca* and *sitiens* cv Rheinlands Ruhm and *notabilis* cv Hellfrucht and the ethylene perception mutant *never ripe* of tomato cv Ailsa Craig and the corresponding wild types were raised to the third leaf stage in vermiculite substrate for 4 weeks. The mutants were generously donated by Prof. Karl Hammer (GenBank, Gatersleben, Germany) and Prof. Don Grierson (University of Nottingham, UK). Uniformly developed plants of *G. aparine* and tomato were transferred into 320-mL glass vessels in one-half-strength Linsmaier and Skoog medium (according to Scheltrup and Grossmann, 1995) in 16/8 h light/dark cycles at 25°C/20°C and 75% relative humidity (three plants per vessel, five replications). Light (530 μmol m^{-2} s^{-1}, 400–750 nm) was provided by Osram Powerstar HQI-R 250W/NDL and Osram Krypton 100 W lamps (München, Germany). The solution was aerated throughout the experiments. After 1 d of adaption, IAA, dicamba, picloram, NAA, quinmerac, or fluridone were added, alone or in combination, to the medium in acetone solution (0.1% [w/v] final concentration of acetone). Controls received corresponding amounts of acetone alone, with no adverse effect on the growth of the plants. Naproxen, sodium tungstate, cobalt chloride, AVG, and ACC were added to the medium from aqueous stock solutions. At various times after treatment, shoot fresh weight as growth parameter and ethylene formation were measured. Shoots from parallel vessels were harvested, immediately frozen in solid CO_2, and stored at $-80°C$. Plant material was powdered under liquid nitrogen. The osmotic potential of the cell sap, which was obtained from samples of powdered plant material, was measured with a freezing point osmometer (Roebling Messtechnik, Berlin) according to Capell and Dörffling (1993).

Determination of Ethylene Production

After treatment in hydroponic vessels, detached shoots of plants were transferred into 100-mL glass cylinders with 10 mL of one-half-concentrated Linsmaier and Skoog medium (one shoot per cylinder; six replications) according to Scheltrup and Grossmann (1995). The cylinders with plants were sealed with ruber caps. After incubation for a further 3 h under light, a 1-mL gas sample of the head space was withdrawn and ethylene was measured by GC.

Determination of ACC

Samples of powdered plant material (100 mg; three replications) were extracted with 70% (v/v) aqueous ethanol. Following conversion to ethylene, the ACC content was assayed by GC (Lizada and Yang, 1979; Scheltrup and Grossmann, 1995).

Determination of ACC Synthase Activity

Powdered plant material (1.5 g; two replicates) was extracted in 100 mM EPPS/potassium hydroxide buffer (pH 8.5) containing dithiothreitol (5 mM), pyridoxal phosphate (6 μM), leupeptin (10 μM), and Pefabloc SC (10 μM) and

assayed as described previously (Grossmann and Scheltrup, 1998). The extract was centrifuged at 25,000g for 10 min (4°C) and the supernatant was passed through a Sephadex G 25 column (Pharmacia, Uppsala), which had been equilibrated with 5 mM EPPS buffer (pH 8.5) containing dithiothreitol (1 mM), pyridoxal phosphate (6 μM), and Pefabloc SC (10 μM). The ACC synthase assay mixture, with a total volume of 0.6 mL, contained 0.3 mL of enzyme preparation in EPPS buffer (80 mM) with pyridoxal phosphate (20 μM) and *S*-adenosyl Met (100 μM). After an incubation period of 2 h at 37°C, the reaction was stopped by adding 20 μmol mercury (II) chloride. The ACC produced was determined subsequently by chemical conversion to ethylene (Lizada and Yang, 1979). All assays were performed with four replicates.

Extraction and Isolation of Pigments

Pigments were extracted and analyzed by the method of Büch et al. (1994) and Scheltrup (1997). Powdered plant material (200 mg) was extracted twice in 5 mL of an organic solvent mixture containing petroliumbenzine (100°C–140°C), acetone, and chloroform (1.0:1.7:0.2, v/v), which was buffered by 6 mg of $NaHCO_3$. After centrifugation at 18,000g for 5 min at 2°C, the combined extracts were passed through a filter funnel, and the filtrate was concentrated by rotary evaporation (50°C) to dryness and redissolved in 400 μL 100% acetone. After a further centrifugation step at 13,800g for 5 min, neoxanthin and violaxanthin were analyzed in 20 μL of the extracts using a 1090 Series Liquid Chromatograph (Hewlett-Packard, Palo Alto, CA) with the Diode Array Detector Series 1040. A ODS-Hypersil column (4.6 × 60 mm, 3-μm particle size, Hewlett-Packard) was used in combination with a guard column (4.0 × 20 mm, 5-μm particle size of ODS-Hypersil). Neoxanthin (4.5–4.8 min), violaxanthin (5.4 min), antheraxanthin (6.3 min), and β-carotene (14.1 min) were detected by their absorbances at 445 nm using a linear gradient of 60% (v/v) acetone in aqueous buffer (1 mM $NaHCO_3$) to 98% (v/v) acetone. The flow rate was 1.2 mL min^{-1}. Pigment concentrations were calculated as described (Büch et al., 1994).

Determination of ABA, PA, Xanthoxal, and Conjugates

Powdered plant material (1 g) was extracted with 80% (v/v) aqueous methanol containing 10 mg L^{-1} butylated hydroxytoluene (three replicate extractions). To avoid non-physiological increases in xanthoxal levels through breakdown of carotenoids and to minimize isomerization and degradation of xanthoxal and ABA, the extracts were immediately passed through a C_{18}-reversed phase prepacked column (SEPPAK; Waters, Königstein, Germany) under dim light conditions at 4°C (Weiler et al., 1986; Grossmann et al., 1987; Scheltrup and Grossmann, 1995). This step removes quantitatively carotenoids from the extract (Feyerabend and Weiler, 1988a). For ABA and PA determination, the effluent was concentrated in vacuo and dissolved in 3 mL of double-distilled water. The extract was acidified to pH 2.5 with 1 M HCl, and partitioned three times into

ethyl acetate (3 mL). The organic solvent was evaporated to dryness under a N_2 stream and samples were dissolved in 2 mL of 5% (v/v) methanol in 0.1 M acetic acid. Separation of ABA and PA in a 1-mL aliquot of the extract was performed by HPLC on a reverse-phase Nucleosil 120 5-μm C_{18} column (250 × 10 mm, Machery-Nagel, Düren, Germany) using a linear gradient from 5% (v/v) methanol in 0.1 M acetic acid to 95% (v/v) methanol. The fractions containing ABA (26.0–27.0 min) and PA (20.0–21.0 min) were collected, concentrated in vacuo to dryness, and dissolved in a solution of 50 μL 100% methanol and 950 μL Tris-HCl (50 mM, pH 7.8) for ELISA.

Monoclonal antibodies for ABA (Mertens et al., 1983) and PA (Gergs et al., 1993), 100% reactive against their respective antigens, were used for analyses according to a standard procedure described by Weiler et al. (1986). The quantification of ABA and PA conjugates after NaOH hydrolysis was performed according to a modified method of Sembdner et al. (1987). The effluent from C_{18}-SEPPAK-columns of methanolic extracts of the plant material was evaporated to dryness and redissolved in 4 mL 70% (v/v) aqueous methanol. Two milliliters of the extract were concentrated in vacuo, diluted with 2 mL of double-distilled water, and hydrolyzed with 0.1 M NaOH (pH 13) at 60°C for 1 h. After alkaline hydrolysis, the samples were adjusted to pH 2 with 1 M HCl and extracted three times with equal volumes (3 mL) of diethyl ether. The organic fractions were combined and concentrated under a N_2 stream to dryness. ABA and PA were determined after HPLC separation, as described above. In the remaining 2 mL of the extract, ABA and PA were quantified by the same method without alkaline hydrolysis. The concentrations of ABA and PA conjugates were calculated from the difference between ABA or PA concentrations measured in the hydrolyzed part of the sample and those measured in the non-hydrolyzed part.

For determination of xanthoxal, the effluent from C_{18}-SEPPAK-columns after methanolic extraction of the plant material was concentrated by rotary evaporation to dryness and the residue was redissolved in 2 mL of 5% (v/v) methanol in double-distilled water. Separation of xanthoxal in an aliquot of 1 mL of the extract was performed by HPLC as described above. In this case, the gradient did not contain acetic acid because the epoxy-group of xanthoxal is sensitive to acids. The fraction containing cis- and trans-xanthoxal (25.5–27.5 min) were collected, reduced under a N_2 stream to dryness, and redissolved in 1 mL of 5% (v/v) methanol in Tris-HCl (pH 7.8) for ELISA, as described by Feyerabend and Weiler (1988a, 1988b). Monoclonal antibodies for xanthoxal were cross-reactive with 2-cis-(-)-xanthoxal (100%), 2-trans-(-)-xanthoxal (111%), 2-cis-(-)-xanthoxal-alcohol (133%), 2-trans-(-)-xanthoxal-alcohol (67.7%), ABA (0.1%), violaxanthin (12.3%), neoxanthin (11.5%), and zeaxanthin (9.4%), as determined by Feyerabend and Weiler (1988a, 1988b). No cross-reactivity with ABA-alcohol and ABA-aldehyde was found (J. Kwiatkowski and K. Grossmann, unpublished data). The cross-reactivity of xanthoxal antibodies with xanthoxic acid was not determined. Interference of ELISA

analyses with carotenoids was prevented by their quantitative removal using C_{18}-SEPPAK-columns for purification of the methanolic extracts (Feyerabend and Weiler, 1988a) and subsequently HPLC separation. The antibodies for ABA, PA, and xanthoxal were kindly provided by Prof. Elmar W. Weiler (University of Bochum, Germany). The detection limit for ABA, PA, and xanthoxal was 0.1 pmol, as estimated from standard curves. All samples were assayed at least in triplicate. Internal performance controls of assay accuracy and reliability were carried out as described by Weiler et al. (1986) and Grossmann et al. (1987). Recovery of ABA and xanthoxal, as checked with internal radiolabeled standards added to the methanolic extracts of the ground tissues, were above 90% in both cases. Confirmation of identity of ABA and xanthoxal in the immunoreactive HPLC fractions of the plant extracts was obtained by GC-MS (Finnigan 4600, Finnigan, San Jose, CA) and LC-MS (Sciex API III, Sciex, Concord, Ontario, Canada), respectively.

Feeding Experiments with [³H] ABA

Four-day-old cell suspensions of *Galium mollugo* were cultivated as described by Häuser et al. (1990), and transferred into fresh medium (30 mL of cell suspension added to 50 mL of medium) and incubated for 2 h in the presence of 0.01 mM (±)-ABA containing 0.5 MBq [³H] (±)-ABA (2.9 TBq/mmol (Amersham Buchler, Braunschweig, Germany). The cells were collected by filtration, intensively washed, and resuspended in 60 mL of medium. Aliquots (2 mL with 200 mg of cell fresh weight) were incubated with 0.03 mM quinmerac in sterile plastic tubes. The tubes were kept on a rotary shaker at 400 rpm for 4 and 21 h at 25°C in the dark. After centrifugation, the cells were immediately frozen at −20°C. Five replicates were used. For determination of ABA and its catabolite, PA, the frozen cell mass of each sample was immediately extracted for 1 h in 8 mL of 100% methanol and twice for 1 h in 8 mL of 80% (v/v) aqueous methanol at 4°C. Both extraction media contained 10 mg L^{-1} butylated hydroxytoluene. After further partitioning into ethyl acetate and evaporation to dryness, the samples were redissolved in 0.5 mL 10% (v/v) aqueous methanol. Then, 0.35 mL was subjected to HPLC as described above and the radioactivity of the fractions was determined by scintillation counting. The fractions containing ABA and PA were identified by their chromatographic retention and were quantified by ELISA. Approximately 97% of radioactivity taken up by the cells (34% of applied [³H] ABA) was recovered after HPLC separation.

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