Studies on arabiopsides using two *Arabidopsis thaliana* ecotypes

Nilay Peker

Practical work, July - August 2010, 15 hp
Department of Plant and Environmental Sciences
University of Gothenburg
Abstract

In plants, the oxylipin jasmonic acid (JA), a plant hormone, is involved in defence responses against herbivores, pathogens and other environmental conditions that are damaging to plants. Jasmonic acid is produced by the reduction and chain shortening of 12-oxo-phytodienoic acid (OPDA) and dinor-oxo-phytodienoic acid (dn-OPDA). In Arabidopsis thaliana OPDA not only occurs in free form but also esterified in molecules denoted arabidopsides. In this study, the levels of arabidopsides were measured in several different Arabidopsis thaliana ecotypes. The analysis was also performed in different in plants of different ages, in response to both pathogens and wounding. The highest level of arabidopside accumulation was observed in the ecotype, Col-0 and the lowest in the ecotype C24. Furthermore, the levels of arabidopsides in F2 plants from a cross of Col-0 and C24 was determined and was used in a map based cloning approach to identify the gene responsible.

Examiner: Bente Ericsson
Supervisors: Anders Nilsson, Mats Ellerström and Mats Andersson
**Introduction**

All living organisms have their own specialized defence system against pathogens and other environmental damaging stresses. In contrast to animals, plants have an innate immune system based on two mechanisms. These are pattern-triggered immunity (PTI) and effector-triggered immunity (ETI). In the first one, plants recognize pathogen elicitors for example pieces of the cell wall of pathogens. These pathogen components are called pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs). After reception of PAMPs or MAMPs, defence mechanism is activated. In the effector-triggered immunity system, resistance proteins act as a receptor for pathogenic virulence factors. These receptors specifically recognize the effectors [1].

Hypersensitive response (HR) is one of the most effective defence systems. In this system, a host resistance (R) protein specifically recognizes a pathogen-derived avirulence (Avr) protein. This recognition leads to a rapid and localized cell death as well as defence gene expression and fortification of cell walls. Mainly two hormones, salicylic acid (SA) and jasmonic acid (JA), are involved in plants local defence responses. When the plant recognizes a pathogen or when wounded, generally jasmonic acid is formed. [2] Jasmonic acid is an oxylipin. Oxylipins are lipid metabolites and they are formed from oxidation of unsaturated fatty acids by enzymatic or chemical reactions. Formation of the oxylipins is initiated by lipoxygenases (LOX) and JA synthesis occurs in the following pathway; first fatty acid is transformed into 13-hydroperoxy α-linolenic acid (13-HPOT) by lipoxygenases. Then allene oxide synthase (AOS) and in the following allene oxide cyclase (AOC) generates 12-oxo phytodienoic acid (OPDA). And finally, jasmonic acid is formed from dn-OPDA and OPDA, which is are derived from 16:3 and 18:3 unsaturated fatty acids, respectively. When dn-OPDA and OPDA are bound to monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) in different combinations, new structures called arabidopsides (Arabidopside A, B, C, D, E, F and G which are currently reported) are formed. These structures are accumulated in *Arabidopsis thaliana* plants in during HR and wounding. It is reported that arabidopsides have role in the inhibition of the growth of pathogens [3, 4].

In this study, the aim was to investigate arabidopside formation in the *Arabidopsis thaliana* ecotypes, Col-0 and C24. And also, level of arabidopsides was determined in a F2 population of a cross between Col-0 and C24. We found that the gene responsible for
the difference between arabidopside accumulation in Col-0 and C24 are located on chromosome 4.

**Material and Methods**

**Extraction and fractionation of lipids from plant material**

*HPLC method;* 5 leaf discs, approximately 0.025g, were taken from each plant. For freeze/thaw induction of arabidopside formation, leaf discs were put into test tubes and frozen in liquid nitrogen. Then they were thawed and left in room temperature for 1 hour, subsequently the leaves were boiled for 5 minutes in 3-4 ml of 2-propanol and dried under N₂. 2 ml of one phase solution (contents of that solution) was added and incubated in fridge for 30 minutes. Then they were extracted in ultrasonic bath for 30 minutes (until the leaf discs were depigmented). Phase separation was provided by adding 500 μl CHCl₃ (with 0.05% BHT) and 500 μl K₂SO₄ solutions, vortexed and left until the phase were separated. The lower phase was then transferred to new tube and 1ml CHCl₃ (with 0.05%BHT) was added, vortexed and the phases were separated. The second lower phase was added to the first and then the pooled chloroform phase was dried under N₂. The dried samples were dissolved in 100μl CHCl₃. A 500mg Si60 SPE columns was placed in a glass tubes and washed with 2.5ml of CHCl₃. The samples were added to the columns and washed with 2×2.5ml CHCl₃. The columns were moved to new tubes and glycolipid fractions were eluted with 2×2.5ml of acetone:MeOH (9:1). After these fractions were dried under N₂ they were dissolved in 35μl acetonitrile and transferred to the vials and became ready for HPLC (high performance liquid chromatography) analysis.

*For gas liquid chromatography (GC) method;* 2 leaf discs were taken and 5μl of 2μg/μl di-l9:0-PC solution was added before freezing in liquid nitrogen. 1 hour later, 2ml isopropanol is added and let to boil for 5 minutes. And samples were dried under nitrogen. After that, 0.5ml of 0.5M sodium methoxide and 0.25ml of heptane added. In the following, samples were let to extract in sonic bath and 50μl acetic acid and 1 ml
water was added. After vortex and phase separation, the upper phase was transferred to GC-vials and run.

**Genomic DNA isolation, PCR and mapping**

For genomic DNA isolation, small pieces were taken from the youngest leaves into a 1.5ml eppendorf tube and where left in the freezer, -20°C, for 1 hour. 750μl of EDM-buffer (200 mM Tris-HCL, 250mM NaCl, 25mM EDTA and 0.5% SDS) was added and incubated at 95°C for 5 minutes. Next the samples were left in room temperature overnight. In the following, 750μl of isopropanol was added, mixed and the samples were centrifuged at full speed for 5 minutes. After the supernatant was removed, the pellet was washed with 1ml of 70% EtOH, inverted several times and centrifuged at full speed for 2 minutes and dried at 37°C. The pellet was resuspended in 200μl of TE-buffer and shaken for approximately 10 minutes. Before the use as PCR template, they were centrifuged 1 minute at full speed.

In the following, 10μl PCR reaction was prepared for each sample and chemicals for master mix were taken for each sample as in the following amounts: 4.955μl of water, 1μl of 10x buffer, 0.4μl of 5mM dNTP, 0.1μl of BSA, 2.5μl of primer, 0.05μl of Taq polymerase and 1μl of template. After PCR reaction was completed, 2μl 6x loading buffer and 1μl gelstar were added to each well and 5μl of 100bp DNA ladder, mixed with 1μl gelstar, was used for gel electrophoresis. Samples were run on 3.5% agarose gel, at 130V for approximately 50 min. For mapping, following primers were used: GOT4, GOT9, GOT7, GOT8 and GOT16.

**Results**

**Formation of arabidopsides with freeze-thawing:**

Arabidopside levels of Col-0×C24 plants were measured with both HPLC and GC method. The genotype of the plants was determined by the use of PCR. As can be seen in figure1, highest amount of arabidopsides was found in Col-0 plants and the lowest in C24 plants.
Figure 1: Levels of arabidopside E and G in F2 population (Col-0xC24). Levels was determined with HPLC method

The same samples were also analysed with GC method, amount of OPDA for each sample are illustrated in figure 2. Highest OPDA formation is seen in Col-0 plants and lowest one is in C24.

Figure 2: synthesized OPDA level after freeze-thawing in F2 mapping population plants.

This arabidopside measurement was also done in some species closely related to Arabidopsis thaliana. Some of the HPLC results are shown below.
Figure 3: HPLC results for Col-0. The first arrowed peak shows the amount of arabidopside A, second one is arabidopside E, third one is arabidopside G and fourth one shows the amount of acyl-MGDG.

Figure 4: HPLC results for Arabidopsis lasiocarpa. The peak indicated with arrow shows the acyl-MGDG level. There is no peak seen in the region of arabidopside.
Figure 5: HPLC results for *Capsella rubella* MTE. First arrowed peak shows the arabidopside A, second shows the arabidopside E and third one shows the arabidopside G.

These experimental procedures have been repeated many times for different series of F2 populations (Col-0×C24). By using different makers in PCR, the following map has been created.

Table 1: Genetic mapping with different markers.
Discussion

In this study, the genes responsible for the difference in the accumulation of arabidopsides and acyl-MGDG, between the Arabidopsis ecotypes Col-0 and C24 genes was determined to be located on a fragment on chromosome 4 between the markers GOT9 and GOT16. Arabidopside levels were determined using both a HPLC and a GC method. The mapping using PCR correlated with the HPLC results. Thus, the HPLC was found to be more reliable than the GC method. It was determined that the highest accumulation occurs in Col-0 and the lowest in C24. Our results showed that, some of the F2 plants had less arabidopside than even C24. This most likely reflects the existence of more than one gene responsible for the arabidopside accumulation. Still even if more than one loci influence arabidopside formation the mapping study with different markers showed that, the most influential gene are located between GOT9 and GOT16 on chromosome four (table 1).

Comparison between the Arabidopsis ecotype Col-0 and related species showed that there is no correlation with the evolutionary distance to *Arabidopsis thaliana* and levels of arabidopside formation. As it can be seen from HPLC results (figure 3, 4, 5), although *Capsella rubella* MTE is not an arabidopsis species, arabidopside accumulation is seen in this plant. And this accumulation is not seen in *Arabidopsis lasiocarpa* even if it is a species closely related to Arabidopsis.
References


