Journal of
Experimental
Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Hormonal control of root development on epiphyllous plantlets of *Bryophyllum* (*Kalanchoë*) *marnierianum*: role of auxin and ethylene

Richard G. Kulka*

Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel

Abstract

Epiphyllous plantlets develop on leaves of *Bryophyllum marnierianum* when they are excised from the plant. Shortly after leaf excision, plantlet shoots develop from primordia located near the leaf margin. After the shoots have enlarged for several days, roots appear at their base. In this investigation, factors regulating plantlet root development were studied. The auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA) abolished root formation without markedly affecting shoot growth. This suggested that auxin transport from the plantlet shoot induces root development. Excision of plantlet apical buds inhibits root development. Application of indole-3-acetic acid (IAA) in lanolin at the site of the apical buds restores root outgrowth. Naphthalene acetic acid (NAA), a synthetic auxin, reverses TIBA inhibition of plantlet root emergence on leaf explants. Both of these observations support the hypothesis that auxin, produced by the plantlet, induces root development. Exogenous ethylene causes precocious root development several days before that of a control without hormone. Ethylene treatment cannot bypass the TIBA block of root formation. Therefore, ethylene does not act downstream of auxin in root induction. However, ethylene amplifies the effects of low concentrations of NAA, which in the absence of ethylene do not induce roots. $Ag_2S_2O_3$, an ethylene blocker, and $CoCl_2$, an ethylene synthesis inhibitor, do not abolish plantlet root development. It is therefore unlikely that ethylene is essential for root formation. Taken together, the experiments suggest that roots develop when auxin transport from the shoot reaches a certain threshold. Ethylene may augment this effect by lowering the threshold and may come into play when the parent leaf senesces.

Key words: Auxin, *Bryophyllum*, development, epiphyllous, ethylene, *Kalanchoë*, plantlet, roots.

Introduction

The formation of miniature plants on the leaves of *Bryophyllum* species has intrigued biologists since Goethe (1820). In some species such as *B. daigremontianum*, plantlets develop spontaneously on leaves attached to the plant (Henson and Wareing, 1977) whereas in other species such as *B. calycinum* (Goethe, 1820), *B. fedschenkoi*, and *B. marnierianum* (Kulka, 2006), plantlets develop only when the leaves are detached from the plant. The latter type of species is convenient for studying the regulation of plantlet formation as the development can be induced at any time by leaf excision. During the last century plantlet development in *B. calycinum* (also known as the 'Goethe plant') was studied in a number of laboratories (Goebel, 1902, 1916; Loeb, 1915; Reed, 1923; Mehrlich, 1931; Freeland, 1933; Heide, 1965; Karpoff, 1982; Houck and Riesberg, 1983). It was found that when the leaf is plucked, plantlets develop from primordia pre-formed at the leaf margin (Howe, 1931; Naylor, 1932; Yarbrough, 1932, 1934). The crucial question was how plantlet primordium dormancy is maintained while the leaf is attached to the plant. Although some papers, when taken together, hinted at an answer to this question (Loeb, 1915; Henson and

Wareing, 1977; Yazgan and Vardar, 1977), no definitive solution was reached. In this laboratory, a related species, *B. marnierianum*, was used to study the question of plantlet primordium dormancy further (Kulka, 2006). The experiments indicated that cytokinins originating in the plant stem maintain plantlet dormancy. It was suggested that when the leaf is plucked, cytokinins are depleted and plantlet primordium development can proceed. The plantlet roots develop several days after the appearance of the plantlet shoot. In the work described here, factors regulating plantlet root development were studied. Clues to the mechanism came from the dramatic effects of two substances on root outgrowth. 2,3,5-Triiodobenzoic acid (TIBA), an auxin transport inhibitor, completely inhibits plantlet root development but has virtually no effect on shoot growth. The plant hormone ethylene, on the other hand, causes precocious development of plantlet roots. The experiments described below indicate that auxin transport initiates root development while ethylene amplifies the effect of auxin.

Materials and methods

Materials

Amino-oxyacetic acid, (AOA), amphotericin B, benzylaminopurine (BAP), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), myo-inositol, kinetin, naphthalene-1-acetic acid (NAA), and TIBA were purchased from Sigma. Ethephon and L-α-(2-aminoethoxy-vinyl)glycine (AVG) were obtained from Riedel de Haen. *N*-1-Naphthylphthalamic acid (NPA) was purchased from Greyhound Chromatography and Allied Chemicals, UK. Stock solutions used were as follows: BAP, kinetin and NPA, 50 mM in dimethylsulphoxide; ethephon, 100 mM in water. Stocks of other hormones were 50 mM or 100 mM in ethanol, and TIBA was 10 mM in ethanol. The hormones were diluted to the appropriate concentrations in water. Controls for equivalent concentrations of solvents were included in experiments. Silver thiosulphate ($Ag_2S_2O_3$) was prepared by mixing 2 vols of 0.1 M $AgNO_3$ with 8 vols of 0.1 M $Na_2S_2O_3$.

Plants

Plants of *B. marnierianum* (Jacobsen) were grown in a greenhouse illuminated by natural light, maintained above 15 °C in winter but not thermally regulated in summer. Plants were harvested all the year round. Fully developed leaves, 25–35 mm long and ~2 mm thick, from mature plants, were used in the experiments.

Excised leaves

For experiments without hormone or other chemical treatment, excised leaves or other plant parts were placed in Petri or other transparent dishes lined with moist Whatman 3MM filter paper. They were incubated in a growth chamber at 25 °C illuminated with white light at 80 μmol photon m^{-2} s^{-1} for 16 h, and kept in the dark for 8 h.

Chemical treatment of leaves

Plants without their roots were cut into several pieces, washed three times for 30 s in 0.005% Tween-20, and then sterilized in 5% bleach containing 0.005% Tween-20 for 5 min followed by three washes of 30 s each in sterile distilled water. All steps were accompanied by gentle agitation. [One hunded per cent bleach contained 3% (w/v) sodium hypochlorite and 0.8% (w/v) NaOH.] The leaves, with or without attached stems, were then excised. The petiole or basal end of the stem section was immersed in distilled water (5 ml) containing the appropriate additions in a well of a 6-well dish (Nunc) under sterile conditions. For acidic hormones and TIBA, 1 mM phosphate buffer pH 6.5 was used instead of water. The covers of the dishes were raised with plasticine at each corner to avoid contact with the leaf. The edge of the dish was wrapped with Parafilm and the dishes were incubated in a growth chamber as described above.

Organ culture of leaf explants

Plants without their roots were cut into several pieces and washed three times for 30 s in 0.005% Tween-20. Then they were sterilized in 10% bleach containing 0.005% Tween-20 for 10 min followed by three washes of 30 s each in sterile distilled water. All steps were accompanied by gentle agitation. Triangular sections of leaf ~30 mm^2 surrounding a plantlet primordium were excised. These were placed in the well of a 12-well tissue culture dish (Nunc) containing 2 ml of MS (Murashige–Skoog) agar with the appropriate additions. Two explants were placed in each well. The dishes were incubated in a growth chamber as described above. The MS medium contained MS salts (Murashige and Skoog, 1962), 0.1 mg l^{-1} thiamine–HCl, 0.5 mg l^{-1}, nicotinic acid, 0.5 mg l^{-1} pyridoxine, 2 mg l^{-1} glycine, 100 mg l^{-1} myo-inositol, 3% sucrose, and 0.5% agar; pH 5.7. The medium also contained 5 μM kinetin which was essential for shoot growth. This was used rather than BAP, which inhibits root outgrowth at the same concentration. A 20 μl aliquot of Amphotericin B, 250 μg ml^{-1}, was spread on the surface of each well before the start of the experiment.

Plantlet apical bud excision and auxin treatment

Plantlets that had formed on leaves 10 d after removal from the plant were used. The apical bud was excised with fine ophthalmic surgeons' scissors. Where indicated, auxin was applied to the cut end as a lanolin paste. This was prepared by mixing 10 μl of 100 mM NAA or IAA in ethanol with 100 mg of lanolin. Controls were treated with a mixture of 10 μl of ethanol in 100 mg of lanolin.

Treatment with ethylene

Leaf or explant cultures prepared as described above were placed in a desiccator (with a volume of 8.0 l). Ethylene was released from 25 ml of a solution of ethephon (concentration as indicated in individual experiments), by adding phosphate buffer pH 6.5 to a final concentration 5 mM. For explants, the ethephon concentration was 20 μM. The maximum concentration of ethylene which could be released from this solution, which contained a total of 0.5 μmol of ethephon, was 1.4 μl l^{-1} of air. The ethephon solution was renewed every three days or each time the desiccator was opened.

Measurements and statistics

Plantlet development and growth were followed with a dissecting microscope. Plantlet shoot growth was followed by measuring the widest leaf pair span of each plantlet ('leaf pair span'). Root growth was determined by measuring the length of the longest root of each plantlet ('longest root length' on the *y*-axis of the figures). Individual leaf pair span or maximum root length values were entered into a Microsoft Excel spread sheet and the standard deviation was calculated using the STDEV function. Plantlets

wthout roots were recorded as zero root length. Error bars in the figures show the standard deviation. As root outgrowth was not synchronous and root length was strongly affected by various treatments, the results were also expressed as '% of plantlets with roots'. This value indicates the extent of root initiation independently of root length. All experiments were repeated at least three times.

Results

Plantlet roots develop several days after plantlet shoot emergence from the leaf

When leaves of *B. marnierianum* are detached from the plant stem, plantlets develop from primordia present at their margins (Kulka, 2006). Plantlet shoots emerge and grow for several days before roots are formed (Fig. 1). Shoots develop mainly as a cohort, with a few stragglers, reaching similar stages of development at around the same time. Root development is less synchronous. The first roots usually emerge 10–15 d after leaf detachment at the junction of the plantlet shoot with the leaf (Fig. 1A). In the experiment summarized in Fig. 1, roots first appeared 13 d after leaf excision when the mean plantlet leaf pair span was ~4 mm. Unlike shoot outgrowth, the time of initiation and elongation of roots differs markedly from one plantlet to another even if they are on the same leaf (Fig. 1A). There appears to be a certain minimum plantlet size necessary for root development. However, the largest plantlets do not necessarily produce roots first. Also, plantlets of a similar size do not form roots at the same time (Fig. 1A). The lack of synchrony of root growth is reflected in the very large standard deviation of root length on the root length curve (Fig. 1B). Several days after first root emergence, most plantlets produce roots of widely different lengths (Fig. 1A, B). For this reason most of the observations on root development are expressed as the percentage of plantlets with roots (Fig. 1C) as well as in terms of mean root length. This comparison is important as some treatments result in a high percentage of plantlets with short roots. There are considerable differences in the times of first appearance of roots between different batches of leaves.

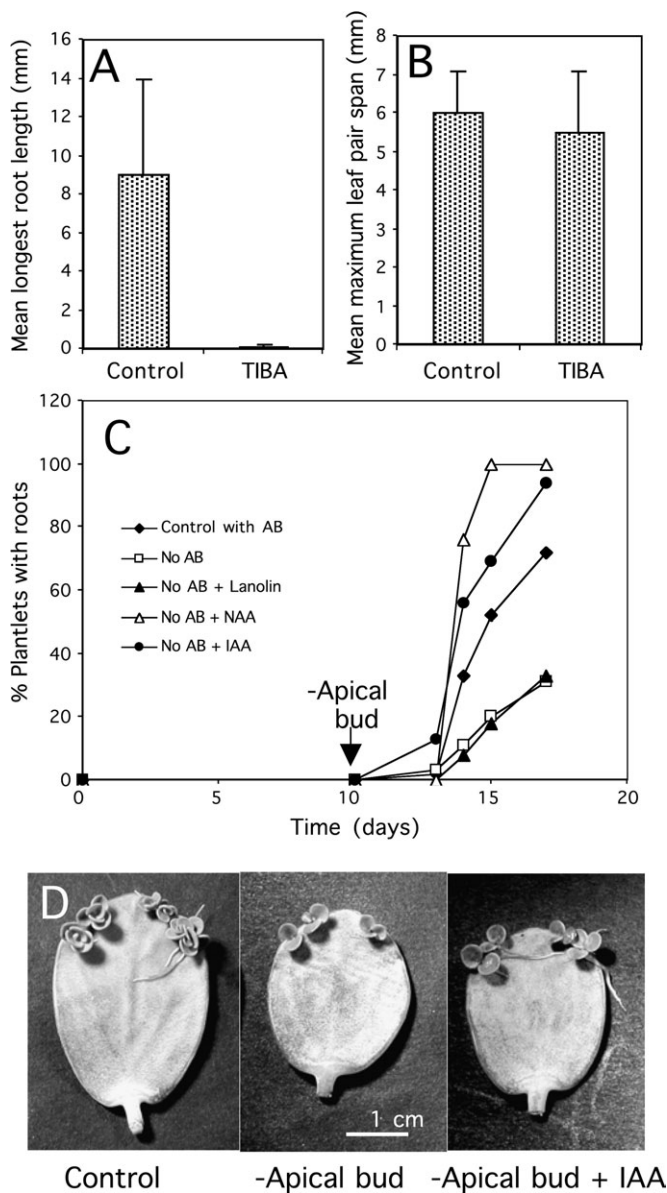### Role of auxin in plantlet root development

A striking finding is that plantlet root emergence from plantlets on leaves is completely inhibited by TIBA, a polar auxin transport inhibitor (Fig. 2A) (Taiz and Zeiger, 2006). In contrast, shoot growth is not significantly inhibited by TIBA (Fig. 2B). A possible explanation is that auxin transport from the plantlet shoot induces root development as in other plants (Sachs, 1991; Taiz and Zeiger, 2006).

Removal of the apical buds of plantlets 10 d after plucking the leaf inhibits root formation in most plantlets (Fig. 2C). Root development of almost all decapitated plantlets is restored by applying auxin (NAA or IAA) in
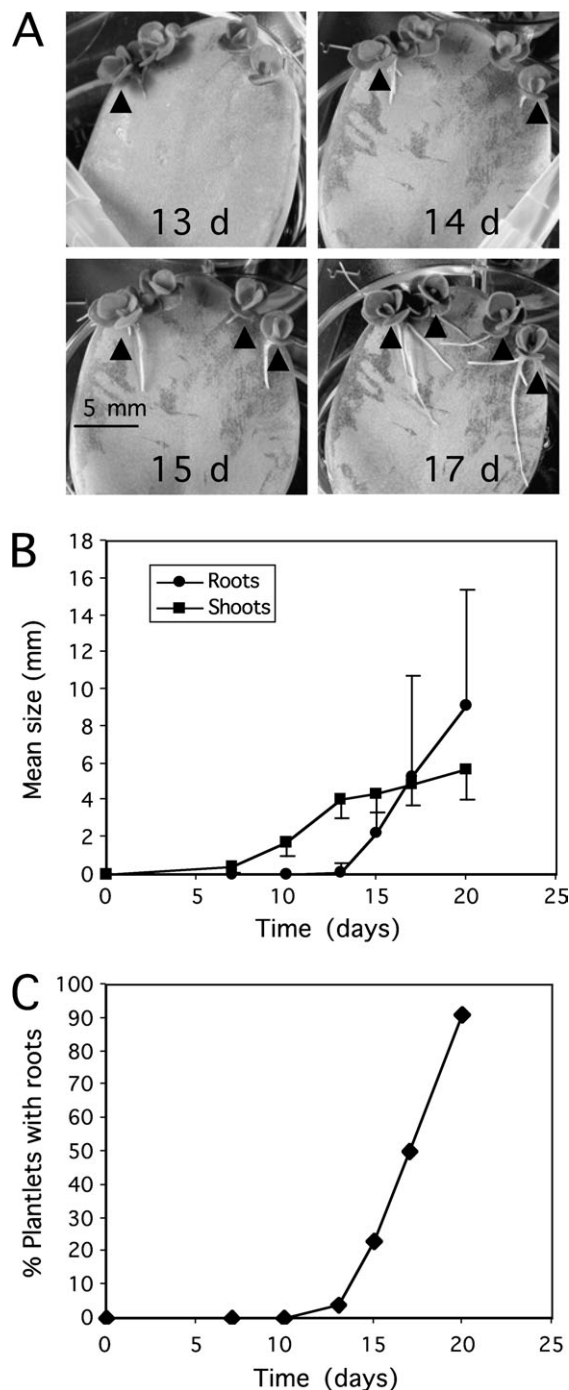
Fig. 1. Normal plantlet root development. Excised leaves were placed in 6-well dishes with petioles immersed in water. Leaf pair span and maximum root length were measured at intervals. (A) Root development on a single leaf after 13, 14, 15, and 17 d. Arrows mark plantlets with roots. (B) Mean shoot leaf pair span (mm) (filled squares) and mean longest plantlet root length (mm) (filled circles) at different times after leaf excision. Downward error bars, shoots; upward error bars, roots. $n=52$. (C) Percentage of plantlets with roots at different times after leaf excision.

lanolin paste to their decapitated ends. IAA induces the growth of elongated roots similar in length to those of the untreated control (Fig. 2D). NAA induces normal

Fig. 2. Inhibition of plantlet root development on leaves by TIBA or apical bud excision. Leaves were incubated with petioles immersed in 1 mM phosphate buffer with or without 5 μM TIBA. (A) Mean longest plantlet root length 15 d after leaf excision; n=37. (B) Mean maximum plantlet leaf pair span 15 d after after leaf excision; n=10. (C) Effect of apical bud excision and treatment with auxin. Excised leaves were incubated in Petri dishes for 10 d. Apical buds were removed from all plantlets except the controls (filled diamonds). Treatment of decapitated plantlets: none (open squares); lanolin (filled triangles); NAA in lanolin (crosses); IAA in lanolin (filled circles). AB, apical bud. For details see Materials and methods. (D). Using the same protocol as in (C), leaves were photographed 15 d after their removal from the plant. Ten days after leaf excision, plantlet apical buds were removed from the central and right hand leaf, and IAA in lanolin was applied to the decapitated ends of plantlets on the right hand leaf.

elongated roots somewhat shorter than those of the control (not shown). These observations support the view that the polar transport of auxin in the plantlet shoot induces root development.

In order to avoid possible complications due to auxin produced by the leaf, further experiments to test the effects of auxin were carried out with leaf explants. The question arose of whether exogenous auxin could reverse the effect of TIBA. Because of its hydrophobic nature and relative stability, the synthetic auxin NAA was used. NAA was successfully used in other laboratories to overcome genetic lesions of auxin import (Casimiro *et al.*, 2001; Marchant *et al.*, 2002). NAA was found to bypass the auxin influx carrier which is inhibited by TIBA (Marchant *et al.*, 1999). Addition of NAA to explants treated with TIBA restores root formation (Fig. 3A). However, the roots formed under these conditions are short and stubby (Fig. 3B, C), indicating that NAA is effective in reversing the inhibitory effects of TIBA on root initiation but does not promote root elongation. Exogenous IAA did not reverse the inhibition of root outgrowth by TIBA. As TIBA inhibits both the auxin influx and efflux systems (Kleine-Vehn *et al.*, 2006), this result is not surprising.

The polar auxin transport inhibitor NPA does not prevent plantlet root development

Surprisingly, NPA, another widely used auxin transport inhibitor (Taiz and Zeiger, 2006), has no effect on plantlet root development either on leaves (not shown) or on explants, even at a concentration of 25 μM (Fig. 4A). A lower concentration of NPA than that used with the explants (10 μM) completely inhibits adventitious root growth at the basal end of cuttings but does not prevent it at positions nearer the apex (Fig. 4B). TIBA (10 μM) completely inhibits all adventitious root growth. These observations imply that different auxin transport pathways function in plantlet root induction and adventitious root induction in cuttings.

Ethylene causes precocious plantlet root development

When isolated leaves are exposed to ethylene, roots develop on plantlets several days earlier than on control leaves (Fig. 5A, B). In the presence of ethylene, the roots develop when plantlets are smaller than usual. Ethylene causes premature leaf senescence and promotes the detachment of plantlets. This makes it difficult to study its effect on plantlets on leaves over extended periods of time. Also substances released by the senescing leaf may affect plantlet development.

In order to avoid complications due to leaf senescence, further experiments to elucidate the action of ethylene were done with explants. Plantlet shoots growing on explants treated with ethylene form roots several days earlier than the controls (Fig. 5C, D). The maximum leaf pair span of plantlets has been used as an approximate measure of plantlet size at different stages of development (Fig. 5E). Inspection of Fig. 5D and E reveals that
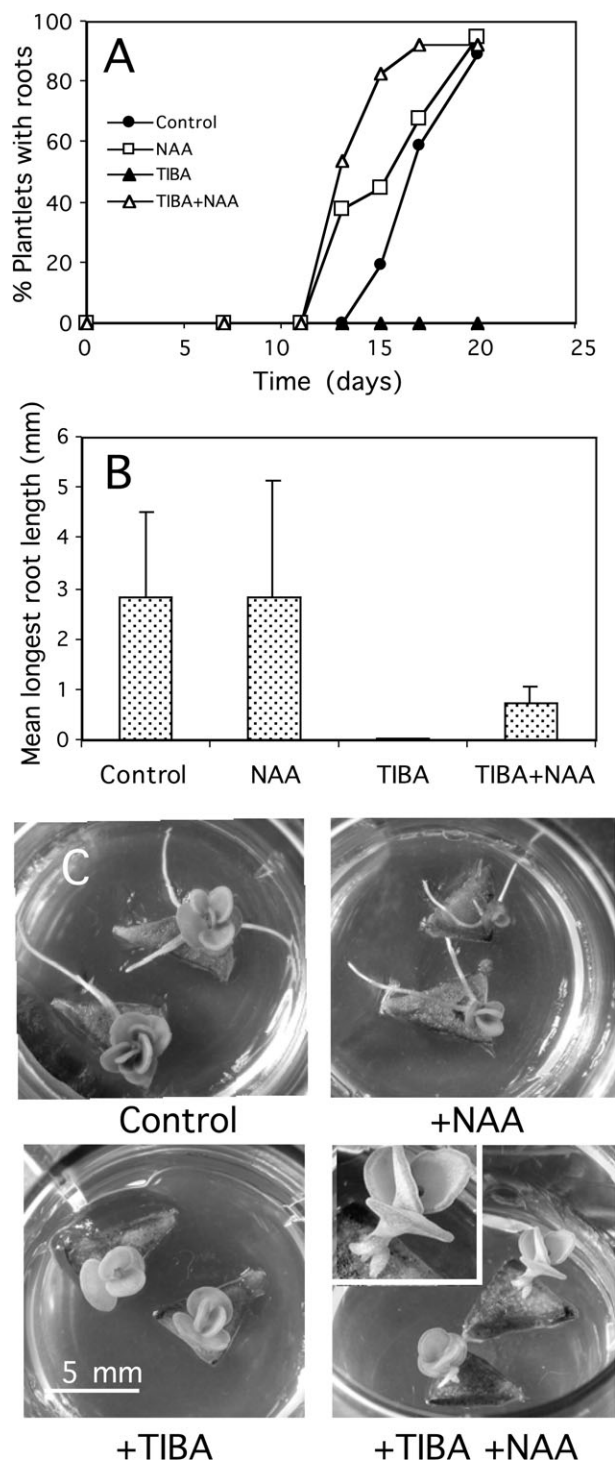
Fig. 3. Rescue of TIBA inhibition of plantlet root outgrowth on leaf explants by NAA. (A) Percentage of plantlets with roots at different times after leaf excision. Control (filled circles); 5 μM NAA (open squares); 2 μM TIBA (filled triangles); 2 μM TIBA plus 5 μM NAA (open triangles). (B) Mean maximum plantlet root length after 20 d; $n=18$. (C) Photos taken after 18 d of the same treatments in a different experiment from that shown in (A) and (B). Inset bottom right hand panel: upper plantlet enlarged to show roots.

ethylene shifts the onset of root outgrowth to a leaf pair span about half that of the control.

In addition to causing precocious root development, ethylene affects plantlet morphology in several ways. The roots induced by ethylene are hairy (Fig. 5C). In the presence of ethylene, plantlets usually produce more roots (3–6 per plantlet) than control plantlets, which usually have one or two roots (Fig. 5C). Although measurements of leaf pair span do not reveal this, ethylene changes the morphology of the leaves. Leaves of plantlets developing in the presence of ethylene have a distinct petiole, giving the leaf pair a propeller-like appearance (Fig. 5C, right panels). Petioles are usually not visible in control plantlets (Fig. 5C, left panels). Stimulation of petiole growth by ethylene has been observed in other plant species (Millenaar *et al.*, 2005). After prolonged incubation with ethylene, a large percentage of plantlets develop more than one tier of leaves (Fig. 5C). In the experiment shown in Fig. 5, after 15 d of ethylene treatment 80% of plantlets had two tiers of leaves whereas only 30% of control plantlets had two leaf tiers. This difference is not significantly reflected in the leaf span curve (Fig. 5E) but indicates that ethylene-grown plantlets on average are bigger at this stage of development. However, the premature root development induced by ethylene cannot be attributed to a larger plantlet size as at the time of root emergence ethylene-treated plantlets and control plantlets have only a single pair of leaves plus a bud, and are similar in size (Fig. 5C).

In other plant systems, ethylene was found to be a mediator of auxin action (Hansen and Grossman, 2000; Y Tanaka *et al.*, 2006). The question therefore arose whether ethylene is an obligatory intermediate of plantlet root induction acting downstream of auxin. If ethylene acted downstream of auxin, a strong precocious response to ethylene would be expected even when TIBA is present. To test this, explants treated with TIBA were exposed to ethylene. In the presence of TIBA, only a slow response of some of the explants to ethylene was observed, 5 d after the response to ethylene alone (Fig. 6A). The limited root development under these conditions was probably due to leakiness of the TIBA block of auxin transport. Many of the roots formed under these conditions were 'ectopic', emerging above the first pair of leaves (Fig. 6C, arrows). These observations indicate that ethylene cannot bypass the inhibitory effect of TIBA and therefore does not act downstream of auxin.

The question arose of whether the effect of ethylene is dependent on the presence of auxin. Preliminary experiments showed that low concentrations of NAA (500 nM) on their own slightly stimulated root development but were insufficient to reverse inhibition by TIBA. Figure 6A shows that when explants were treated with TIBA plus a low concentration (500 nM) of NAA, no roots were formed. However, when explants with TIBA and 500 nM
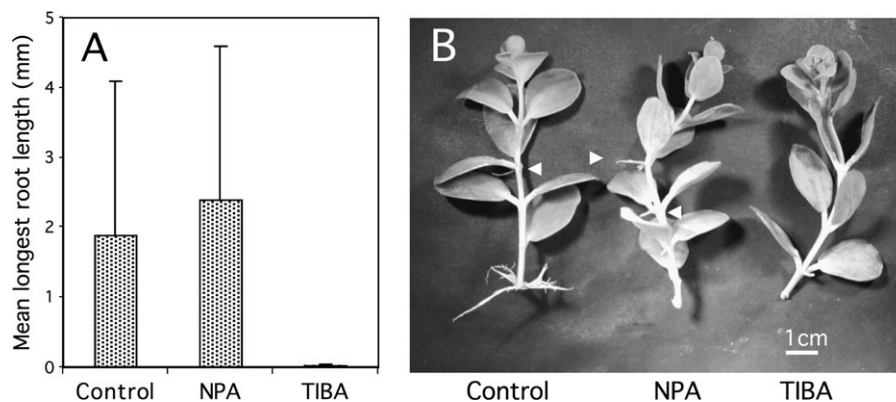
Fig. 4. Effect of NPA on growth of plantlets or cuttings. (A) Lack of effect of NPA on root development of plantlets growing on explants. Explants were incubated with no additions (control), 5 μM TIBA, or 25 μM NPA (*n*=16). (B) Inhibition of adventitious root growth of cuttings by NPA. Cuttings were incubated with their basal ends immersed in 1 mM phosphate buffer pH 6.5 with no additions (control), 10 μM TIBA, or 10 μM NPA. Arrows show non-basal adventitious roots.

NAA were also exposed to ethylene, short roots were formed by many of the plantlets (Fig. 6A, B). These results are consistent with the hypothesis that there is a threshold concentration of auxin for root induction and that ethylene lowers this threshold. As in the absence of ethylene (Fig. 3) NAA is not sufficient to compensate fully for the inhibition of endogenous auxin transport by TIBA, as only short roots are formed when very low concentrations of NAA and ethylene are both present (Fig. 6B, C).

Effect of inhibitors of ethylene action and synthesis

Although the above results indicate that ethylene does not act downstream of auxin, the simultaneous action of both could be necessary for root induction. $Ag_2S_2O_3$ is a known blocker of ethylene action (Taiz and Zeiger, 2006). If ethylene is a mandatory signal for the development of plantlet roots, Ag^+ should prevent root formation. Figure 7A shows that although Ag^+ inhibits the precocious root development induced by ethylene, it does not abolish root growth in either the presence or absence of ethylene. In the presence of ethylene, Ag^+ shifts root emergence from an earlier time when the leaf pair span is about half that of the control to a later time when leaf pair span is the same as that of the control (not shown). This is the result expected if Ag^+ blocked ethylene action. Additional evidence that Ag^+, at the concentration used, was effective is that it prevented the elongation of petioles caused by ethylene (not shown). The slight inhibition of root growth by Ag^+ alone may be due to the partial inhibition of shoot growth (not shown). The initiation of roots under these conditions occurs at approximately the same leaf pair span as in the control.

CoCl$_2$ (50 μM), an inhibitor of ethylene synthesis, did not prevent root outgrowth, although it delayed it slightly (Fig. 7B). This concentration slightly inhibited plantlet shoot growth (not shown). Other inhibitors of ethylene synthesis, AOA and AVG, were toxic to explants. As root growth was not prevented by either Ag^+ or Co^{2+}, an obligatory role for ethylene in plantlet root development is unlikely, but is not unequivocally excluded.

Discussion

The strong and specific inhibition of root development by the polar auxin transport inhibitor TIBA suggests that auxin is involved in root induction in *B. marnierianum* plantlets. As roots are formed by plantlet shoots on small leaf explants, as well as on whole leaves, it is likely that the origin of the auxin is in the plantlet shoot. The finding that plantlet apical bud removal delays the onset of root outgrowth is also consistent with the plantlet origin of the auxin (Fig. 2C). The hypothesis that auxin induces plantlet roots is further supported by the rescue of root development of decapitated plantlets by application of IAA or NAA (Fig. 2C). Reversal by NAA of TIBA inhibition of root outgrowth in explants (Fig. 3) is also in line with this view. As rescue of TIBA inhibition by NAA produces stubby roots rather than normal long roots, it is possible that NAA can rescue root initiation but cannot support root elongation under these conditions. This may be due to the fact that TIBA inhibits both auxin influx and efflux carriers (Kleine-Vehn *et al.*, 2006) but NAA can only bypass the influx carrier (Marchant *et al.*, 1999). Another possibility is that the entry of lipophilic NAA into cells of the root elongation zone inhibits their growth (Taiz and Zeiger, 2006). As IAA causes the growth of normal elongated roots on decapitated plantlets, it seems that intact polar auxin transport is required for root elongation.

Polar auxin transport is involved in many aspects of plant growth and development including embryogenesis, phototropism, gravitropism, and lateral root development (Taiz and Zeiger, 2006). In *Arabidopsis*, a number of auxin carrier molecules have been implicated in these
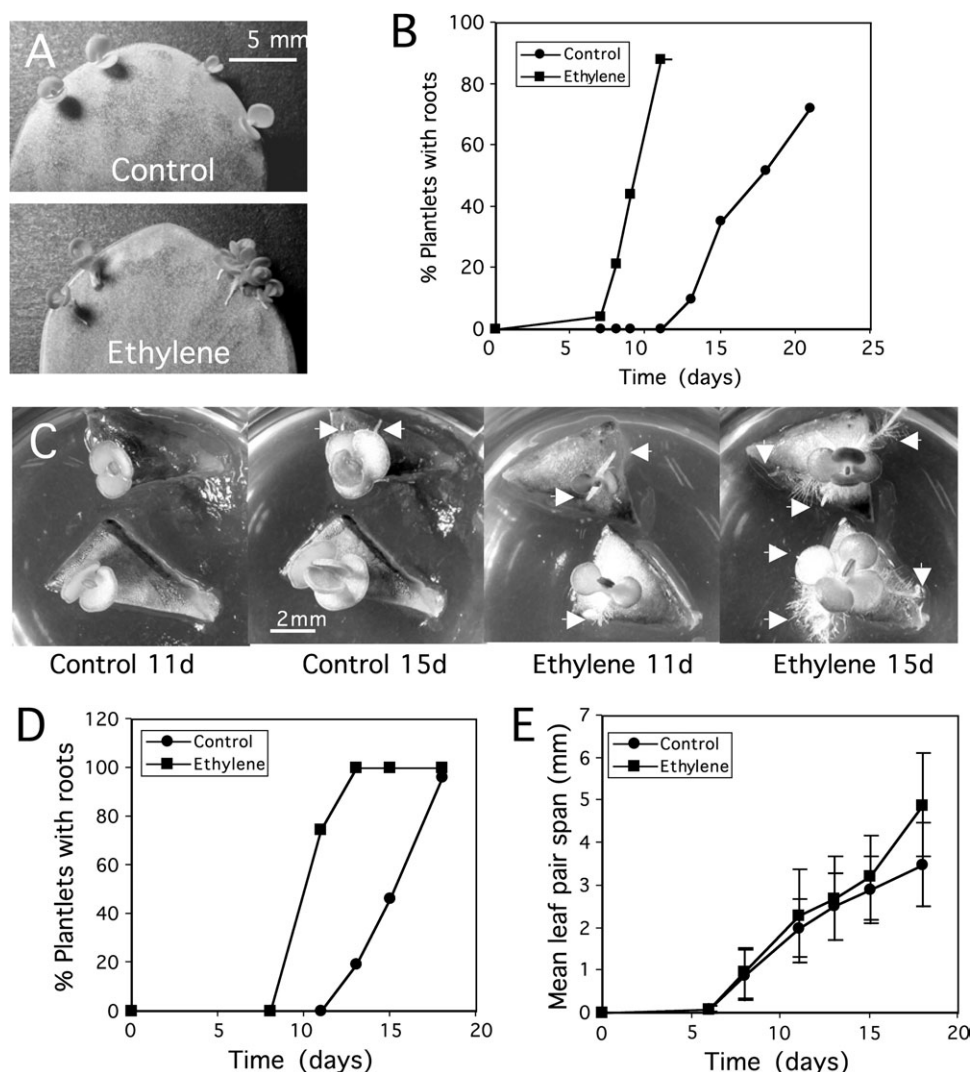
Fig. 5. Ethylene induces precocious root development in plantlets on leaves and explants. (A) Comparison of plantlet root development 8 d after leaf excision in the absence and presence of ethylene. Ethylene was generated from 25 ml of 10 μM ethephon in 5 mM phosphate buffer pH 6.5 in an 8.0 l desiccator. (B) Percentage of plantlets with roots on leaves without (filled circles) (n=60) or with ethylene (filled squares) (n=40) generated from 25 ml of 20 μM ethephon in 5 mM phosphate buffer pH 6.5 in an 8.0 l desiccator. (C) Comparison of plantlets developing on explants in the presence and absence of ethylene for 11 d or 15 d. Photographs at 10 d and 15 d are of the same plantlets. Arrows mark roots. (D) Percentage of plantlets with roots in the presence (filled squares) or absence (filled circles) of ethylene. (E) Mean maximum leaf pair span of plantlets in (D) developing in the presence (filled squares) or in the absence (filled circles) of ethylene; n=13.

functions including the auxin influx carrier AUX1, five species of polar auxin efflux facilitator (PINs), and members of the phosphoglycoprotein (PGP/MDR) family (reviewed by H Tanaka *et al.*, 2006). Different subsets of the PIN efflux facilitator proteins are implicated in the various auxin functions (H Tanaka *et al.*, 2006). PGPs may also play a role in auxin action, but their mode of function is still unclear (H Tanaka *et al.*, 2006). There is also evidence that different auxin carrier molecules co-operate with distinct subcellular trafficking systems (Jaillais *et al.*, 2006; Kleine-Vehn *et al.*, 2006) Thus, each function of auxins may involve a characteristic and different subset of auxin carrier and trafficking molecules.

Auxin is known to induce lateral root development in *Arabidopsis* (reviewed by Casimiro *et al.*, 2003; Fukaki *et al.*, 2007). It therefore seemed possible that the root development of *B. marnierianum* plantlets occurs by a similar mechanism. However, plantlet root induction is strongly inhibited by TIBA, but is insensitive to NPA (Fig. 4A), whereas lateral root growth of *Arabidopsis* is sensitive to NPA (Casimiro *et al.*, 2001). In contrast, adventitious root growth on cuttings of *B. marnierianum*, unlike plantlet root growth, is sensitive to both TIBA and NPA (Fig. 4B) and, in this respect, resembles lateral root development. The results suggest that plantlet root development and adventitious root development in cuttings involve different subsets of auxin transport molecules.
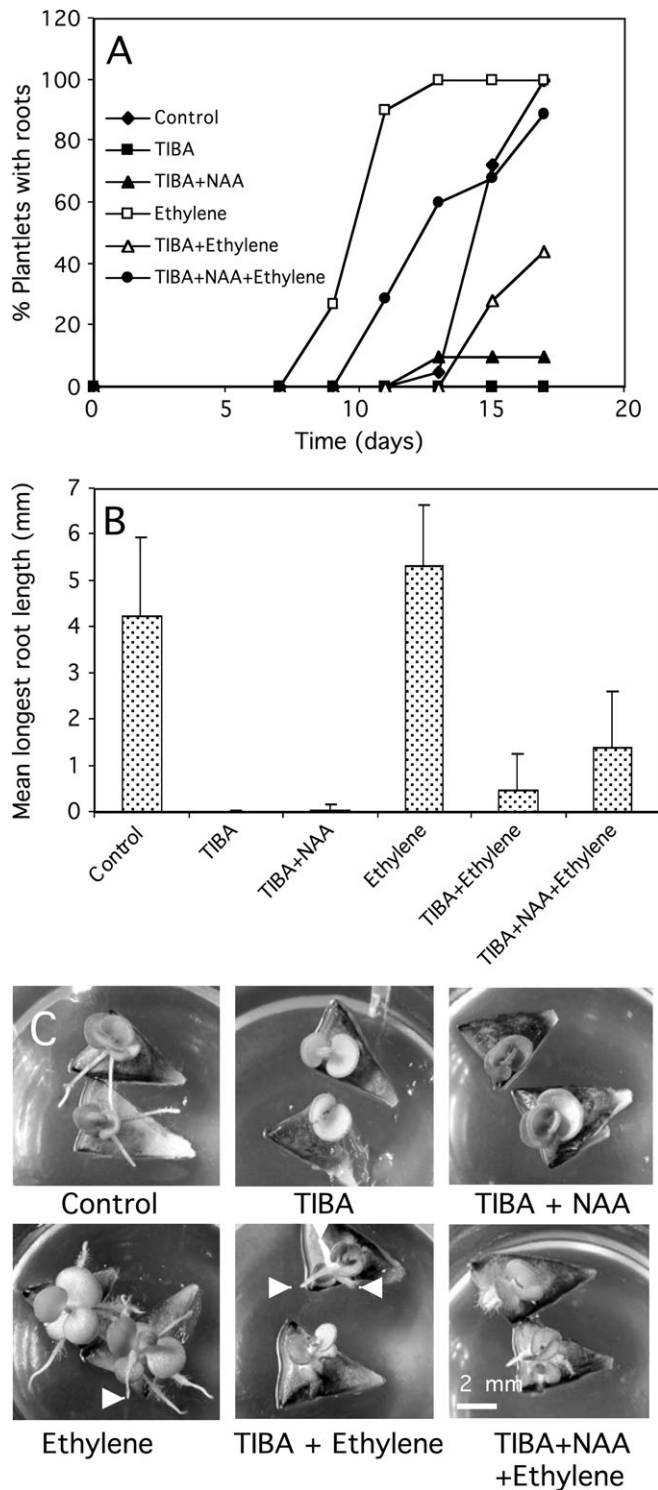
Fig. 6. Reversal of TIBA inhibition of plantlet root development on explants by ethylene plus low NAA. (A) Percentage of plantlets with roots: control (filled diamonds); 2 μM TIBA (filled squares); 2 μM TIBA plus 500 nM NAA (filled triangles); ethylene (open squares); 2 μM TIBA plus ethylene (open triangles); 2 μM TIBA plus 500 nM NAA plus ethylene (filled circles). Ethylene was generated from 25 ml of 20 μM ethephon in 5 mM phosphate buffer pH 6.5 in an 8.0 l dessicator. (B) Mean longest root length after 17 days; n=16. (C) Photographs 17 d after the start of culture. Arrows mark ectopic roots.
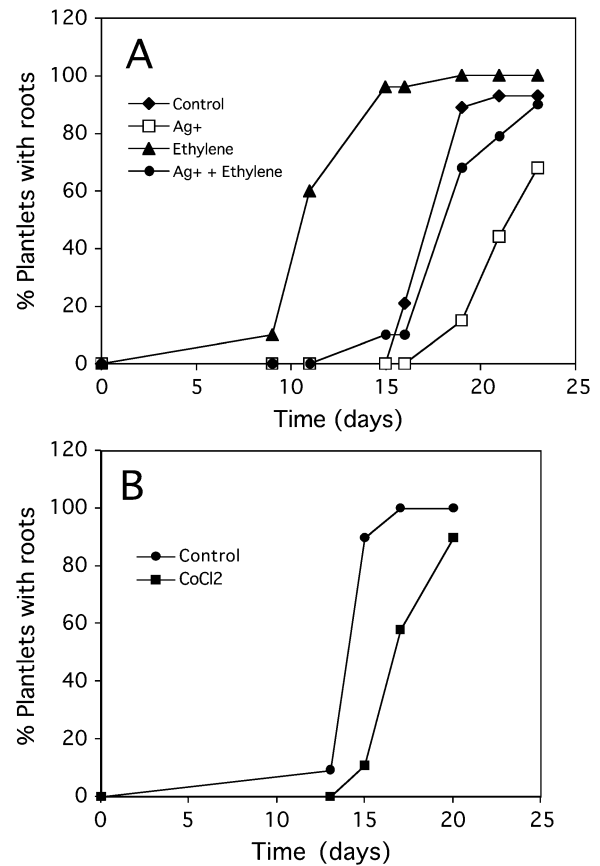
Fig. 7. Effect of $Ag_2S_2O_3$ or $CoCl_2$ on plantlet root development on explants. (A) Effect of $Ag_2S_2O_3$. Percentage of plantlets with roots: control (filled diamonds); 5 μM $Ag_2S_2O_3$ (open squares); ethylene (filled triangles); ethylene plus 5 μM $Ag_2S_2O_3$ (filled circles). (B) Effect of $CoCl_2$: control (filled circles); 50 μM $CoCl_2$ (filled squares).

Auxin import and export carriers of *Arabidopsis* are inhibited by both TIBA and NPA (Kleine-Vehn *et al.*, 2006). However, some auxin-regulated processes respond differently to TIBA and NPA. These include cell elongation, actin polymerization (Rahman *et al.*, 2007), gravitropic response, and root elongation (Fujita and Syono, 1996; Lu and Fedoroff, 2000). TIBA and NPA have previously been suggested to interact with different target proteins (Michalke *et al.*, 1992; Fujita and Syono, 1996; Lu and Fedoroff, 2000). One may speculate that components of different subsets of the auxin carrier molecules mentioned above may have different sensitivities to individual auxin transport inhibitors. However, as the present work is purely physiological, it can shed no light on the identity of the molecules which cause different responses to the inhibitors.

The question arises how root outgrowth is initiated only when the plantlet shoots reach a certain stage of development. Plantlet roots first appear when the mean shoot leaf pair span is 4–5 mm on leaves and 2.5–3 mm on explants. It seems, therefore, that auxin is a reporter of plantlet developmental stage, and when a certain auxin

threshold is reached root outgrowth starts. The observations do not fit the hypothesis that auxin flux is an indicator of plantlet size. Smaller plantlets frequently form roots before larger ones, and plantlets of similar size form roots at different times (Fig. 1A). How auxin indicates developmental progression thus remains unclear.

The dramatic premature root outgrowth caused by ethylene (Fig. 5) needs to be explained. In many plant developmental systems, auxin and ethylene act co-ordinately. The co-operation may be at a number of levels. In some systems, auxin acts by activating the synthesis of ethylene (Hansen and Grossman, 2000; Swarup *et al.*, 2002; Y Tanaka *et al.*, 2006). In other cases, the effect of ethylene requires the presence of auxin (Liu and Reid, 1992: Visser *et al.*, 1996). Ethylene has been found to stimulate auxin biosynthesis (Stepanova *et al.*, 2005) and to enhance the auxin response (Li *et al.*, 2004). In the present case it seems unlikely that ethylene acts downstream of auxin. If it did, it would be expected to bypass the inhibition of root development by TIBA, but it does not do so (Fig. 6). As ethylene greatly enhances the effect of a low concentration of NAA, which is inactive in its absence (Fig. 6), it is likely that it has an amplifying effect on auxin action. A similar mechanism of ethylene action has been proposed for adventitious root formation in other plant systems (Liu and Reed, 1992; Visser *et al.*, 1996). This model is consistent with the finding that ethylene shifts the point of root emergence to a smaller plantlet size than that of the controls (Fig. 5). As smaller plantlets would be expected to produce less auxin, enhancement of the effect of auxin by ethylene could explain this finding.

The experiments summarized in Fig. 6 do not eliminate the possibility that ethylene, or its downstream effectors, is essential for plantlet root development. As neither $Ag_2S_2O_3$, an ethylene blocker, nor $CoCl_2$, an ethylene synthesis inhibitor, prevent plantlet root outgrowth (Fig. 7), it is unlikely that ethylene is essential for the process. However, in the absence of ethylene signalling mutants of this plant, it is difficult to decide unequivocally if this is the case. It is likely that under particular circumstances, such as leaf senescence, ethylene acts as an enhancer of plantlet root induction.

The following model could explain most of the above results. Auxin transported from the plantlet shoot induces root formation at the base of its stem. Ethylene enhances the effect of auxin and lowers its threshold for root induction. This mechanism would make biological sense. Auxin flux from the shoot could be an indicator of plantlet stage of development. When auxin flux is above a certain threshold, root development is initiated. When the isolated leaf starts to senesce, ethylene may be released (Lim *et al.*, 2007), signalling the end of nutrient supply from the leaf to the plantlet and accelerating the growth of roots which under these circumstances become essential for plantlet survival.

References

Casimiro I, Beekman T, Graham N, Bhalerao R, Zhang H, Casero P, Sandberg G, Bennett MJ. 2003. Dissecting *Arabidopsis* lateral root development. *Trends in Plant Science* **8,** 165–171.

Casimiro I, Marchant A, Bhalerao RP, et al. 2001. Auxin transport promotes *Arabidopsis* lateral root initiation. *The Plant Cell* **13,** 843–852.

Freeland RO. 1933. Some morphological and physico-chemical changes accompanying proliferation of *Bryophyllum* leaves. *American Journal of Botany* **20,** 467–480.

Fujita H, Syono K. 1996. Genetic analysis of the effects of polar auxin transport inhibitors on root growth in *Arabidopsis thaliana*. *Plant and Cell Physiology* **37,** 1094–1101.

Fukaki H, Okushima Y, Masaka M. 2007. Auxin-mediated lateral root formation in higher plants. *International Review of Cytology* **256,** 111–137.

Goebel K. 1902. Ueber Regeneration im Pflanzenreich. *Biologisches Zentralblatt* **22,** 385–397.

Goebel K. 1916. Zur Jaques Loeb's Untersuchungen uber Regeneration bei *Bryophyllum*. *Biologisches Zentralblatt* **36,** 193–206.

Goethe JW. 1820. Goethe and Schiller Archive, Weimar, documents: Signatur GSA 25/W 16,7; Signatur GSA 26/ LIV 5,31.

Hansen H, Grossmann K. 2000. Auxin-induced ethylene triggers abscisic acid biosynthesis and growth inhibition. *Plant Physiology* **124,** 1437–1448.

Heide OM. 1965. Effects of 6-benzylaminopurine and 1-naphthaleneacetic acid on the epiphyllous bud formation in *Bryophyllum*. *Planta* **67,** 281–296.

Henson IE, Wareing PF. 1977. Changes in the levels of endogenous cytokinins and indole-3-acetic acid during epiphyllous bud formation in *Bryophyllum daigremontianum*. *New Phytologist* **79,** 225–232.

Houck DF, Riesberg LH. 1983. Hormonal regulation of epiphyllous bud release and development in *Bryophyllum calycinum*. *American Journal of Botany* **70,** 912–915.

Howe DM. 1931. A morphological study of the leaf notches of *Bryophyllum calycinum*. *American Journal of Botany* **18,** 387–398.

Jaillais Y, Fobis-Loisy I, Miege C, Rollin C, Gaude T. 2006. *AtSNX1* defines an endosome for auxin-carrier trafficking in *Arabidopsis*. *Nature* **443,** 106–109.

Karpoff AJ. 1982. Hormones and early *in vitro* development of epiphyllous propagules on *Bryophyllum calycinum*. *American Journal of Botany* **69,** 348–355.

Kleine-Vehn J, Dhonukshe P, Swarup R, Bennett M, Friml J. 2006. Subcellular trafficking of the *Arabidopsis* auxin influx carrier *AUX1* uses a novel pathway distinct from *PIN1*. *The Plant Cell* **18,** 3171–3181.

Kulka RG. 2006. Cytokinins inhibit epiphyllous plantlet development on leaves of *Bryophyllum (Kalanchoë) marnierianum*. *Journal of Experimental Botany* **57,** 4089–4098.

Li H, Johnson P, Stepanova A, Alonso JM, Ecker JR. 2004. Convergence of signaling pathways in the control of differential cell growth in *Arabidopsis*. *Developmental Cell* **7,** 193–204.

Lim PO, Kim HJ, Nam HG. 2007. Leaf senescence. *Annual Review of Plant Biology* **58,** 115–136.

Liu JH, Reid DM. 1992. Auxin and ethylene-stimulated adventitious rooting in relation to tissue sensitivity to auxin and ethylene production in sunflower hypocotyls. *Journal of Experimental Botany* **43,** 1191–1198.

Loeb J. 1915. Rules and mechanisms of inhibition and correlation in the regeneration of *Bryophyllum calycinum*. *Botanical Gazette* **60,** 249–276.

Lu C, Fedoroff N. 2000. A mutation in the Arabidopsis HYL1 gene encoding a dsRNA binding protein affects responses to abscisic acid, auxin and cytokinin. *The Plant Cell* **12,** 2351–2365.

Marchant A, Bhalerao R, Casimiro I, Eklof J, Casero P, Bennett MJ, Sandberg G. 2002. *AUX1* promotes lateral root formation by facilitating indole-3-acetic acid distribution between sink and source tissues in the *Arabidopsis* seedling. *The Plant Cell* **14,** 589–597.

Marchant A, Kargul J, May ST, Muller P, Delbarre A, Perrot-Rechenmann C, Bennett MJ. 1999. *AUX1* regulates root gravitropism in *Arabidopsis* by facilitating auxin uptake within root apical tissues. *EMBO Journal* **18,** 2066–2073.

Mehrlich FP. 1931. Factors affecting growth from the foliar meristems of *Bryophyllum calycinum*. *Botanical Gazette* **92,** 113–140.

Michalke W, Katekar GF, Geissler AE. 1992. Phytotropin-binding sites and auxin transport in *Cucurbita pepo*: evidence for two recognition sites. *Planta* **187,** 254–260.

Millenaar FF, Cox MC, van Berkel YE, Welschen RA, Pierik R, Voesenek LA, Peeters AJ. 2005. Ethylene-induced differential growth of petioles in *Arabidopsis*. Analyzing natural variation, response kinetics and variation. *Plant Physiology* **137,** 998–1008.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15,** 473–497.

Naylor E. 1932. The morphology of regeneration in *Bryophyllum calycinum*. *American Journal of Botany* **19,** 32–40.

Rahman A, Bannigan A, Sulaman W, Pechter P, Blancaflor EB, Baskin TI. 2007. Auxin, actin and growth of the *Arabidopsis thaliana* primary root. *The Plant Journal* **50,** 514–528.

Reed E. 1923. Hypothesis of formative stuffs as applied to *Bryophyllum calycinum*. *Botanical Gazette* **75,** 113–142.

Sachs T. 1991. *Pattern formation in plant tissues*. Cambridge, UK: Cambridge University Press.

Stepanova AN, Hoyt JM, Hamilton AA, Alonso JM. 2005. A link between ethylene and auxin uncovered by two root-specific ethylene-insensitive mutants in *Arabidopsis*. *The Plant Cell* **17,** 2230–2242.

Swarup R, Parry G, Graham N, Allen T, Bennett M. 2002. Auxin cross talk: integration of signaling pathways to control plant development. *Plant Molecular Biology* **49,** 411–426.

Taiz L, Zeiger E. 2006. *Plant physiology*, 4th edn. Sunderland, MA: Sinauer.

Tanaka H, Dhonukshe P, Brewer PB, Friml J. 2006. Spatiotemporal asymmetric auxin distribution: a means to coordinate plant development. *Cellular and Molecular Life Sciences* **63,** 2738–2754.

Tanaka Y, Sano T, Tamaoki M, Nakajima N, Kondo N, Hasezawa S. 2006. Cytokinin and auxin inhibit abscisic acid-induced stomatal closure by enhancing ethylene production in Arabidopsis. *Journal of Experimental Botany* **57,** 2259–2266.

Visser EJW, Cohen JD, Barendse GWM, Blom CWPM, Voesenek LACJ. 1996. An ethylene-mediated increase in sensitivity to auxin induces adventitious root formation in flooded *Rumex palustris* Sm. *Plant Physiology* **112,** 1687–1692.

Yarbrough JA. 1932. Anatomical and developmental studies of the foliar embryos of *Bryophyllum calicinum*. *American Journal of Botany* **19,** 443–453.

Yarbrough JA. 1934. History of leaf development in *Bryophyllum calycinum*. *American Journal of Botany* **21,** 467–484.

Yazgan M, Vardar Y. 1977. Studies on the effect of auxin–kinetin applications on epiphyllous budding of *Bryophyllum daigremontianum* Berg. *Zeitschrift für Pflanzenphysiologie* **84,** 203–211.