Video Article

Monitoring Plant Hormones During Stress Responses

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Abstract

Plant hormones and related signaling compounds play an important role in the regulation of plant responses to various environmental stimuli and stresses. Among the most severe stresses are insect herbivory, pathogen infection, and drought stress. For each of these stresses a specific set of hormones and/or combinations thereof are known to fine-tune the responses, thereby ensuring the plant's survival. The major hormones involved in the regulation of these responses are jasmonic acid (JA), salicylic acid (SA), and abscisic acid (ABA). To better understand the role of individual hormones as well as their potential interaction during these responses it is necessary to monitor changes in their abundance in a temporal as well as in a spatial fashion. For the easy, sensitive, and reproducible quantification of these and other signaling compounds we developed a method based on vapor phase extraction and gas chromatography/mass spectrometry (GC/MS) analysis (1, 2, 3, 4). After extracting these compounds from the plant tissue by acidic aqueous 1-propanol mixed with dichloromethane the carboxylic acid-containing compounds are methylated, volatilized under heat, and collected on a polymeric absorbent. After elution into a sample vial the analytes are separated by gas chromatography and detected by chemical ionization mass spectrometry. The use of appropriate internal standards then allows for the simple quantification by relating the peak areas of analyte and internal standard.

Video Link

The video component of this article can be found at <http://www.jove.com/details.php?id=1127>

Protocol

1. First we need to prepare the 2ml screw-cap vials for extraction: Add 400ul extraction buffer (1-propanol:H₂O:concentrated HCl 2:1:0.002 vol/vol/vol) and 10 µl of the respective internal standard (10ng/µl) to a vial. Freeze in liquid nitrogen and then add the ceramic beads.
2. Add the plant material (between 50 and 200mg) while the vials are still positioned in the liquid nitrogen. Note that the weight of the added plant material has to be estimated before further processing to allow later for exact quantification, which is based on the fresh weight.
3. Make sure that all liquid nitrogen is evaporated before tightly closing the vial with a cap. Note that the cap needs to have a rubber seal to prevent spills of solvents and thus, extracted compounds during the homogenization.
4. Homogenize the tissue in a FastPrep or Precellys bead grinder homogenizer at a speed of 6000 (for Precellys) for 30 sec.
5. Remove vials from homogenizer individually, carefully open the cap and add 1ml of dichloromethane to each sample. Close vials again tightly and homogenize again for 10-15sec at 6000 (Precellys).
6. Transfer vials to a tabletop centrifuge and separate the organic phase from the aqueous phase at 10.000xg for 60sec.
7. After phase separation transfer the lower green layer (organic phase, contains the hormones and the internal standards) from individual vials to a clean 4ml glass vial. Avoid the transfer of water (upper phase). Alternatively, ethyl acetate can be used instead of dichloromethane. In that case the ethylacetate phase will be the upper layer (green) and is now easier to remove and transferred to a fresh 4ml glass vial. As before, avoid the transfer of water.
8. Blow off the solvent with air through a manifold for about 10-25 min (depends on sample); check with a single airflow tip if samples are dry (carefully). Do not overdry samples, for this might cause losses of compounds.
9. Once the samples are dried we can start the methylation of the carboxylic acid-containing compounds in our samples. First, 100 µl of a diethylether/methanol mixture (9:1 vol/vol) are added to each vial followed by 4µl of a 2M trimethylsilyldiazomethane solution (in hexane, Sigma Aldrich). Vials are immediately closed with an open-top screw cap fitted with a Teflon-lined silicone septum. Shake gently and incubate at RT for 25 to 30min.
10. After finishing step 9, turn on a heating block and set at the desired collection temperature. The volatility of the methylated compounds depends on their size, but also on other, more polar groups like =O, -OH, and -NH. For jasmonic acid and salicylic acid methyl esters a collection temperatures of around 80°C are sufficient, whereas for example other compounds like C18 fatty acids, jasmonic acid-amino acid conjugates, coronatine, and 12-oxo-phytodienoic acid methyl ester require temperature between 180-200°C to effectively volatilize.
11. After a 30min incubation the methylation reaction needs to be stopped to avoid unwanted secondary reaction. This is done by adding 4 µl of a 2M acetic acid-solution (in hexane) to each vial. After closing the vials with caps and a short vortexing, the samples are allowed to incubate for another 30min.
12. The filters used for this vapor phase extraction are handmade and in the presented form not commercially available. They consist of an outer Teflon tube (about 7-8cm long), into which from one side a well-fitting glass tube of about 4cm length is inserted. On the other side of the Teflon tube a stainless steel mesh disc is pushed inside the tube until it reaches the inner glass tube and onto that about 20-30mg of adsorbent (either SuperQ 80/100 (discontinued) or HayeSep® Q80/100) are added. Another stainless steel mesh disc is inserted and finally a glass tip is pushed into the Teflon tube, thereby carefully pressing the stainless steel mesh disc on the absorbent. A detailed picture a filter is shown in figure 1.

Figure 1

Figure 1.

13. During the incubation time of step 11, the filters for the collection of the methylated and thus, volatile plant hormones, have to be washed using first 200ul of methanol, and then 2 washes with 200ul dichloromethane each. Solvent remaining in the filters is then blown off by air. The filters can now be used for the trapping of volatile hormones through vapor phase extraction.
14. After 30min of incubation the rubber septa of the 4ml vial caps are cut with a scalpel. To collect the plant hormones by vapor phase extraction the cleaned filters are connected to individual vacuum lines at a flow rate of about 800ml/min. The filters are then inserted into the vial through the cut septa. A small pipet tip is also inserted to act as an air inlet. During this initial part of the procedure the vials with the inserted filters have to be held in an upright position to avoid any of the liquid inside the vial to get in contact with the filter tip, which would contaminate the filter and subsequently also the GC/MS. The vials are then held by hand until all the liquid is evaporated through the filter. Then the vials with the attached filters are placed on the heating block and heat-evaporated compounds collected for 3min. The principle components of the system as well as the setup are depicted in figure 1. After this final vapor phase extraction step the vials with the filters still attached are placed on a rack and allowed to cool down.
15. When the filters have reached room temperature again they are eluted with 150ml of dichloromethane into a 1.5 ml GC-vial fitted with an insert. After blowing out the remaining solvent from the filter the vials are capped and analyzed by GC/MS.

A gas chromatograph equipped with a split/splitless injector (splitless mode 250°C, injection volume 1µl) interfaced to a mass spectrometer operated in chemical ionization mode (CI) should be used for the analysis. Compounds are separated on HP-1MS column (30m x 0.25mm x 0.25µm) held at 40°C for 1min after injection, and then temperature programmed at 15°C/min to 250°C (for 10min), with helium as the carrier gas (constant flow 0.7ml/min). For the MS-based analysis both, a quadrupole (e.g. Agilent HP5973) and an ion trap-based (e.g. Varian Saturn 2200) mass spectrometer can be used. As a CI gas either isobutane (comes in gas tanks) or methanol (vapors from a liquid reservoir are used) can be used, however, in each case the fragments of the ionization reaction should be checked before the selected ion count (SIC) programs are created. We have used the following program for a Varian GC 3900/Saturn MS 2200 system and methanol as the CI reagent: 5.00-11.30min ions (M+1)⁺ 153-158 (methyl salicylate 153, methyl ²H₅-salicylate); 11.30-13.30min ions (M+1)⁺ 207-228 (methyl jasmonate 207 and 225, methyl dihydrojasmonate 209 and 227); 13.30-16.30min ions (M+1)⁺ 237-300 (methyl abscisic acid 261, methyl ²H₆-abscisic acid 267 (abscisic acid produces predominantly [M-H₂O+1]⁺ ions). All compounds should be identified by comparison with authentic commercially available standards. Quantification of JA, SA, and ABA is done by correlating the peak area (extracted ions) with the peak area (extracted ions) of the respective internal standard and is also based on the fresh weight of the plant material used.

Discussion

The method presented here has been proven to work reliably for a wide array of plant signaling compounds provided that the size and the chemical nature of the compound does not prevent it from being extracted, methylated, and vaporized. Also, the methylation procedure as it is described herein is not the only way of derivatization. Silylation and acetylation are alternative methods and protocols can be easily found online.

If the mass spectrometer is not capable of single ion monitoring the mass range that is recorded during a run should be limited to exclude too many unwanted ions from the process. For example, jasmonic acid (JA) and its internal standard dihydro jasmonic acid (dhJA) produce two major (M+1)⁺ ions, 207 and 225 for JA, and 209 and 227 for dhJA during chemical ionization with methanol. Because these two major (M+1)⁺ masses (207 and 225 for JA; 209 and 227 for dhJA) do not have the same abundance ratio for JA and dhJA, they should both be extracted and used for quantification. Therefore, a mass range from 207 to 228 should be recorded and the specific ions should be extracted later.

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