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The Arabidopsis 1-Aminocyclopropane-1-Carboxylate Synthase Gene 1 Is Expressed during Early Development

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The temporal and spatial expression of one member of the Arabidopsis 1-aminocyclopropane-1-carboxylate (ACC) synthase gene family (*ACS1*) was analyzed using a promoter-β-glucuronidase fusion. The expression of *ACS1* is under developmental control both in shoot and root. High expression was observed in young tissues and was switched off in mature tissues. *ACS1* promoter activity was strongly correlated with lateral root formation. Dark-grown seedlings exhibited a different expression pattern from light-grown ones. The ACC content and the in vivo activity of ACC oxidase were determined. ACC content correlated with *ACS1* gene activity. ACC oxidase activity was demonstrated in young Arabidopsis seedlings. Thus, the ACC formed can be converted into ethylene. In addition, ethylene production of immature leaves was fourfold higher compared to that of mature leaves. The possible involvement of *ACS1* in influencing plant growth and development is discussed.

INTRODUCTION

Ethylene is involved in several aspects of plant development from germination and seedling growth to flowering, fruit ripening, leaf abscission, and organ senescence. It also plays a key role in the response to environmental factors (Abeles, 1973; Yang and Hoffman, 1984; Moore, 1989; Van Der Straeten and Van Montagu, 1991).

With the recent cloning of genes encoding the two most important enzymes in the biosynthesis of ethylene, 1-aminocyclopropane-1-carboxylate (ACC) synthase (Nakajima et al., 1990; Van Der Straeten et al., 1990; Dong et al., 1991; Huang et al., 1991; Olson et al., 1991; Rottmann et al., 1991) and ACC oxidase (ethylene-forming enzyme or EFE) (Hamilton et al., 1990; Spanu et al., 1991; Wang and Woodson, 1991), more information about the molecular regulation of the synthesis of this hormone has been made available. The main focus has been on the regulation of ACC synthase gene expression because of its key regulatory role in the pathway. This implies that diverse inducers of ethylene production are also inducers of de novo synthesis of ACC synthase (Yang and Hoffmann, 1984; Van Der Straeten and Van Montagu, 1991).

ACC synthases have been cloned from tomato (Van Der Straeten et al., 1990; Olson et al., 1991; Rottmann et al., 1991; Yip et al., 1992), winter squash (Nakajima et al., 1990), zucchini (Huang et al., 1991), Arabidopsis (Liang et al., 1992; Van Der Straeten et al., 1992), mung bean (Botella et al., 1992; Kim et al., 1992), carnation (Park et al., 1992), orchid (O'Neill et al., 1993), tobacco (Bailey et al., 1992), and apple (Dong et al., 1991; Kim et al., 1992). In several cases, it has been shown that ACC synthase genes belong to a multigene family and that they are differentially responsive to various ethylene-inducing factors and conditions such as wounding, fruit ripening, and auxins, although in certain cases some level of coordination was demonstrated (Nakajima et al., 1990; Van Der Straeten et al., 1990; Dong et al., 1991; Olson et al., 1991; Rottmann et al., 1991; Yip et al., 1992). These studies have provided information about general aspects of ACC synthase gene inducibility. However, our knowledge about tissue and cell specificity of expression of ACC synthase genes during plant development remains poor.

Ethylene is a plant hormone the effects of which have been thoroughly studied at the physiological and molecular levels in relation to ripening, senescence, and associated processes (Roberts et al., 1985a; Broglie and Broglie, 1991; Van Der Straeten and Van Montagu, 1991). On the other hand, the involvement of ethylene has been documented in some aspects of development of vegetative tissue as in the case of stem extension in deep-water rice or aerenchyma formation in maize

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Figure 1. Expression of the *ACS1* Gene in Arabidopsis Seedlings during Development and in Dark Conditions.

roots (Raskin and Kende, 1984; Salter and Kende, 1985; Jackson, 1991; Van Der Straeten and Van Montagu, 1991). Furthermore, the effect of ethylene is also dependent on cell and tissue type, indicating the presence of different processes of recognition and/or different levels of responsiveness of these cells and tissues (Osborne et al., 1985; Raskin, 1991). Another effect, ethylene-induced radial cell expansion, points to the involvement of this hormone in the change of the orientation of cellulose microfibrils in the cell wall from the transverse to the longitudinal direction (Raskin, 1991). It has been hypothesized that this change is a direct consequence of a change in the orientation of microtubules. This has been supported in part by the observation that a net reorientation of microtubules in the longitudinal direction is a consequence of ethylene treatment (Raskin, 1991).

In this paper, we report on the developmental regulation of a member of the Arabidopsis ACC synthase gene family, *ACS1* (previously referred to as *At-ACC1* by Van Der Straeten et al. [1992] or *ACC2* by Liang et al. [1992], respectively). The promoter–β-glucuronidase (*gus*) fusion technique (Jefferson, 1987; Jefferson et al., 1987) has been used to study the expression of *ACS1*.

The results indicate that the promoter of the Arabidopsis *ACS1* gene is strongly regulated developmentally and associated with tissue that is undergoing differentiation. Although no direct evidence is presented, a possible role for *ACS1* in determining the cell length/width ratio (via ethylene), which ultimately leads to control of shape of plant organs and of plant growth, is proposed. In addition, the expression seems to be clearly correlated with tissues that are an auxin source or where auxins are known to act.

RESULTS

Developmental Control of the *ACS1* Promoter

We have analyzed the expression of the *ACS1* promoter by qualitative and quantitative measurements of the GUS activity at several stages of Arabidopsis development.

Figure 1 presents the progression of *ACS1* gene expression during development. Low levels of GUS activity were first detected in 3-day-old seedlings mainly in a restricted zone of the root (Figure 1A). Two to four days later, expression was apparent in the hypocotyl–root junction, at a lower level in the root vascular tissue, in the root tip, and in the young cotyledons (Figure 1B). With aging, this pattern evolved for both root and shoot. The expression was detectable from 5 to 12 days in the primary root tip and decreased after this period (compare Figures 1B and 1D). The activity in the roots increased steadily until full development of the root system was reached at ~30 to 35 days in vitro (Figure 1M). Figures 1K and 1L show that the expression was strongly correlated with the initiation and/or formation of lateral roots. Generally, elongated lateral roots did not show any expression at the root tip level, although in some cases expression near the root elongation zone was detected. In older roots, the overall expression visibly declined (Figure 1N). At this stage, it was limited to the younger and active branches where new lateral roots were still being formed.

In the shoot, the expression increased steadily to a maximum at ~30 to 35 days and was consistently stronger in the youngest tissues and the vegetative meristem (Figures 1B to 1F). The bottom part of the trichomes in leaves was heavily

Figure 1. (continued).

Representation of macroscopic histochemical analysis of transgenic pACC10-J+.
(A) Three-day-old seedlings.
(B) Five-day-old seedling. Arrows indicate the root–hypocotyl junction and root tip.
(C) Shoot from 9-day-old plant; trichomes.
(D) Fifteen-day-old seedling.
(E) Rosette from 21- to 25-day-old plant.
(F) Rosette from 35-day-old plant.
(G) Rosette of a flowering plant at 40 to 45 days.
(H) Flowering stem.
(I) Young flower buds and immature silique.
(J) Young flower.
(K) Lateral root formation, 9 to 12 days. Arrows indicate site of expression associated with lateral root formation.
(L) Lateral root formation.
(M) Roots from a 35-day-old plant.
(N) Roots from a plant 40 to 45 days after flowering.
(O) Three-day-old dark-grown plant.
(P) Five-day-old dark-grown plant.
(Q) Nine-day-old dark-grown plant.
(R) Plant grown for 7 days in the dark and then transferred to white light and grown for 7 days.

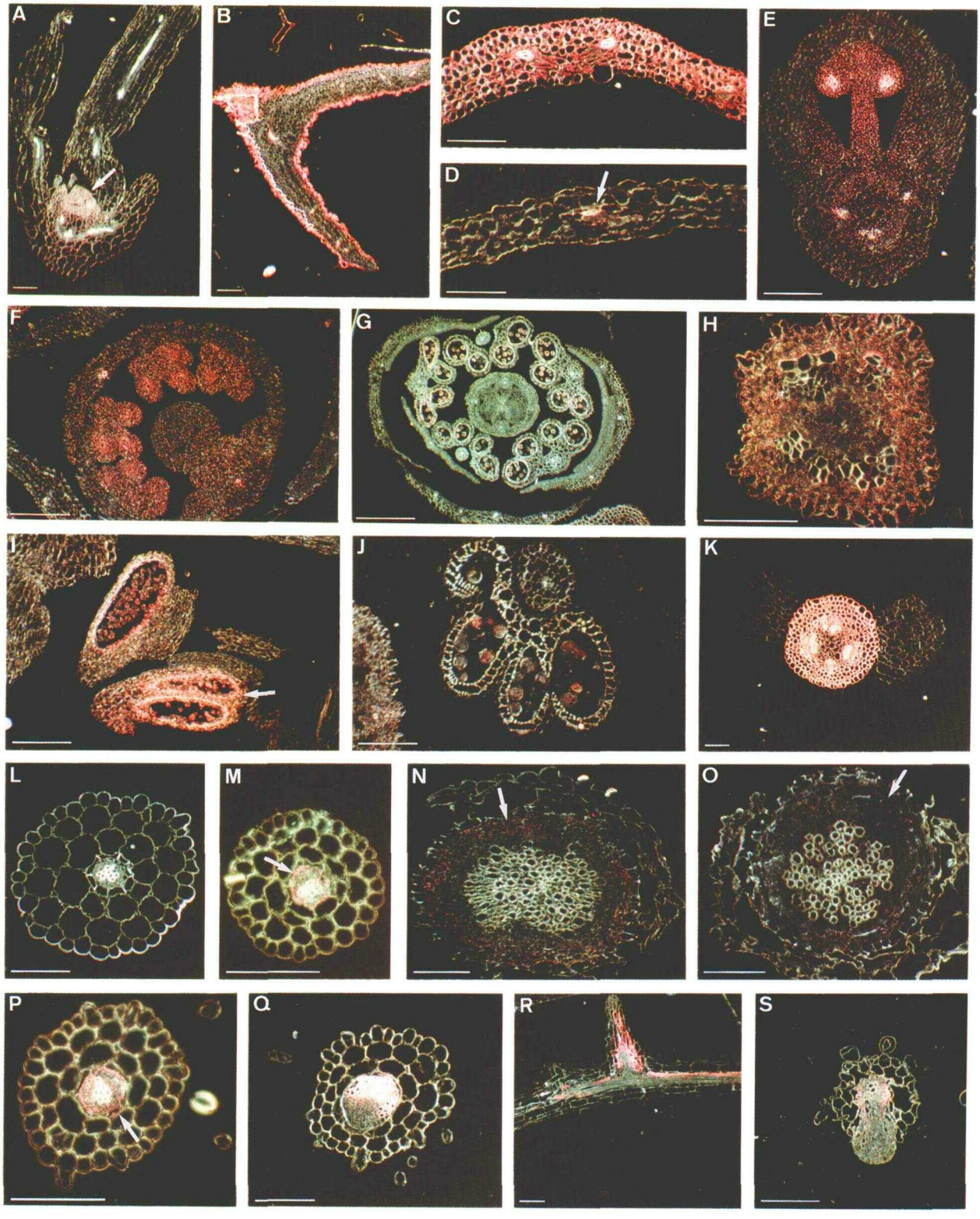

Figure 2. Expression of *ACS1* Gene in Arabidopsis during Development.

stained, resulting in a spotted pattern on the leaves (Figure 1C). A gradient of increased activity from older to younger leaves was observed (Figures 1D to 1F). This pattern of expression remained until the transition to the plant's reproductive cycle and coincided with a reduction of expression in most rosette leaves. Upon flowering, GUS activity was detected only in the youngest tissues, namely the youngest rosette leaves, young stem leaves, floral buds, and immature siliques (Figures 1G to 1J). At seed setting, the overall levels were even more diminished, especially in the rosette (data not shown). Expression was then highest near the inflorescence and floral meristems, in young flower buds (compare Figures 1H to 1J with Figures 2E and 2F, representing cross-sections of young flowers), correlating well with the age of the tissue involved. In older flowers, low expression was observed in the sepals and was even lower in petals and anthers, although visible in the pollen grains. Figure 2 gives additional evidence of a clear association of expression with young tissues. In young anthers, expression was detected in the tapetum and subtapetum layers, as well as in the pollen grains (Figure 2I), whereas in older anthers expression was only detected at a low level in the pollen grains (Figure 2J). In the pistil or immature silique, high expression was observed in the stigma and also in the receptacle (Figures 2H and 2K). No *ACS1* promoter activity was detected in mature siliques and seeds.

Additional information on tissue and cell type specificity was obtained from sections of different Arabidopsis organs after GUS staining (Figure 2). Expression in young leaves was observed in all tissues (Figure 2C), whereas in young cotyledons, it was mainly epidermal and low in the vascular bundles (Figure 2B). In older leaves (when detectable), sepals, and petals, expression was mainly vascular (Figure 2D). In contrast to the root apical meristem, the shoot vegetative apex showed a much higher activity, which was maintained throughout plant development (Figure 2A). Expression in hypocotyl sections was completely absent, confirming the data of Figure 1 (compare Figure 2L with Figures 1B and 1D).

In roots, expression was confined to the vascular bundle (pericycle layer and cambium; Figure 2M) and to the initiation of a lateral root appearing as an internal "knot" before emerging through the epidermal layer, as well as at the very early stages of elongation (compare Figures 2R and 2S with Figures 1F and 1H). This increased level of expression subsided eventually with further elongation of the emerging lateral root (data not shown). The expression in the root–hypocotyl junction (and root hair zone) was mostly visible in the vascular tissues (Figure 2P). An interesting observation was that two endodermis cells opposing the xylem poles, at the level of the root hair zone, were highly active (Figure 2P). As soon as a developing meristem (which will eventually lead to the emergence of a lateral root) appeared at the side of the xylem pole, the staining disappeared in the corresponding endodermis cell (Figure 2Q). An increased staining in the vascular bundle of the root–hypocotyl junction is concomitant with the development of this lateral meristem. The latter shows a drastic reduction of staining, which is in agreement with the phenomenon observed in lateral root formation (compare Figure 2S with Figure 2Q). In this case, the expression in the pericycle layer was limited to two opposing zones on the axis perpendicular to that of the two xylem poles. As the root aged, the expression

Figure 2. (continued).

Representation of thin sections of different tissues.
(A) Shoot apex of a 12-day-old seedling. Arrow indicates associated *ACS1* expression.
(B) Cotyledon of a 12-day-old seedling.
(C) Young leaf.
(D) Old leaf. Arrow indicates vascular bundle and associated *ACS1* expression.
(E) Young flower bud.
(F) Young flower.
(G) Old flower.
(H) Stigma.
(I) Young anthers. Arrow indicates tapetum and subtapetum layers.
(J) Old anther.
(K) Flower receptacle.
(L) Hypocotyl of a 15-day-old seedling.
(M) Root of a 15-day-old seedling. Arrow indicates *ACS1* expression associated with pericycle and cambium.
(N) Old root of a nonflowering plant at 30 to 35 days. Arrow indicates region of detectable expression.
(O) Old root of a flowering plant at 40 to 45 days. Arrow indicates region of detectable expression.
(P) Root–hypocotyl junction of a 15-day-old seedling. Arrow indicates endodermis cell that presents associated *ACS1* expression prior to appearance of lateral root.
(Q) Lateral root formation in the root–hypocotyl junction zone.
(R) Lateral root formation, longitudinal section.
(S) Lateral root formation, transversal section.
Bars = 100 μm.

was reduced and confined to the pericycle layer and phloem (Figure 2N). In flowering plants it was almost completely absent from the oldest parts (Figure 2O).

Table 1 presents quantitative GUS activities as determined by fluorometric assays. The results demonstrate that the activity of *ACS1* in roots of flowering plants is very low, ~0.25 units per g fresh weight. When expressed in units per milligram protein, reflecting the metabolically active parts, 5.4 units per mg were found, a level that was almost as high as that for young leaves (6 units per mg of protein). Nevertheless, one can note from Table 1 that the expression (when GUS activity is represented in units per gram fresh weight of tissue) in young leaves is ~70-fold higher than in roots. The level of expression is in the same range in all young tissues (19.9, 17.4, and 21.5 units per g fresh weight for young leaves, young stem leaves, and young flowers and meristems, respectively) and ~10-fold higher than that in old leaves. These data correlated well with the histochemical results. These measurements were repeated independently in plants of the same age, and although some variation was observed, the relative ratios between young and old tissues were maintained. The degree of strength of the promoter can be estimated by comparison to the activities detected in transgenic plants carrying the cauliflower mosaic virus 35S–driven *gus* gene construct. These activities were ~2 to 3 kilounits of GUS per mg of protein in 15-day-old seedlings, thus ~1000-fold higher than in light-grown seedlings carrying the pACC construct. In six independent lines of transgenic plants containing a promoterless *gus* gene, no sign of activity was detected (negative control; data not shown).

After wounding of young rosette leaves, no difference in the expression was observed at the histochemical level. In addition, no induction was observed upon wounding of rosette leaves from flowering plants (data not shown).

Table 1. Expression of *ACS1* in Different Tissues of Arabidopsis

	GUS Activities			
	Units per Gram Fresh Weight		Units per Milligram of Protein	
Tissue Type	Mean	SD	Mean	SD
Roots	0.25	0.06	5.42	0.42
Young rosette leaves	19.92	1.64	5.99	0.72
Old rosette leaves	1.83	0.56	2.11	0.28
Young stem leaves	17.44	0.69	4.75	0.07
Stems	4.40	0.62	3.34	0.42
Young flowers and inflorescence meristems	21.54	2.75	2.08	0.18

All material is derived from a bolting plant (40 to 45 days old). The values are the means of three determinations with standard deviations (SD) indicated.

Table 2. *ACS1* Expression during the Development of Young Arabidopsis Seedlings

Age (days after vernalization)	GUS Activity (units per milligram of protein)			
	Light Grown		Dark Grown	
	Mean	SD	Mean	SD
3	5.36	1.63	3.59	1.59
5	13.21	1.43	11.69	1.10
7	14.48	4.23	6.84	1.04
9	14.82	4.09	5.36	0.09
11	16.81	3.09	6.61	0.71
13	20.64	1.64	6.25	0.82
15	17.12	2.29	4.43	0.73

The values are the means of three determinations with standard deviations (SD) indicated.

Light Control of the *ACS1* Promoter

When comparing expression in dark-grown and light-grown seedlings, a different pattern became apparent, which was again age dependent. Figures 1O to 1R show that, in general, very low expression was observed in the cotyledons of dark-grown seedlings at all ages studied. In elongated hypocotyls, the pattern of expression changed with age: in 3-day-old seedlings expression was undetected (Figure 1O), from 5 to 7 days it became rather high (from the root–hypocotyl junction up to 80% of the hypocotyl; Figure 1P), and after 7 days, it decreased to very low levels (Figure 1Q). In the root, expression was initially visible (3-day-old dark-grown seedlings) in a pattern similar to that observed in 3-day-old light-grown seedlings (Figure 1O, compare with Figure 1A). Within 2 to 4 days, this was reduced to lower levels (Figures 1P and 1Q).

Table 2 represents the evolution of GUS activities with seedling age in light and dark conditions as obtained from fluorometric measurements. A clear difference between dark- and light-grown seedlings was observed, confirming the histochemical data (compare Figures 1A to 1C with Figures 1O to 1Q). The expression was approximately equal in 3-day-old seedlings in both conditions, but it progressed differently. Whereas in light-grown seedlings it increased after 5 days and remained at this level for the period studied, it peaked at about 5 days in dark-grown seedlings and was reduced twofold at later stages (Table 2).

When 5-day-old dark-grown seedlings were exposed to light for another 7 days, the expression pattern became almost the same as for light-grown seedlings, in both the root and the leaves (Figure 1R). As no induction by a red pulse could be demonstrated from either histochemical or fluorometric assays, we concluded that phytochrome is not directly involved in the regulation of *ACS1* expression (data not shown).

Measurements of ACC Contents, in Vivo ACC Oxidase Activities, and Ethylene Production in Arabidopsis

To support the physiological significance of the expression of the *ACS1* gene in young tissues, we measured internal levels of ACC and checked the activity of ACC oxidase which is able to convert ACC into ethylene. From the data presented in Table 3, it can be concluded that ACC is available in 2-week-old and 24-day-old Arabidopsis plants. A positive correlation could be found between the expression of the *ACS1* gene and ACC concentrations. Young leaf tissues had higher ACC concentrations than older leaves (compare contents in apex with first leaf pair and cotyledons in 2-week-old seedlings, and apex and fourth leaf pair with older leaf pairs in 24-day-old plantlets). Overall, younger seedlings presented a higher ACC content than older plantlets. In addition, the presence of an active ACC oxidase was confirmed in Arabidopsis plants. Upon incubation in a buffer containing 10 mM ACC and 10 mM aminooxyacetic acid, an ethylene emanation of 0.31 to 0.33 $\mu L\ hr^{-1}\ g^{-1}$ of fresh weight of tissue was observed in 21-day-old seedlings and young rosette leaves from bolting plants. These activities were approximately twofold higher than in older mature leaves (0.15 $\mu L\ hr^{-1}\ g^{-1}$ fresh weight of tissue). This suggests that the ACC produced can be converted into ethylene in young tissues. Furthermore, ethylene emanations from young leaf tissues (3.1 $nL\ hr^{-1}\ g^{-1}$ fresh weight of tissue) are approximately fourfold higher than in fully matured leaves (0.8 $nL\ hr^{-1}\ g^{-1}$ fresh weight of tissue). This result was, as in the case of ACC contents, in agreement with the expression pattern of *ACS1*.

Influence of Exogenous Ethylene Treatment on Cell Size in Arabidopsis Leaves

To attribute biological relevance to the expression of *ACS1*, and thus ethylene, in the control of cell shape and size, the following experiment was conducted. The 24-day-old plantlets were flushed with 10 ppm of ethylene in air for a period of 3 days and compared to air controls (see Methods). Figure 3A demonstrates that ethylene treatment dramatically affects the cell size in developing Arabidopsis leaves. Cells appear up to five times smaller than control (Figure 3B) and are extremely compacted. On the other hand, no change was observed in mature leaves of the same plants upon this treatment (compare Figure 3C with the control, Figure 3D). This result indicates that the expression of *ACS1*, and consequently the ethylene resulting from its activity, may play a role in the control of cell size and shape in young developing tissues.

Table 3. Measurement of ACC Concentrations in Different Tissues of Arabidopsis Plants in Two Developmental Stages

	ACC Content (nmol g^{-1} f.w.)	
Tissue Type	Seedlings (2-week-old)	Plantlets (24-day-old)
Root	11.7	1.2
Cotyledons	3.9	3.9
First leaf pair	13.8	2.4
Second leaf pair	–	2.3
Third leaf pair	–	2.1
Fourth leaf pair	–	3.7
Apex	25.9	4.1

All values are the average of two independent determinations. f.w., fresh weight of tissue.

DISCUSSION

ACC synthase, the key regulatory enzyme of the ethylene biosynthetic pathway, has been shown to be responsive to a series of environmental and developmental stimuli (Abeles, 1973; Yang and Hoffmann, 1984; Moore, 1989; Van Der Straeten and Van Montagu, 1991). In this paper, we report on the expression of one member of the Arabidopsis ACC synthase gene family (Van Der Straeten et al., 1992), as analyzed by the promoter–*gus* fusion technique. The *ACS1* promoter is strongly regulated during the development of Arabidopsis from the seedling stage to the mature flowering plant, both in the root system and in the aerial part. Figures 1 and 2 and Tables 1 and 2 show that the expression is associated with young tissues, whereas those completely differentiated have a considerably lower level of expression. These results are in good agreement with previously published data in which *ACS1* expression was studied by reverse transcription–polymerase chain reaction (RT-PCR) (Van Der Straeten et al., 1992).

The physiological role of ethylene and the changes in ACC synthase gene expression in late development (ripening and senescence) have been extensively documented (Roberts et al., 1985a; Broglie and Broglie, 1991; Van Der Straeten and Van Montagu, 1991). However, studies on the regulation of ethylene biosynthesis in vegetative cells and tissues have been limited. The analysis of expression of an Arabidopsis ACC synthase gene which is associated with early development provides some molecular data on this aspect of ethylene biology.

Expression of *ACS1* Is Associated with Young Tissues

The expression of the *ACS1* gene was found to be developmentally regulated in the shoot. The activity of GUS correlated well with the age of leaves. Thus, cotyledons stained at a very early stage (5 days old), but this staining decreased with the appearance of the first leaf pair (Figure 1). A gradient of decreased activity from younger to older leaves became clear from then on (Figure 1). This pattern remained until the

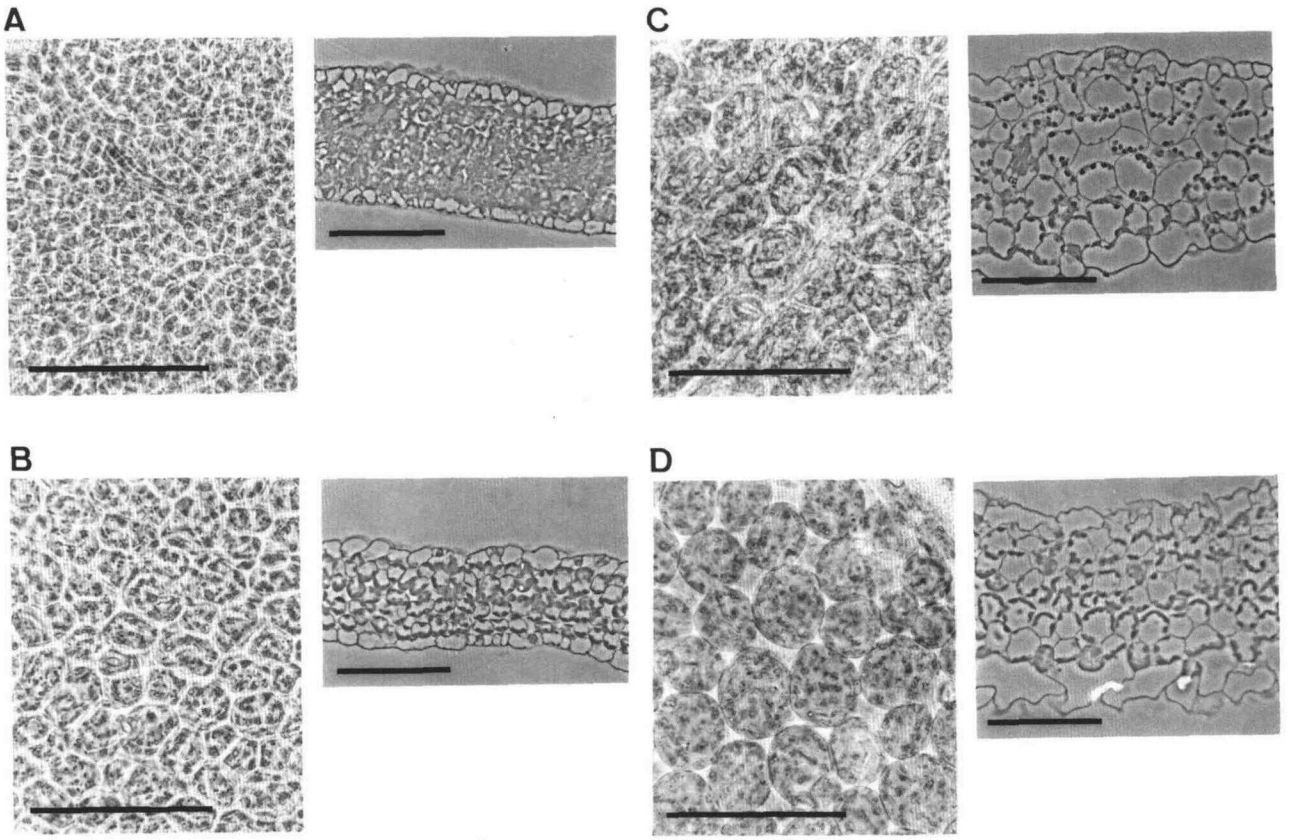

Figure 3. Effects of Ethylene on the Cell Size of Young or Mature Arabidopsis Leaves.

All material is from 24-day-old plantlets.
(A) NaOH cleared and thin section of ethylene-treated young leaf.
(B) NaOH cleared and thin section of air-treated (control) young leaf.
(C) NaOH cleared and thin section of ethylene-treated mature leaf.
(D) NaOH cleared and thin section of air-treated (control) mature leaf.
Bars = 100 μm.

transition into the reproductive phase, at which a total switch-off was observed in the older rosette leaves. These observations were substantiated by quantitative fluorometric assays (Table 1). In contrast to root apical meristems, the shoot vegetative apex showed a much higher activity that was maintained throughout plant development. These data confirmed the results obtained earlier by RT-PCR (Van Der Straeten et al., 1992). Thus, from these two independent analyses, it can be concluded that *ACS1* gene expression is generally confined to young tissue.

In several plant species it has been shown that different members of the ACC synthase gene family are expressed more strongly in response to certain factors, such as environmental stress, auxins, ripening and senescence, and physical wounding (Nakajima et al., 1990; Van Der Straeten et al., 1990; Dong et al., 1991; Olson et al., 1991; Rottmann et al., 1991; Yip et al., 1992). The importance of the expression of the *ACS1* gene in normal Arabidopsis leaves during development and its inverse correlation with aging indicates that this gene is probably not associated with tissue senescence (Abeles, 1973; Moore, 1989; Lawton et al., 1990; Raghothama et al., 1991; Van Der Straeten et al., 1993). Furthermore, the fact that wounding of young or old vegetative light-grown tissue had no effect on the *ACS1* promoter (data not shown) implies the involvement of another ACC synthase gene in wound responses. The results of RT-PCR using conserved primers indicated that other Arabidopsis ACC synthases are induced by wounding, as well as by ethylene, and in a more complex manner when compared to *ACS1* (Van Der Straeten et al., 1992).

In view of the strong correlation of *ACS1* expression and immature tissue, we suggest that *ACS1* is probably involved in the control of cell expansion and consequent determination of size and shape, where ethylene is known to have a definite role (Goeschl et al., 1966; Abeles, 1973; Moore, 1989; Guzmán

and Ecker, 1990; Ortuño et al., 1991; Sánchez-Bravo et al., 1992; Kieber et al., 1993). Moreover, quickly dividing and expanding tissues show relatively high rates of ethylene production (Osborne, 1991). Usual rates for expanding leaves range from 0.1 to 0.4 nL per g fresh weight per hr, falling below 0.1 nL per g fresh weight per hr in fully matured leaves. Interestingly, young leaves are also good sources of auxin, which is a well-known mediator of certain ethylene effects. It is possible that the expression of *ACS1* in immature tissues of Arabidopsis is related to the presence of auxin. Moreover, the expression at the level of the epidermis in cotyledons (Figure 2) can be correlated with the fact that maize coleoptiles transport auxins at the epidermal layer (Jones, 1990). However, it remains to be proved that auxins are also transported in the epidermal layer of cotyledons in dicotyledonous plants. Whether auxin has any direct influence on *ACS1* expression in leaves remains to be investigated.

ACC Contents, in Vivo ACC Oxidase Activities, and Ethylene Production in Young and Old Arabidopsis Leaves Are Correlated with *ACS1* Expression

The above hypothesis that presents *ACS1* as an ACC synthase gene involved in early development of vegetative tissues is obviously dependent on the existence of the active gene product and its reaction product, ACC, as well as on ACC oxidase, which converts ACC into ethylene. Therefore, we investigated whether ACC levels correlated with *ACS1* expression levels and whether ACC oxidase was present in young Arabidopsis tissues. Table 3 presents data indicating that ACC is detected in Arabidopsis. ACC levels correlated well with *ACS1* gene expression. Younger tissues had a higher ACC content than older tissues, and these trends were maintained in different developmental stages of the plant (Table 3). These data fit well with earlier observations by Osborne (1991) in Euonymus and in tomato leaf discs (Woodrow and Grodzinski, 1993). The high ACC content of the cotyledons might be explained by the fact that they were in a senescent stage when the plantlet had reached the age of 24 days. As the ACC content in cotyledons is approximately sixfold lower than in the apex at an early age (2-week-old seedlings; Table 3), it is possible that another member of the Arabidopsis ACC synthase family (Liang et al., 1992; Van Der Straeten et al., 1992), is involved in senescence.

In vivo ACC oxidase assays demonstrated the presence of this enzyme in young tissues leading to twofold higher activity than in older, mature tissues. This implies not only that the expression of the *ACS1* gene can lead to production of ethylene but also that the capacity of ACC oxidase is correlated with *ACS1* expression. Further evidence was obtained by the fact that ethylene production from young developing leaves was fourfold higher than from older mature leaves. These data fit well with results obtained in tomato leaf discs (Woodrow and Grodzinski, 1993).

Ethylene Influences Cell Size in Arabidopsis Leaves

One major biological question is related to the function of ethylene in young tissues. At present, one can consider that the effects of exogenous ethylene treatment on development are a clue to the function of the endogenous hormone. There is some experimental evidence suggesting that modifications of internal ethylene production can govern leaf growth in vivo. This is indicated by the transient enhancement of expansion following a red light pulse (Goeschl et al., 1967). Also, promotion of leaf expansion has been observed in the presence of Co^{2+} ions, known as ACC oxidase inhibitors (Lau and Yang, 1976).

In Arabidopsis, exogenous ethylene has a dramatic effect on the cell size of young leaves (Kieber et al., 1993), whereas it does not seem to affect the size of the cells in leaves already matured before the treatment (Figure 3). This result is in agreement with those obtained in Gramineae (meadow grass and oat), where the effect of exogenously supplied ethylene on cell enlargement seems dependent on the state of cell maturity (Van Andel and Verkerke, 1978). In young leaves, expansion is inhibited by ethylene but in mature leaves enlargement can be enhanced. These data and our present findings indicate that ethylene could play a role in the determination of cell shape and size, and subsequently of an organ, via the control of cell elongation at very early stages perhaps by affecting cell wall elasticity (due to increased levels of hydroxyproline-rich proteins) (Lamport, 1965; Ridge and Osborne, 1971; Sadava and Chrispeels, 1973; Sadava et al., 1973) or orientation of cell wall cellulose microfibrils, which itself is a consequence of changes of microtubule orientation (Steen and Chadwick, 1981; Eisinger, 1983; Roberts et al., 1985b).

ACS1 Expression Is Developmentally Regulated in the Root and Correlated with Lateral Root Formation

Our data indicated that the expression of *ACS1* in roots is equally associated with young tissues. In addition, the expression detected in the root tip was restricted to a period of time between 4 and 12 days. The expression in the root tip at early stages of root development could possibly be correlated with the suggestion that auxins are produced in this small zone (Van Overbeek, 1939; Thurman and Street, 1960). It is also possible that an inhibitory factor produced in the root tip controlling the formation of the branching roots, as proposed by Torrey (1976) and Schiefelbein and Benfey (1991), could be involved in the control of the expression of the *ACS1* gene, resulting in this transient pattern in the root tip. Interestingly, the appearance of laterals is concomitant with the disappearance of expression in the root apex.

Expression of *ACS1* in the root gradually increased up to maturation. With the maturation of the plant, the establishment of a fully developed root system, and the transition into the flowering stage, GUS activity decreased and became mostly observable in the young (active) parts where lateral branching

still occurred. This "active" region constitutes a small part of the entire root system. When fluorometric measurements are expressed in terms of units per gram fresh weight of tissue or units per milligram of protein (Table 1), two different views were obtained. This implies that one has to take into account the tissue and cell specificity of *ACS1*, which may cause an under- or overestimation of the quantitative data, and hamper the comparison of the results. In our case, there was clearly a better correlation between results expressed in units of GUS per gram fresh weight of tissue (Table 1) and histochemical observations (Figure 1). This was in good agreement with the weak signal detected by RT-PCR experiments in mature roots (Van Der Straeten et al., 1992).

An explanation for the significance of the expression of the *ACS1* gene in Arabidopsis roots is hindered by the lack of sufficient physiological information about the role of endogenous ethylene in root development. With the exception of the long-known effect of exogenously supplied ethylene in the inhibition of longitudinal root growth (Harvey and Rose, 1915) and the possible interference in root geotropic response (Jackson, 1991), few correlations can be drawn. There is also a lack of convincing evidence that elongation in unstressed roots is stimulated by inhibitors of ethylene action or synthesis (Jackson, 1991). This could suggest that the "basal" ethylene (in unstressed conditions) is insufficient to be physiologically active. Alternatively, it is possible that low concentrations of ethylene may enhance rather than restrain root growth (Jackson, 1991). Concentrations of ethylene well below 1 μL per L have been found to promote root elongation in several plant species in conditions that avoided possible artifactual accumulation of ethylene (Smith and Roberston, 1971; Kays et al., 1974; Goeschl and Kays, 1975; Konings and Jackson, 1979; Bucher and Pilet, 1982). The expression of the *ACS1* gene in vascular tissues of young roots could perhaps be related to ethylene-promoted root growth.

Furthermore, our observations point to a relation between the emergence of lateral roots and the *ACS1* expression. A predominant role of auxins has been accepted in the process of lateral root formation (Torrey, 1976; Schiefelbein and Benfey, 1991). It is suggested that preexistent lateral primordia are initiated in the elongation zone of the root tip (Torrey, 1976). Nevertheless, although indirectly, ethylene also has been correlated with the initiation of lateral root meristems (Torrey, 1976). In view of the results in this paper, lateral root elongation through the epidermal layer seems to be correlated with expression of an ACC synthase gene, and could possibly suggest a more significant involvement of ethylene in root development. With the appearance of laterals, the intensity of the GUS-dependent histochemical staining in the root tissue increases. A small zone of vascular tissue shows increased expression whenever a lateral root is at its early stage of meristem development and emergence through the main root (Figures 1 and 2). However, the meristems developing in the root–hypocotyl junction and in the root itself are almost devoid of any expression, although an increased expression is observed in the vascular bundle of this tissue near the meristematic region (Figure 2). More detailed studies on the hormonal control of the Arabidopsis *ACS1* gene will be pursued to further clarify the role of ethylene in lateral root formation.

Expression Pattern of *ACS1* Changes during Growth in the Dark

Because some aspects of light and ethylene growth responses are similar during seedling development (Goeschl et al., 1967; Pjon and Furuya, 1967; Mohr, 1972; Abeles, 1973; Guzmán and Ecker, 1990) and considering the contradictory reports relating light and ethylene (Bassi and Spencer, 1982; Grodzinski et al., 1982; Rohwer and Schierle, 1984; Decoteau and Craker, 1987; Parsons and Mattoo, 1991), the effect of darkness on the control of the *ACS1* promoter was investigated.

In the absence of light, a different pattern of expression of the *ACS1* gene was observed both by histochemical and fluorometric assays (Figure 1; Table 2). This was to a certain degree dependent on the age of the seedlings. Early stages showed a higher expression in the hypocotyl and in the root, whereas in older seedlings (more than 7 days old), expression was very low in all tissues (Figure 1; Table 2). The fact that reasonable levels of activity were detected by fluorometric assays in dark-grown seedlings older than 7 days (Table 2) is due to the higher sensitivity of this method. These results, again, confirmed previous data obtained by RT-PCR (Van Der Straeten et al., 1992).

It has been found that in dark-grown seedlings of lupine, most of the ACC present in the basal part of the hypocotyl is in the conjugated form as malonyl-ACC (Pérez-Gilabert et al., 1991). If this would also be the case in Arabidopsis, this could perhaps explain why the expression of the *ACS1* gene is not correlated with an inhibition of elongation in these conditions (5- to 7-day-old dark-grown seedlings) because ACC could readily be converted into the inactive conjugate. On the other hand, it is possible that the sensitivity of this tissue to ethylene is different. Alternatively, low endogenous levels of ethylene could enhance hypocotyl growth in the dark, in a manner similar to what has been hypothesized for roots. The expression in the hypocotyl was completely cancelled by light. Furthermore, light-grown hypocotyls are significantly shorter than dark-grown ones, possibly due to phytochrome-controlled inhibition of elongation independent of ethylene (Laskowski et al., 1992).

Another aspect to be considered is that no expression is detected in the apical hook of dark-grown seedlings. Hook formation in dark-grown seedlings is known to be ethylene dependent (Goeschl et al., 1966; Abeles, 1973; Taylor et al., 1988; Guzmán and Ecker, 1990). Therefore, one can speculate that either another ACC synthase gene is leading to the ethylene responsible for this effect or that the sensitivity of the tissue to ethylene is enhanced (Guzmán and Ecker, 1990). In addition, the possible interorgan transport of ACC to target tissues cannot be excluded because this phenomenon has been demonstrated in various cases (Amrhein et al., 1982; Fuhrer and Fuhrer-Fries, 1985; Mor et al., 1985; Nichols and

Frost, 1985; Kiss and Koning, 1989; Woltering, 1990; Tudela and Primo-Millo, 1992; O'Neill et al., 1993).

When dark-grown seedlings (5 days old) are transferred to light for a period of 2 to 7 days, the normal pattern of expression observed in white light conditions, including the age-dependent response of the shoot, is fully recovered (Figure 1R).

Taken together, these results suggest that there is developmental control of the Arabidopsis *ACS1* gene by light, although probably indirect. Because the CACGTG hexanucleotide (G-box core) present in this promoter (Van Der Straeten et al., 1992) has been found in many other light-regulated promoters (Giuliano et al., 1988; Schulze-Lefert et al., 1989; Donald and Cashmore, 1990; Guiltinan et al., 1990; Williams et al., 1992), it is not unlikely that this sequence is relevant in the observed responses. Future experiments should provide evidence for this hypothesis.

Our data indicate that *ACS1* expression in vegetative tissue is age dependent with a clear association with young tissues and lateral root formation. An additional control is exerted by light. Obviously, the promoter–*gus* analysis has its limitations and, as already stressed by Jefferson et al. (1987), a careful interpretation of the results is necessary mainly when comparing stain intensities in different cell types. Other possible controlling mechanisms (e.g., exonic or intronic sequences, 3′-untranslated region) cannot be determined by promoter–*gus* analysis (Douglas et al., 1991; Dietrich et al., 1992).

A dissection of the *ACS1* promoter, including a study of the protein–DNA interactions governing the promoter activity during plant development, as well as the study of the hormonal regulation of the *ACS1* gene, particularly the possible relation with auxins, will be the scope of future research. This may lead to a more detailed view of the molecular patterns governing ethylene production in plant life.

METHODS

Constructs

The 1432 bp of the 5′ region upstream of the ATG start codon of the 1-aminocyclopropane-1-carboxylate (*ACS1*) gene (Van Der Straeten et al., 1992) was modified by polymerase chain reaction (PCR) to allow easy subcloning in the NcoI fusion site of the β-glucuronidase (*gus*) gene that is present in pGUS1 (Plant Genetic Systems, Gent, Belgium). After replacement of the internal BglII-ClaI fragment (1300 bp) of the PCR product by original sequences followed by a sequence checkup to exclude PCR-induced mutations, a cassette containing the fusion and the 3′ untranslated region of the octopine synthase gene was transferred as a SmaI-XbaI fragment in the T-DNA vector pGSV4 (Plant Genetics Systems) and introduced into *Agrobacterium tumefaciens* C58C1 (pGV2260) by normal triparental mating (Van Haute et al., 1983). A promoterless *gus* construct (negative control) was made in the same vector. In both cases, a single colony was streaked on media containing triacillin, rifampicin, and spectinomycin as selectable markers, and the presence of the intact T-DNA was checked by DNA gel blot analysis of total bacterial DNA (Dhaese et al., 1979) (data not shown). Preliminary results from DNA gel blot analysis indicate that up to three T-DNA insertions are present in the Arabidopsis genome of the transgenic line pACC10-J+. The p35S-*gus* construct was kindly provided by Jürgen Denecke (Plant Genetic Systems).

Plant Material, Growth Conditions, and Stress Treatments

Arabidopsis thaliana (ecotype C24 and Columbia) plants were grown at 22°C and 60% relative humidity under white fluorescent light (photoperiod 16-hr light/8-hr dark; fluence rate, 75 μmol m^{-2} sec^{-1}).

Root transformations were done as described elsewhere (Valvekens et al., 1988). Sixteen transformants were analyzed of which four showed detectable *gus* expression. The transformant line pACC10 was selected. A homozygous T_2 line named pACC10-J+ was used for all the studies presented in this report. Seeds were germinated, after 24 hr vernalization at 4 to 8°C, and grown in vitro on kanamycin-containing media (Valvekens et al., 1988), at 21°C and 60% relative humidity, and under the same light regime as mentioned above (fluence rate, 50 μmol m^{-2} sec^{-1}). Dark-grown pACC10-J+ Arabidopsis plants were grown in the same conditions of temperature and humidity and used for experiments at the age of 3 to 12 days. All ages indicated are considered after the vernalization treatment.

For wounding experiments, leaf tissue at different ages was severely punctured with a needle or cut into small pieces. This treatment was followed by incubations varying between 30 min and 2 hr.

Ethylene and control treatments were done as follows. The 24-day-old plantlets were placed inside a 2.5-L flask. Subsequently, 10 ppm of ethylene or air (organic carbon free) was flushed through at a flux rate of 250 mL min^{-1}. The flasks were left in these conditions for 3 days under a photoperiod of 16-hr light/8-hr dark (fluence rate, 75 μmol m^{-2} sec^{-1}). Immature and mature leaves of the plantlets treated with air or ethylene were cut, fixed in a 3:1 mixture of ethanol and acetic acid for 24 hr, and washed three times for 2 hr with ethanol. Finally, samples were washed three times for 2 hr with 1.5 M NaCl, 0.5 M Na_2HPO_4, $2H_2O$, 0.015 M $KHPO_4$, pH 7.4 and then softly cleared during 16 hr with 0.1 M NaOH. Photographs of the leaves were taken at low-power magnification on a light microscope. Thin sections of air- or ethylene-treated leaves were prepared according to Peleman et al. (1989). Photographs were taken with a Leitz-DIAPLAN microscope under bright-field optics.

Protein Extraction, Histochemical, and Quantitative GUS Assays

Protein extractions were as described previously (Jefferson, 1987; Jefferson et al., 1987). Protein measurements were according to Bradford (1976), and spectrophotometric measurements were done in a computer-directed microtiter plate reader (340-ATTC colorimetric reader and SOFT 2000 software, SLT Lab Instruments, Grödig/Salzburg, Austria).

Histochemical assays were performed as described previously (Jefferson et al., 1987), except that all the material analyzed was prefixed in cool 90% acetone for 20 min at room temperature and then incubated for 24 to 48 hr at 37°C in 50 mM sodium phosphate buffer, pH 7.2, containing 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (Biomol, Hamburg, Germany). Photographs from whole mounts were taken at low-power magnification using bright-field microscopy. Thin sections of positively stained plant material were prepared as indicated in the previous section. Photographs were taken with a Leitz-DIAPLAN microscope equipped with dark-field optics.

Quantitative GUS activity measurements were performed as reported previously (Jefferson, 1987; Jefferson et al., 1987), using 4-methylumbelliferyl glucuronide as a substrate in a buffer containing 50 mM sodium phosphate, pH 7.0, 10 mM β-mercaptoethanol, 10 mM Na_2 · EDTA, and 0.1% Triton X-100. The fluorometric method was adapted for determination of GUS activities by kinetic analysis at 37°C in a computer-directed microtiter plate reader (Fluoroscan II; Labsystems, Helsinki, Finland) (Breyne et al., 1993). The results are expressed in units of GUS per mg of protein or units of GUS per g of fresh weight of tissue.

Determination of ACC Contents, in Vivo ACC Oxidase Assays, and Measurement of Ethylene Production

ACC concentrations of different tissues of 2-week-old and 24-day-old Arabidopsis plants were determined according to the method of Chauvaux et al. (1992) based on coupled HPLC/mass spectrophotometry.

In vivo ACC oxidase assays were done according to the methods of Hamilton et al. (1991) and Lee and Chu (1992) with slight modifications. Either complete 3-week-old Arabidopsis seedlings of which roots had been removed or detached young or mature rosette leaves of a bolting plant (C24 ecotype) were submerged in 3 mL of a 10 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.5, solution containing 10 mM ACC and 10 mM aminooxyacetic acid in a 10-mL flask and then vacuum infiltrated (10 min under vacuum). After 4 hr of preincubation, the flasks were sealed with a gas-tight cap.

Ethylene production by young or mature Arabidopsis rosette leaves was measured as follows. The leaves were cut and placed in a 10-mL flask on humid glass fiber paper. After a 6-hr preincubation period to avoid wound-induced ethylene production, the flasks were sealed.

Aliquots (500 μL for ACC oxidase assays, 1 mL for ethylene production) were taken from the head space after a certain period (1.5 to 2 hr for ACC oxidase assays, 22 hr for ethylene production) and injected into a gas chromatograph (IGC-120DFL; Intersmat, Pavillons sous Bois, France). The accumulation of ethylene produced was calculated using data processed by a recorder/integrator (ENICA 21; Delsi Instruments, Suresnes, France).

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