Physiological and biochemical traits of dormancy release and growth resumption in Japanese cedar in the warm-temperate zone

Forest Science

Online Resource 4

Measurement of phytohormone contents

Briefly, samples of 0.09–0.17 g of frozen shoots were ground in liquid nitrogen, extracted in 4 mL of solvent (1% [v/v] acetic acid in 80% [v/v] acetonitrile) mixed with internal standards (stable isotope-labeled compounds), and incubated at 4°C for 1 h. The amounts of internal standards were 3.64 ng for D_2-IAA, 3.32 ng for D_6-ABA, 39.64 ng for D_4-SA, 4.46 ng for D_2-JA, 0.214 ng for $^{13}C_6$-JA-Ile, 0.14 ng for D_5-tZ, and 0.36 ng for D_6-iP. After centrifugation at 3000 × *g* for 15 min at 4°C, the supernatants were evaporated off and 1% (v/v) acetic acid was added to obtain a volume of 1.2 mL. These supernatants were then centrifuged at 20 000 × *g* for at least 5 min at 4°C and loaded onto a reversed-phase cartridge (Oasis HLB 1 cc Vac Cartridge [30 mg]; Waters Corp., Milford, MA, USA) that had been washed sequentially with 1 mL of 100% (v/v) acetonitrile, 1 mL of 100% (v/v) methanol, and 1 mL of 1% (v/v) acetic acid. After the cartridges were again washed with 1 mL of 1% (v/v) acetic acid, phytohormones were eluted using 2 mL of 80% (v/v) acetonitrile mixed with 1% (v/v) acetic acid. Next, the eluates were evaporated and loaded onto cation-exchange cartridges (Oasis MCX 1 cc Vac Cartridge [30 mg]; Waters Corpo., Milford, MA, USA). Prior to loading, 1 mL of 100% (v/v) acetonitrile, 1 mL of 100% (v/v) methanol, 1 mL of hydrochloric acid (0.1 mol/L), and 1% (v/v) acetic acid had been added sequentially to fill the entire cartridge. IAA, ABA, jasmonates, and SA were eluted with 2 mL of 80% (v/v) acetonitrile mixed with 1% (v/v) acetic acid, whereas cytokinins were eluted with 2 mL of 50% (v/v) acetonitrile mixed with 5% (v/v) ammonia (extract 1). Prior to elution of cytokinins, the cartridges had been washed with 1% (v/v) ammonia. After the extracts of IAA, ABA, jasmonates, and SA were evaporated, they were loaded onto anion-exchange cartridges (Oasis WAX 1 cc Vac Cartridge [30 mg]; Waters Corp., Milford, MA, USA) to which 1 mL of 100% (v/v) acetonitrile, 1 mL of 100% (v/v) methanol, 0.3 mL of potassium hydroxide (0.1 mol/L), and 1% (v/v) acetic acid had been added sequentially to fill the entire cartridge. IAA, ABA, and jasmonates were then eluted with 2 mL of 80% (v/v) acetonitrile mixed with 1% (v/v) acetic acid (extract 2), and SA was eluted with 97% (v/v) acetonitrile mixed with 3% (v/v) formic acid (extract 3). Prior to the elution of phytohormones, the cartridges had been washed with 1 mL of 1% (v/v) acetic acid and 2 mL of 50% (v/v) acetonitrile. All three extracts

(extracts 1–3) were further quantified by LC-ESI-MS/MS (1260 Infinity LC with 6410 Triple Quad LC/MS; Agilent Technologies, Santa Clara, CA, USA) equipped with a column (CAPCELL PAK ADME-HR S2 [93301]; Osaka Soda, Osaka, Japan). For quantification of the cytokinins in extract 1, pure water containing 0.01% (v/v) acetic acid and methanol containing 0.20% (v/v) acetic acid were used as eluents, and the flow rate was set to 0.25 mL/min. For quantification of IAA, ABA, and jasmonates in extract 2, pure water containing 0.01% (v/v) acetic acid and acetonitrile containing 0.05% (v/v) acetic acid were used as eluents with a flow rate of 0.40 mL/min. For quantification of SA in extract 3, pure water containing 0.10% (v/v) formic acid and acetonitrile containing 0.10% (v/v) formic acid were used as eluents with a flow rate of 0.40 mL/min. The amount of each phytohormone (ng) was calculated proportionally by multiplying the amount of the internal standard (ng) to the area ratio, i.e., area of phytohormone in the sample divided by the area of internal standard. The contents of phytohormones on a dry weight basis (ng g^{-1}) in each sample were calculated by dividing the amounts of each phytohormone by the dry weight of the sample.