Communication

Determination of Abscisic Acid in *Pinus densiflora* by Selected Ion Monitoring

Received for publication February 1, 1988 and in revised form May 24, 1988

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ABSTRACT

Abscisic acid (ABA) in the stem of akamatsu (*Pinus densiflora*) was identified and quantified by gas chromatography-mass spectrometry-selected ion monitoring using hexadeuterated ABA as an internal standard. *tert*-Butyldimethylsilyl ester was used as a derivative of ABA. This derivative had high sensitivity and selectivity for ABA determination. ABA concentrations in cambial region scrapings were independent of the cessation of cambial activity.

The role of ABA in the regulation of cambial activity in conifers remains unclear, because conflicting results have been reported in the relationship between endogenous ABA levels and this activity (6, 8, 15, 18, 21, 22). Thus, more studies which precisely quantitate ABA are required to elucidate its role in the regulation of cambial activity and wood growth in conifers. GC-SIM has been widely used for quantitative analyses of ABA in plants (4, 13, 17), because it has high specificity and sensitivity. ABA has been generally analyzed as the methyl ester after esterification with diazomethane, and the base peak (m/z 190) in the mass spectrum has been used for GC-SIM analyses (4, 17). However, the mass of this ion is low, and the possibility of interference by contaminants is therefore high.

*t*-BDMSi derivatives (1) have been used for recent GC-SIM analyses of endogenous substances, namely, steroids (3, 20), fatty acids (23), terbutalines (9), and other plant hormones (2, 14, 19), because they have good stability and give an intense [M-57]* ion in the mass spectrum (5, 7, 16).

In this paper, the *t*-BDMSi derivative was used for GC-SIM analysis of ABA. The absolute concentration of endogenous ABA (d₀-ABA) in the stem of akamatsu (*Pinus densiflora* Sieb. et Zucc.) was determined using hexadeuterated ABA (d₀-ABA) as an internal standard.

MATERIALS AND METHODS

Materials. A healthy, 20-year-old akamatsu (*Pinus densiflora* Sieb. et Zucc.) tree approximately 9 m in height growing in the Experimental Forest of our University was used. Tissues from a 15 cm² (3 × 5 cm) area including phloem, cambial zone, and differentiating xylem were taken from the trunk at 1.2, 3.2, and 5.2 m above ground on September 17, 1985. They were harvested with knives and chisels.

Preparation of d₀-ABA. The d₀-ABA was synthesized by a procedure similar to that reported by Rivier et al. (17). The deuterium content was 94.3%.

Extraction of ABA Fraction. The tissues were rapidly homogenized after harvest and extracted three times with absolute methanol for 24 h at −20°C in darkness. To the extracts, d₀-ABA (1.2 μg) was added as an internal standard. The extract was concentrated *in vacuo* and the aqueous residue was adjusted to pH 2.8 with 0.5 N HCl. The acidic aqueous solution was extracted three times with diethyl ether. The diethyl ether fraction was concentrated *in vacuo* and extracted three times with 5% NaHCO₃. NaHCO₃ extractions were done below pH 8 so that no isotope exchange would occur (11). The aqueous fraction was then adjusted to pH 2.8 with 0.5 N HCl and again extracted three times with diethyl ether. The diethyl ether fraction was dehydrated over Na₂SO₄ and evaporated to dryness. The residue was dissolved with 50% acetonitrile.

Purification on HPLC. The reversed-phase column (15 × 0.39 cm i.d.) was a NOVAPAK C₁₈ (Waters Associates, Inc.) column was eluted with 25% acetonitrile containing 1% acetic acid at a flow rate of 0.4 mL/min. The fraction corresponding to the Rt of authentic ABA was collected and transferred to a mini-vial.

GC-MS. The fraction in the mini-vial was dried over P₂O₅ *in vacuo*. To the dry residue, absolute acetonitrile (40 μL) and N-(*tert*-butyldimethylsilyl)-N-methyltrifluoroacetamide (Tokyo Kasei Co.; 20 μL) were added. The mixture was heated at 70°C for 15 min. GC-MS was carried out under the following conditions: ionizing energy 20 eV; separating port temperature 280°C; a glass column (1 m × 3 mm, i.d.) packed with 1.5% OV-1; column temperature 195°C; carrier gas He; flow rate 30 mL/min.

Calibration Curve. Various amounts of d₀-ABA (from 0.24 to 1.92 μg) and 1.92 μg d₀-ABA, dissolved in methanol, were used for preparation of the calibration curve. Combined solutions were injected three times at any one molar ratio. The peak heights of the ions at m/z 321 and 327 were measured by a SIM chromatogram. The peak height ratio *versus* the molar ratio was plotted on a graph.

RESULTS

Authentic d₀-ABA and d₀-ABA were mixed in equal quantities, and then their *t*-BDMSi derivatives were prepared. The mass spectrum of the mixed *t*-BDMSi compounds is shown in Figure 1. The ions at m/z 384 and 378 were the molecular ions of the
The ions at m/z 327 and 321 were more intense. The ions at m/z 190 and 194 were the base peaks. For quantitative measurement, a standard curve (m/z peak height ratio as a function of the ABA molar ratio) was prepared using authentic d6-ABA and d0-ABA (Fig. 2). The curve was essentially a straight line ($r = 0.998$).

The 6.6 to 7.6 mL fraction obtained by HPLC was collected on the basis of the Rt of authentic ABA. The total ion recording from GC-MS of the t-BDMSi products from the ABA fraction is shown in Figure 3. The Rt of the peak at 3.2 min was identical with that of the authentic t-BDMSi ABA. The mass spectrum of the peak from the ABA fraction is shown in Figure 4. The ions at m/z 384 and 327 derived from the t-BDMSi d6-ABA were present. In addition, ions at m/z 378 and 321 were also observed; since each ion was 6 a.m.u. lower than that of the t-BDMSi d6-ABA, we conclude that they were derived from the t-BDMSi derivative of endogenous ABA. The full mass spectrum and the Rt on GC also confirmed the presence of endogenous ABA in the samples.

The amounts of endogenous ABA in the samples were calculated after GC-SIM using the ions at m/z 327 and 321 as shown in Figure 5. Peak height ratio of the endogenous ABA to the standard was determined, and the amount of endogenous ABA was calculated from the standard curve shown in Figure 2. The amounts of endogenous ABA with the standard deviation are shown in Table I. ABA amounts in the stem (expressed per unit of area from which scrapings were done) were 16.1, 14.2, and 16.5 ng/cm² at 5.2, 3.2, and 1.2 m above ground, respectively. There was no significant difference between amounts at different positions in the stem.

**DISCUSSION**

Mono t-BDMSi ester derivative was made under mild conditions (at 70°C for 15 min). It has been reported that sterically
hindered hydroxyl groups (such as the 1'-OH in ABA) could not be tert-butylidemethylsilylated (5). Thus, synthesis of the t-BDMSi derivative under these conditions prevents the formation of plural products. In contrast, trimethylsilylation gave two isomeric derivatives (12). It was also reported that trimethylsilyl and oxime derivatives did not have identical stability at the nanogram level (17).

The t-BDMSi ester derivative was suitably volatile for the quantitative analyses of endogenous ABA by GC-SIM. The calibration curve showed a linear relationship between the ratio of peak height and that of concentration. Additionally, this derivative gave a very characteristic fragment ion [M-57]+ which had medium intensity (60%) in a higher mass region, that is, greater than m/z 300. Therefore, the use of the t-BDMSi derivative can reduce considerably the effect of background due to contaminants in samples. Although the pentfluorobenzyl ester of ABA is more sensitive to electron capture detection than the methyl ester of ABA, it does not have intense ions in a high mass region (10), and so is less useful for GC-SIM quantitation. We thus conclude that the t-BDMSi ester is an ideal derivative for accurate and reproducible quantitative analyses of ABA by GC-SIM.

Our use of GC-SIM for quantitation of ABA kept the SD (expressed as percent of the average) less than 10% (Table 1). ABA concentrations found in akamatsu were almost independent of where the sample was taken from the stem. On the day of sampling cell-division activity was still continuing at the 5.2 and 3.2 m positions, whereas it had already ceased at the 1.2 m position, as reported previously (2). Thus, the hypothesis that ABA concentrations can regulate the cessation of cambial activity is not confirmed by our data. Little and Wareing (8) and Savidge and Wareing (18) had earlier reported results indicating that ABA probably is not involved in the regulation of cambial dormancy. Our results corroborate their earlier findings in other conifers.

Acknowledgment—We would like to thank Mr. N. Watanabe for providing us with the experimental trees.

LITERATURE CITED

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