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Hormone Levels and Apical Dominance in the Aquatic Fern *Marsilea drummondii* A. Br.

Gilles Pilate, Lucienne Sossountzov*, and Emile Miginiac

Laboratoire de Physiologie végétale, CNRS (UA 1180), T 53, 5ème étage, Université P. et M. Curie, 4 Place Jussieu, 75252 Paris Cedex 05, France

ABSTRACT

Terminal buds and successively subjacent lateral buds of the water fern, *Marsilea drummondii*, were examined to determine the pattern of hormone distribution in relation to apical dominance. Quantitative levels of indole-3-acetic acid (IAA), abscisic acid (ABA), zeatin and zeatin riboside (Z and ZR), and isopentenyladenosine (iPA) were determined by a solid-phase immunoassay using polyclonal antihormone antibodies. Enzyme-linked immunosorbent assay was used following a one-step HPLC purification procedure to obtain the free hormones. Active shoot apices contained the most IAA and Z-type cytokinins and inhibited buds the least. No significant differences in ABA levels were found leading to the conclusion that ABA did not play any role in apical dominance. The normal precedence of the most rapid outgrowth of the youngest inhibited bud as observed previously in decapitated plants was well correlated with its very high level of iPA observed in this study. The same phenomenon was observed in the median buds but with a weaker amplitude. The presence of this storage form could indicate that a bud at its entry into quiescence eventually looses the ability to hydroxylate iPA to Z-type cytokinins when it is fully inhibited. IAA and Z + ZR are concluded to be essential for lateral bud growth.

The lateral buds of the water fern, *Marsilea drummondii*, are quiescent due to the inhibition produced by the main shoot tip and its young leaf primordia (1). Over the last several years, we have characterized all these buds in intact and decapitated plants in an attempt to elucidate the reasons for the inhibitory role of the terminal bud (2, 3, 11–16). We have shown that release of lateral buds from apical dominance can be achieved by various means: surgical removal of the shoot tip, or, in the presence of the terminal bud, application of synthetic (kinetin) or naturally occurring (auxin) hormones, little prickings at the bud base (2). But the removal or pricking type experiments were difficult to interpret due to the activation effects of wounding. Studies with exogenous hormones, although widely used, have not furthered our understanding of their involvement in these regulatory processes. Auxin to replace totally or partially the main shoot tip or cytokinins to release the axillary buds from the apical inhibition, must be applied directly at very high levels. All these experiments led to the hypothesis that inhibited buds under-produced IAA and/or CKs.[1] We then employed several other approaches including: movements into the buds of nutriments, ions (16), hormones (3, 13), cytochemical characterization (ATPases [15], DNA and RNA synthesis [12]). They have demonstrated the sink role of the terminal bud and the privileged status of the youngest subapical bud: the last bud to enter in quiescence is the first to resume growth after decapitation, although all the older buds are also, but for a short while, able to begin growing, according to an age gradient. These events may be related to mobile hormones which are presumably synthetized in the shoot tip and its young leaves or in the roots and transported downward (auxin) or upward (CKs) in the stem.

Therefore, the possibility that endogenous hormone levels were involved in the progressive inhibition of lateral buds was studied by comparing the IAA, CKs, and ABA contents of the active terminal bud and the inhibited buds inserted along the rhizome. The need for a simultaneous determination of IAA, CKs, and ABA in the same material led us to use immunoassays on HPLC- purified extracts from four types of buds from intact sporophytes differing by their age and activity.

MATERIALS AND METHODS

Plant Material: Growth and Sampling Conditions

Marsilea drummondii sporophytes were axenically grown from cuttings at 23 ± 2°C in a glasshouse cabinet as previously described (12). On the 45th d of growth, terminal buds and lateral buds of successively increasing ages were excised under magnifying glasses from 240 plants blotted with filter paper to remove the attached liquid medium. The buds, all removed between 9 and 10 AM, were immediately dropped in liquid nitrogen. After freezing, the plant material was quickly weighted and freeze-dried for 48 h, and then accurately weighted again to obtain the DW. They were then stored at −40°C as a homogeneous powder until extracted for hormone analysis. The knowledge of the buds given by our previous histological studies (1, 12) led us to take off equivalent tissues: we discarded the nodes for lateral buds and the long internode below the shoot tips.

Quantitative Determinations of Endogenous Hormones

The simultaneous extraction and purification of IAA, ABA, ZR, and its corresponding base Z as well as iPA were performed according to the techniques described in detail earlier (8, 9, 20). Their levels were quantified using ELISA with antihormone antibodies. HPLC fractionation prior to ELISA

[1] Abbreviations: CKs, cytokinins; DW, dry weight; FW, fresh weight; iP, isopentenyladenine; iPA, isopentenyladenosine; Z, zeatin; ZR, zeatin riboside.

test was used to remove interfering materials eventually present in the extracts and the compounds cross-reacting with the polyclonal antibodies used. In short, freeze-dried powdered tissues were extracted with cold 80% methanol containing an antioxidant for 60 h at 4°C in darkness. Extracts, with the added tritiated standards (IAA and ABA from Amersham, UK, and iPA or ZR, gifts from Dr. Laloue, Gif-sur-Yvette, France) were filtered and then passed through a Sep Pak C18 cartridge (Waters Associates, MA). The resulting eluates were reduced to water *in vacuo* with a rotary evaporator. After acidification at pH 3, they were then submitted to a HPLC fractionation through a Licrospher column (Merck, FRG) fitted on a Beckman apparatus and eluted with a 3.3% acetic acid/methanol gradient. The fractions corresponding to the standards were collected and reduced to dryness. The CK fractions were taken up with PBS without post-treatment and submitted to ELISA test with anti-ZR and with anti-iPA antibodies. The fractions containing IAA and ABA were methylated with etheral diazomethane prior to ELISA quantitation with anti-IAA and anti-*cis*-(+) ABA antibodies. The level of each hormone in each sample was measured five times, the values being corrected for recovery and diminished of the corresponding amount of the recovered standards. The results were analyzed with calibration curves corresponding to the different fractions with an Apple MacIntosh computer system. The results are expressed as the means ± SE on the DW and per organ basis. Because the anti-iPA antibodies cross-react with iP, the iPA contents correspond to iP + iPA not separated during the HPLC purification.

RESULTS

As shown in Table I, the main shoot apex weighed about five times more than the lateral buds on a FW basis and four times on the DW basis. The arrest of growth and age of the lateral buds caused a significant loss of water content and a rise in dry weight.

Comparative Hormone Levels in Active and Inhibited Buds

The most striking result was the finding that IAA, ABA, and CKs occurred in all the buds, in the active or the resting state, with the exception of basal buds in which ZR was not detected (Figs. 1–4).

Table I. *Some Characteristics of the Buds of Marsilea drummondii Submitted to the Quantitation of Hormones*

Nature of the bud	FW	DW	Water Content
	μg/bud		%
TB[a]	2050	177	91.4
SAB	336	31.3	90.7
MB	392	41.7	89.4
BB	394	43.8	88.9

[a] The values were obtained from 240 terminal buds (TB) or subapical (SAB), median (MB), and basal (BB) lateral buds.

IAA Contents

The highest concentration was found, as expected, in the terminal bud (Fig. 1), whatever the criteria used for calculations. There was along the main axis an apico-basal gradient of the IAA contents corresponding to that of the age of the buds and was considered the DW basis. But it is noteworthy that the youngest subapical bud which started to enter in a resting state contained about half of the IAA in the terminal bud. On per organ basis, lateral buds contained only 10% of the IAA present in the leading shoot apex. It is noticeable that the IAA levels dropped abruptly in the lateral buds; however, no distinct effect was found in relation with their relative activity nor their position on the main axis, *i.e.* their age.

ABA Contents

The distribution of ABA (Fig. 2) in the buds was rather homogeneous whatever the criteria used for calculations: the corresponding differences were small when differences in activity were high. The terminal bud grew well, despite an ABA content equal to that found in the older buds. The fact that along the rhizome the maturation of the bud corresponding to a slight decrease in water content was not accompanied by a rise in ABA is possibly to be connected with the aquatic habit of *M. drummondii*, which can also explain its relatively low concentrations in all buds. No concentration gradient

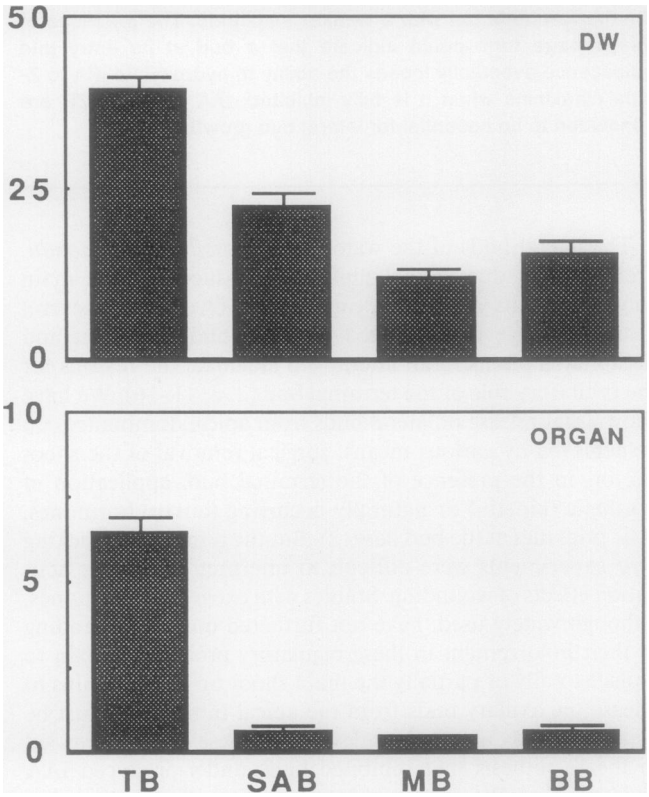

Figure 1. Comparative endogenous IAA levels in the active terminal bud (TB) and in the inhibited subapical, median, and basal lateral buds (SAB, MB, and BB). Results are expressed as nmol/g DW and as pmol/organ, as mean of 5 replicates ±SE.

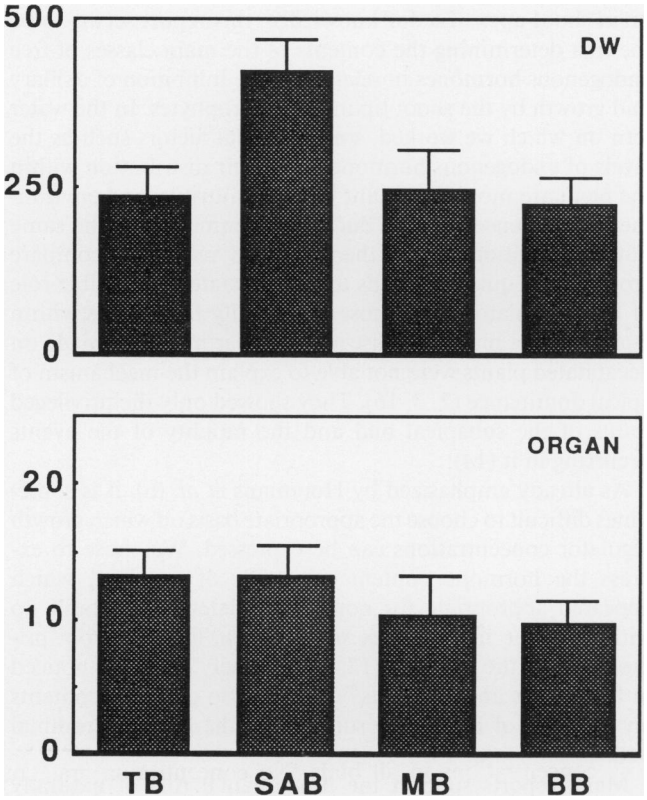

Figure 2. Comparative endogenous ABA levels in the active terminal bud (TB) and in the lateral inhibited buds (SAB, MB, and BB). Results are expressed as pmol/g DW and as fmol/organ, as mean of 5 replicates ±SE.

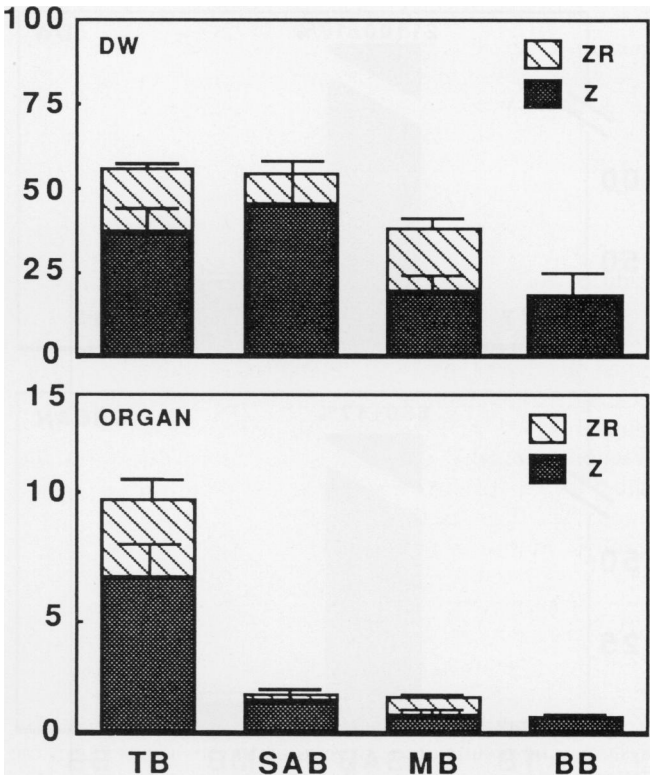

Figure 3. Comparative Z and ZR levels in the active terminal buds (TB) and in the lateral inhibited buds (SAB, MB, and BB). Levels are expressed as pmol/g DW and as fmol/organ. The bars represent the superposed values of Z and ZR, as mean of five replicates +SE.

was observed. The only noticeable result was the higher concentration of ABA, on the basis of DW, in the subapical bud, *i.e.* during the course of the process of its inhibition, while ABA level was equal to that measured in the terminal bud if expressed in terms of per bud.

CK Contents

Z contents, when expressed on a DW basis, decreased with bud age but peaked in the terminal and subapical buds (Fig. 3). But, if the organ as a whole was taken as criterion for calculations, there was a drastic drop of Z content in the not fully quiescent subapical bud when compared to the active shoot tip, and an about 10-fold decrease in the older buds. The concentrations of ZR in the different buds were not relevant to their relative activity. The contents were equivalent in the main shoot apex and in the quiescent median bud, but were depressed in the subapical bud and were undetectable in the basal buds. Here again, an analysis of the results on a per organ basis was more correlated with the known state of the buds: higher ZR content in the growing tips than in the inhibited buds.

CK concentrations, expressed as the sum of Z + ZR, were higher in the terminal bud, and a clear decrease with increasing bud age was observed. On the per organ basis, the two CKs were also distributed as a function of bud age and clearly according to a steep apico-basal gradient.

iPA Contents

The iPA contents, corresponding to iP + iPA as noted in "Materials and Methods," showed clearly, according to the two criteria used, the privileged nature of the subapical bud and, to a lesser extent, of the median bud (Fig. 4): the former was by far richer in iPA content than the main shoot apex and than the older sibling buds. It is noticeable that this bud, the first to resume growth in decapitated plants, contained a very high level of iPA whereas the amount in the terminal buds was low.

Hormonal Balance

It is often assumed that hormonal balance, which supposes that regulators are present simultaneously in the same organ with agonistic or antagonistic effects, is more important than the level of a single hormone to explain morphogenetical processes. This is the reason we have calculated the IAA to ABA and to Z + ZR ratios, eliminating the case of iPA with its very high value in the subapical bud. As shown in Table II, the most striking feature was the great difference between the growing main apex and the inhibited buds when considering the IAA/ABA ratio. All the buds contained about the same level of ABA but differed in their IAA contents. The active bud contained more IAA as compared to ABA. The ratio was then about nine-fold less in the inhibited buds. A comparatively low ratio was well correlated with the quies-

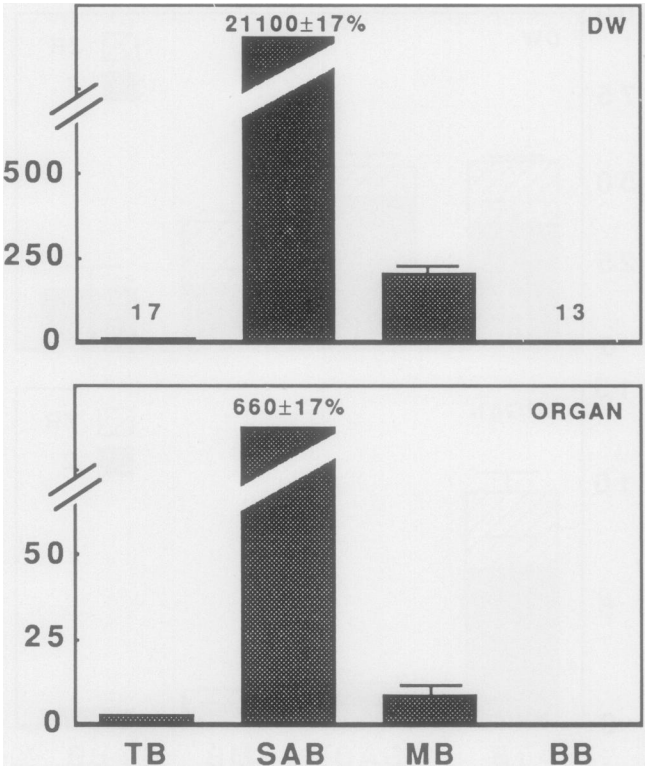

Figure 4. Comparative iPA levels in the active terminal buds (TB) and in the lateral inhibited buds (SAB, MB, and BB). Levels are expressed as pmol/g DW and as fmol/organ, as mean of 5 replicates ±SE.

Table II. *Ratios of IAA to ABA and Z + ZR*

Nature of the Bud	IAA/ABA[a]	IAA/Z + ZR
TB[b]	485	656
SAB	53	418
MB	50	324
BB	68	816

[a] The values used to calculate the ratios are given by Figures 1 to 3, expressed as femtomoles per organ. [b] The values were obtained from 240 terminal buds (TB) or subapical (SAB), median (MB), and basal (BB) lateral buds.

cence of the buds while the pattern of changes in ABA levels was not clear.

In the case of the relation between IAA and CKs of the Z-family, the calculated ratios were distributed according to an apico-basal gradient and appeared consistent with growing capabilities of the buds: there was a clear decrease with increasing age, except for the oldest basal buds. There was a discrepancy between bud activity and this ratio: the highest ratio corresponding to the lowest absolute values was found in the oldest fully inhibited buds.

DISCUSSION

The aim of this investigation was to delineate the role of plant hormones on the growth arrest of lateral buds by the main shoot apex. To our knowledge, this report seems to be the first determining the contents of the main classes of free endogenous hormones in relation to the inhibition of axillary bud growth by the shoot tip in fern sporophytes. In the water fern on which we worked, some internal factors such as the levels of endogenous hormones and their distribution within the plant are more important than the nutritive and environmental influences, all the buds being immersed in the same nutrient solution. Thus, there is good reason to compare growing and quiescent buds to demonstrate the possible role of these regulators. We chose to quantify their levels within buds of intact plants because all the experiments we made on decapitated plants were not able to explain the mechanism of apical dominance (2, 3, 16). They showed only the privileged status of the subapical bud and the rapidity of the events occurring in it (14).

As already emphasized by Horemans *et al.* (6), it is sometimes difficult to choose the appropriate basis on which growth regulator concentrations can be expressed. We chose to express the hormone contents in terms of per bud, which appeared appropriate for nongrowing lateral buds built up with the same tissues: apex *stricto sensu*, leaf, and root primordia, and the bud axis (12, 15) precisely cut off as noticed in "Materials and Methods." But we also gave the contents on the basis of DW more suitable for the growing terminal bud.

Many reports support the fundamental role of naturally occurring IAA on apical dominance: its synthesis and its polar transport are the clues about the mechanism of its control (4, 5, 21). Our data have clearly indicated that not only IAA, but also CKs, played a key role in the regulation of bud growth. Variations in their contents, with very rare exceptions, were well correlated to variations in growth inhibition.

The most striking feature was that the concentrations of free IAA in the actively growing shoot apex were higher than those in inhibited lateral buds where they decreased about 3-fold with age according to a very evident concentration gradient, and with a nearly 10-fold drop if expressed per bud. CKs of the Z-type traced a pattern almost identical to that of IAA, *i.e.* decreasing within the inhibited buds and peaking in the terminal bud when represented on the per organ basis: inhibited buds contained 6- to 10-fold less CKs of the Z type than the terminal bud. Here again levels appeared to be a function of bud age: the lowest concentrations were found in the median and basal buds. The relative distribution of Z + ZR per bud also showed a steep downward gradient as for IAA. We have recently shown (18) by immunocytochemical methods that tomato inhibited buds are well correlated with very low contents of endogenous CKs in their quiescent meristematic cells, and we concluded that CKs are necessary for axillary bud growth. Thus, as for IAA, the distribution of Z-type CKs within the buds fits well with growth activity: actively growing buds contained the highest levels of these hormones. Our results are also suggestive of a connection between these contents and the ability of buds for further growth upon decapitation. Furthermore, they can be considered as physiologically important because inhibited buds were able to respond to applications of synthetic CK-like kinetin (11) or naturally occurring IAA (1). Therefore, in intact plants

of *M. drummondii*, we can assume that the levels of IAA and Z-type CKs in the main shoot apex play an important role in the correlative inhibition between buds.

No such positional differences were observed with the iP-type CKs. It is of interest that the subapical bud was found to contain an enormous level of iPA and that the median bud contained 10 times more iPA than the terminal bud. But, there was a lack of positive correlation between iPA content and bud age: the terminal and the basal buds were very similar. These results could explain that these buds have different capabilities to grow upon decapitation. The high iPA content of the subapical bud possibly pointed out its ability for rapid and continuous growth, whereas the median bud starts growing briefly and then stops. The basal buds, with their very low content of iP-type CKs, are the first to be quiescent again. We do not know what happened to the high iPA content in the subapical bud when it became a median and then a basal bud. Possibly, it was transported to the terminal bud acting as a strong sink (3, 13) where it was rapidly converted to the more active Z-type CKs, since in this actively growing bud, the iP-type CK content was low. This result supports the hypothesis that the subapical and median buds, deficient in a specific enzymatic step, were unable to hydroxylate iP to Z, but could accumulate iPA. They had the capacity to store sufficient CK, as observed in some higher plants (23, 24), to assure their rapid outgrowth upon decapitation, the rapid growth arrest of the basal buds being due to the fact they have no iP-type CKs to convert to Z-type CKs. Interestingly, recent works on intact plants of *Pisum sativum* (7, 10) showed that zeatin, but not iPA, is very efficient to release axillary buds from the apical control.

On the contrary, we found no clear relationship between bud inhibition and ABA levels. The immunoassays revealed the presence of almost equal amounts of ABA within all but the subapical buds. It was difficult to be sure that this regulator present at higher concentration in the subapical bud played a key role in the control of its starting inhibition: on a per organ basis, its content was equal to that of the actively growing shoot apex. It is worthy to note that ABA, classified as an inhibitor counteracting the effects of growth promoters, was in higher proportion relative to IAA in nongrowing buds. Thus, the absolute values were less interesting with regard to the bud growth potentials than the relative contents. We are aware, of course, that the control of a growth process by a molecule is not only dependent on its concentration, but also on a change in sensitivity of the tissues to this molecule according to Trewavas (22). We know also that its compartmentation in the buds depends on their age and affects selected tissues of these structurally heterogeneous organs (17, 19). Therefore, it seems important to analyze the subcellular localization of the hormone. This important question can be answered by the use of immunocytochemical methods to localize plant hormones as initiated in our laboratory (17–19) to delineate the possible role of a regulator not based on its tissue or organ concentration.

Significant differences in the IAA and Z-type CK levels may explain the apical control of the main shoot apex on the lateral buds it has initiated. On the contrary, there is no correlation between ABA content and bud inhibition. Interestingly, the subapical bud precedence and, to a lesser extent, the following activation of the median bud in decapitated plants could be related to their high iPA content playing the role of a storage CK form.

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