Levels of Endogenous Indole-3-acetic Acid in Achenes of *Rosa rugosa* during Dormancy Release and Germination

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ABSTRACT

Indole-3-acetic acid (IAA) in highly purified extracts of rose achenes (*Rosa rugosa* var *rubra*) was quantified by means of ion-pair reverse-phase high performance liquid chromatography with spectrofluorimetric detection. Changes in IAA content were determined during a 14-week 4°C stratification, which leads to dormancy breakage, and during subsequent germination at 17°C. IAA was also determined in achenes stratified in parallel at 17°C, which does not induce release from dormancy. IAA decreased during the first 2 weeks of stratification both at 4°C and at 17°C. IAA remained low during the remaining 12 weeks of stratification at 4°C, whereas it continued to decrease in achenes kept at 17°C. An immediate increase in IAA during germination was followed by transients in the IAA level. The results suggest that IAA is without a regulating role in dormancy release although it seems to be involved in the germination process.

Hormonal regulation of dormancy breakage and germination are believed to be affected by an interplay between endogenous promotors and inhibitors. As these two processes commonly overlap each other in time, it may be difficult to judge if the observed hormonal changes are linked to one or the other process. Rose achenes, however, seem to be a suitable study material, since the period during which embryo dormancy is broken is well separated from the subsequent germination period (16), which is blocked by germination inhibitors (8). In two recent studies from our laboratory, changes in the levels of cytokinins and ABA in achenes of *Rosa rugosa* have been reported. A transient increase of cytokinin-like substances appeared early during cold stratification and another during germination suggesting that they are involved in both processes (9), whereas ABA was shown to play only a passive role, if any, in the regulation of dormancy release and germination (16). The present report describes changes in IAA levels in the same material and discusses its role in these processes.

There are reports suggesting that IAA is involved as an inhibitor in the regulation of dormancy whereas the effect of auxin on germination has long been in dispute. Decreases in the levels of auxins in apple seeds during a time course were strikingly similar to those of growth inhibitors during cold stratification (10). The endogenous IAA content in *Acer tartaricum* seeds was maintained at a high level throughout cold stratification, but decreased dramatically prior to visible germination (12). Those auxin determinations were made with bioassays and the growth regulating substances were not identified. We have, however, demonstrated by the indolo-α-pyrene method that the level of IAA diminishes both in cold and warm stratified embryos of *Acer platanoides* and have suggested that there is more direct relationship between IAA content and germination-growth than between IAA content and dormancy breakage (17). For these reasons, the possible role of IAA in these processes is controversial. The purpose of the present investigation was to determine the dynamics of IAA during cold stratification at 4°C and during germination induced by transfer to 17°C after a 14-week stratification period. Controls were conducted in parallel at 17°C, which does not break dormancy. As preliminary experiments with the indolo-α-pyrene method gave low yields of IAA, a more sensitive method, HPLC with fluorescence detector, was adopted for the analyses.

MATERIALS AND METHODS

Plant Material. Achenes of *Rosa rugosa* L. var *rubra* obtained from Søren Levinersen, Allerød, Denmark were from the same seed lot, that was used simultaneously for determination of germination ability, and content of ABA, cytokinin, and gibberellin (9,16). The achenes were surface-sterilized and put into sterile Petri dishes with water for stratification at 4°C or 17°C as a control, and for germination at 17°C following an inductive period of chilling for 14 weeks. The procedures were as described by Tillberg (16). At various intervals, triplicate samples were withdrawn for IAA determination and immediately homogenized in liquid N2 with an Ultra-Turrax homogenizer (Janke and Kunkel, Ika-Werk).

Extraction and Prepurification. Weighed amounts (2.0 g) of homogenates were extracted in 50 ml of 80% (v/v) redistilled methanol containing 10 mg -1 of the antioxidant BHT. An internal standard of 0.5 ml [1-14C] IAA containing 15.7 ng was added. The radioactive IAA had a specific activity of 57.6 mCi mmol-1 (2.16 GBq mmol-1) and a purity of 99% on TLC and was supplied in toluene from the Radiochemical Centre, Amersham, UK. The homogenates were stirred for 1 h at 4°C in darkness, and filtered through a Whatman GF/F glass fiber disc. After washing with 25 ml of 80% methanol, samples were evaporated down to the water phase, diluted to a volume of 20 ml with 5 mM phosphate buffer (pH 7) and passed through a PVP column (25 × 13 mm i.d.).

The column was washed with 5 ml of the phosphate buffer mentioned above. The eluate was then applied to a 0.5 g *C*18 column (Bond Elut, Analytical International, Inc.) which had been equilibrated by application of 2 ml 80% methanol, followed by 2 ml distilled H2O and finally by 2 ml 5 mM phosphate buffer (pH 7). When the extract had passed through the column, the column was washed with 2 ml distilled H2O. The eluate was acidified to pH 3.0 with 2.5 M H3PO4 and drawn through a 1.0

1 Supported by the Swedish Natural Science Research Council.

2 Abbreviations: BHT, 2,6-di-tert-butyl-p-cresol; tR, retention time; TBA, tetrabutylammonium hydrogen sulfate; 4Cl-IAA, 4-chloroindole-3-acetic acid.
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g C18 column, which had been equilibrated as above, except that the phosphate solution had a pH of 3 instead of 7. After washing with 2 ml distilled H2O, the column was eluted with a solvent of ethanol:acetic acid:distilled H2O (35:1:64) (v/v/v). The 2.0 to 5.5 ml fraction was collected and, after addition of 1 ml ethanol (spectroscopic) containing 10 μg BHT ml−1, it was evaporated to dryness. Such ‘prepurified’ samples were dissolved in ethanol and transferred to centrifuge tubes and stored at −20°C until required.

HPLC Preparative System. The equipment consisted of a Milton Roy Minipump connected via a Rheodyne 7105 loop injector to a 50 × 4.6 mm i.d. pre-column packed with silica gel (63–200 μm), followed by a 250 × 10 mm i.d. liquid chromatography preparative column of 10 μm Polygosil 60 C18 (Skandinaviska GeneTec) and a UV detector, Spectro Monitor 1 (Laboratory Data Control). The wavelength of the detector was adjusted to 282 nm. The eluent was methanol:acetic acid:distilled H2O (27.5:2.0:61.5, v/v/v) and the flow rate was 4.0 ml min−1. Retention times (tR) of IAA and some indole compounds as described in Table I were determined. IAA had a tR of 31.1 min. The fraction corresponding to IAA was collected from rose extracts for quantitative measurements on the analytical system.

HPLC Analytical System. For analytical separations, a reverse-phase ion-pair HPLC system was adopted mainly according to Sandberg et al. (14). The pump was Altex 110A (Beckman Instrument, Sweden), the injection loop was a 50 μl Rheodyne 7125, and the column was a 5 μm Nucleosil C18, 150 × 4.6 mm i.d. (Skandinaviska GeneTec). Elution was by means of 27.5% methanol and 0.01 m phosphate buffer at pH 6.5 with 0.01 m TBA at a flow rate of 1.0 ml min−1. The spectrofluorimetric detector (Schoeffel FS 970) was adjusted to an excitation light of 282 nm and emission light of 360 nm, which passed through a cut off filter. Elution curves of the same indole compounds tested in the preparative HPLC system were recorded. IAA had a tR of 18.38 min. The IAA determinations were made on the basis of peak height. The detection limit for IAA in analysis of rose extracts was 100 pg/injection. Determination of losses of IAA during the purification of rose extracts was made by liquid scintillation of sample aliquots of 0.5 ml. All values presented are corrected both for losses and for the addition of the internal standard.

GC-MS System. IAA was identified in methanolic extracts of dry rose achenes by GC-MS according to Ek et al. (3). In brief, this procedure is as follows: deuterated IAA was included in the extracts as an internal standard. The extracts were adjusted to pH 2.7, fractionated with ether, and dissolved in 0.1 ml pyridine and 0.1 ml N0,N-bis(trimethylsilyl)trifluoroacetamide to silylate the IAA. The IAA was quantified on a Carbo Erba (Fratcovap MOD 2900) GC-Finnigan MS (3200 F) with an on-line computer system.

RESULTS

Elution curves of some indole compounds in the preparative and analytical HPLC systems used are shown in Table I. The preparative one failed to separate IAA from indole-3-carboxylic acid and indole-5-carboxylic acid. Indole-3-carboxylic acid is of interest, in particular, as a possible endogenous auxin (1). The separation of these three compounds was accomplished in the analytical system. It is also necessary to separate compounds other than acidic indoles from IAA. Separation on the preparative column of an extract from unimbibed dormant rose achenes (Fig. 1) revealed the presence of numerous UV absorbing compounds after the prepurification step, which confirms the need for careful purification of the extracts prior to quantification. The compounds in the extracts from unimbibed achenes having the same retention time as IAA on the preparative column (see Fig. 1) were further analyzed with specific fluorimetric detection in the analytical HPLC system (Fig. 2). The elution curve shows only three peaks, one of them with the same retention time as IAA (18.38 min).

The identity of IAA in rose achenes was verified by GC-MS on three methanolic extracts of dry dormant achenes. The IAA content was 84.3 ± 12.1 ng (g fresh weight)−1. This agrees well with the determinations made by ion-pair reverse-phase HPLC; i.e. 83.1 ± 0.6 ng (g fresh weight)−1.

IAA Content. Figure 3 shows how the amount of IAA found in dry dormant rose achenes changed during stratification. A dramatic fall occurred during the first 2 weeks from 83.1 ng to 19.6 ng at 4°C and to 28.0 ng (g fresh weight)−1 at 17°C. According to the germination data of the same seed lot, isolated embryos from cold stratified achenes were still dormant at this time and did not commence to germinate until after 3 weeks of stratifica-
that to decrease occurred transferred 49.8 at 17°C, (see 16). (Fig. 3). Changes in IAA content (-->) of Rosa rugosa achenes during incubation at 17°C after having been stratified at 4°C for 14 weeks. Otherwise as in Figure 3. radicle increased linearly, there was a transient drop in the IAA content before it began to increase again.

**DISCUSSION**

Studies of plant hormones are severely hampered by the extremely low levels of these compounds in most tissues, and by substances which may interfere in the determination methods. The analytical method employed, ion-pair reverse-phase HPLC, enabled us to detect low IAA amounts in the purified samples. The purification methods were developed to fit the special plant material used, rose achenes. The two HPLC systems used in succession separated the tested auxin-related compounds from IAA. Among those 4-Cl-IAA was of special interest as it has been reported to occur naturally preferentially in immature seeds (5,7) and has been found to be more active than IAA in the Avena straight-growth test (2). Therefore, 4-Cl-IAA might have interfered in earlier investigations using bioassays to determine IAA content during dormancy release in poorly purified extracts from seeds (see "Introduction"). The HPLC results presented in this investigation suggest that IAA is the only fluorescent compound in the IAA peak. This is confirmed by the GC-MS results. Combined, these observations clearly illustrate how the pool of free IAA changes in rose achenes during stratification and germination.

The decline in IAA during the first 2 weeks of cold stratification seems not to be an event involved in dormancy release as a similar reduction was found in rose achenes stratified at 17°C, which does not break their dormancy. Moreover, there were no other pronounced changes in IAA content throughout the course of cold stratification as compared to soaking them at the warm temperature except at the end of the 14 week stratification period (Fig. 3). Then, a pronounced increase in IAA (with large differences between the triplicate samples) was recorded exclusively in the extracts of achenes stored at 17°C. This result was unexpected and lacks support in the literature. Presumably, this high IAA yield is an experimental artefact due to the long-term storage in wet conditions and at a temperature favorable for microorganisms. It is well known that bacterial contamination gives increased IAA levels (11). In accordance with the present investigation, an early decline of IAA occurring both at the cold and warm temperatures has also been reported for embryos of Acer platanoides during the time course of stratification (17). Nikolaeva (12) did not report any reduction of IAA during control conditions (stratification at 20°C for 4 months) of Acer tartaricum seeds. But, she did not present quantitative data for the time course at 20°C, and her report of a high IAA content may also be explained by bacterial contamination. Thus, it seems likely that hormones other than IAA regulate the dormancy breaking process. An early increase in cytokinin-like substances during cold stratification has been recorded (9) for the same lot of rose achenes as used for this study, whereas the dormancy release...
seemed to be unrelated to the endogenous ABA level (16). Our results of gibberellin-like activity have not shown any changes before radicle elongation commences (v. Schirach-Smigiel, personal communication).

In accordance with earlier reports (4,10,12,17), our results clearly indicate that endogenous IAA is low when germinability increases and visible germination starts. The level of IAA increased in rose achenes in a manner similar to that during germination of Pinus silvestris and Acer platanoides seeds, which were released from dormancy (15,17) and of Phaseolus vulgaris and Zea mays seeds, which do not possess dormancy (15). This increase in IAA was preceded by an increase in cytokinin-like activity in extracts from germinating seeds of the same species (9; Julin-Tegelman, personal communication), which is in line with the concept that cytokinins affect auxin levels. It is likely that the initial increase of IAA under germination conditions is due to its release from bound forms. It has been shown that Zea kernels utilize IAA esters to meet the requirements of IAA for germination (6). It has also been demonstrated that IAA and esterified IAA are interconvertible in the growing Zea seedling (13) which together with IAA synthesis may cause the observed fluctuations in IAA levels during germination and growth. Also, for the rose achenes it is desirable to measure the metabolic turnover of IAA and the IAA pool in different seed compartments. However, this is not easily done, due to the toughness of the pericarp, which has already been emphasized in a previous report (16). The data presented here relate only to pool size of the entire rose achenes. They support the idea that in rose achenes endogenous IAA may be an important factor for germination, but exclude a role of endogenous IAA in the control of dormancy release induced by cold stratification.

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