The search for the missing link
Investigation of the levels of arabidopsides in Arabidopsis thaliana

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Abstract
Plants have physical barriers and many different secondary metabolites as protection against pathogens. A build-up of chloroplastic substances called arabidopsides has been found as a response to wounding and during the hypersensitive response in Arabidopsis thaliana. It is still not fully understood what functions arabidopsides may have in wounding and defence responses, but they have been shown to inhibit the growth of both bacterial and fungal pathogens in vitro as well as to be processed to more potent signalling molecules. We have determined that high levels of arabidopside A and B are detected shortly after freezing (5 min) in Col-0, but quickly decreased while arabidopside E and G increased at the same rate. Thus, freezing can be used as high throughput method to determine the accumulation of arabidopside after wounding. We confirmed earlier studies that have shown that there exists a large variation in the levels of arabidopside between the A. thaliana ecotypes Col-0 and C24. Arabidopside levels were measured in F2 plants obtained from a cross between Col-0 and C24 plants and compared to the parental ecotypes Col-0 and C24. F2-plants with the highest and lowest levels of arabidopside, resembling the expression in Col-0 and C24 respectively, were used for mapping the gene responsible. During the course of this investigation we have narrowed the area of interest down to a section on chromosome four, containing approximately 648 genes, whereof 86 were predicted (using ChloroP 1.1) to code proteins with a chloroplastic transfer peptide. Interestingly the lipoxygenase 2 gene earlier reported to be responsible for the accumulation of arabidopside is not present in the interval. Thus we have identified a novel locus crucial for arabidopside accumulation.

Sammanfattning
Växter har fysiska barriärer och många olika sekundära metaboliter som skydd mot patogener. Ackumulering av kloroplastiska substanser kända som arabidopside har visats som respons på skada och under den hypersensitiva responsen hos Arabidopsis thaliana. Det är ännu oklart vilka funktioner arabidopside kan ha vid skada och försvarsrespons, men de har visats kunna inhibera tillväxten hos både bakteriella och fungala patogener in vitro och de kan också processeras till mer potenta signalmolekyler. Vi har konstaterat att höga nivär av arabidopside A och B återfinns kort efter frysning (5 min) i Col-0, men snabbt minskar medan arabidopside E och G ökar i samma hastighet. Frysning kan alltså användas som en metod med hög kapacitet för att bestämma ackumuleringen av arabidopside efter skada. Vi bekräftade tidigare studier som har visat att det finns en stor variation i mängden arabidopside mellan A. thaliana ekotyperna Col-0 och C24. Arabidopside-nivärerna mättes i F2 planter från en korsning mellan Col-0 och C24 och jämfördes med parentala ekotyperna Col-0 och C24. F2-planter med de högsta och lägsta nivåerna arabidopside, liknande ekotyperna Col-0 respektive C24, användes för mappning av den ansvariga genen. Under denna undersökning har vi minskat området av intresse till en sektion på kromosom fyra, innehållande approximalt 648 gener, varav 86 förutsades (med ChloroP 1.1) koda proteiner med en kloroplastisk transferpeptid. Intressant nog så är lipoxygenase 2, genen som tidigare sagts vara ansvarig för akumulering av arabidopside, inte med i detta intervall. Vi har alltså identifierat ett nytt lokus som är avgörande för akumulering av arabidopside.
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Introduction

Plants, like all other living organisms, are constantly under pressure for survival and during the course of evolution plants have acquired many specializations and adaptations to survive. Competition with other plants, herbivores and pathogens that can cause physical damage and potentially lead to death are examples of stressors that plants need to overcome.

Plants in contrast to humans do not have an acquired immune system, but they both have an innate immune system. The innate immune system uses both preformed as well as inducible defences and responses, such as physical barriers and many different secondary metabolites for protection against pathogens [1].

Pathogens like bacteria and fungi must cause damage to the cell to infect and often use different substances to facilitate infection. Plant cells can sense such intruding pathogens by detecting the damages from infection (pieces of cell material) or recognition of non-self molecules, known as pathogen associated molecular patterns (PAMPs; like parts of fungi membrane or bacterial flagella) [2,3]. Plants have numerous receptors to sense PAMPs, and their recognition leads to activation of defence mechanisms [2,4].

Recognition of pathogens generally leads to a build-up of different secondary metabolites and hormones. The defence response in plant tissue may ultimately lead to the affected and neighbouring cells undergoing the hypersensitive response (HR), a type of programmed cell-death to stop further pathogenic infection and spreading [5].

Accumulation of substances named arabidopside have been detected at very high levels during HR in the model plant Arabidopsis thaliana. Different arabidopside (named by alphabetical letters in succession, currently A to G are known) are made when the oxylipins 12-oxo-phytodienoic acid (OPDA) and dinor-oxo-phytodienoic acid (dn-OPDA) are esterified to the galactolipids mono- or digalactosyldiacylglycerol (MGDG/DGDG) in different combinations [6].

Whereas the exact function of arabidopside is not known, they inhibit the growth of bacterial and fungal pathogens in vitro, indicating a protective role. When arabidopside are broken down there is an increase in free OPDA/dn-OPDA and jasmonic acid (JA), indicating an additional function as signal deposit, prolonging the signalling [6,7].

There are many different ecotypes of A. thaliana, all accumulating different levels of arabidopside as response to wounding or pathogen recognition. Until recently it was not known what genes are responsible for this accumulation. In 2009 it was reported that lipoxygenase 2 (LOX2) has connection to the arabidopside response, since the levels of arabidopside increase after wounding in wild type (wt) A. thaliana, but not in LOX2 mutants [8].

The aim of this work was to set up a system for high throughput analysis of arabidopside as well as to investigate any age dependency of arabidopside formation. Moreover a number of A. thaliana ecotypes were rescreened to determine the natural variation in arabidopside formation. For the two selected ecotypes, Col-0 and C24, there exists a large variation in the accumulation of arabidopside as well as a related molecule, acyl-MGDG. Acyl-MGDG was described by Heinz more than forty years ago [9,10] and is made when an acyl group from a galactolipid is transferred to a MGDG molecule.

F2 plants from a cross between Col-0 and C24 were used in this study to by genetic mapping determine the genes responsible arabidopside accumulation.
Materials and methods

Plant material
The wt Col-0 and C24 plants were grown together with F2-plants in growth chamber under short-day conditions (8 h light, 16 h darkness, approximately 200 μE light intensity, 22 °C day temperature and 19 °C night temperature). Samples were cut-out using a cork borer (6 mm diameter) that collected leaf-discs with roughly the same weight (5 mg). Ten leaf-discs were collected from each plant from randomly selected leaves, and placed in double-distilled water (ddH2O) in six-well plates.

Lipid extraction of arabinodipside and Acyl-MGDG
Five discs were collected from each well, dried with paper-tissue, put in test-tubes and boiled in 2-propanol for 5 min (105 °C). The 2-propanol were then dried out under nitrogen on heating-block (all drying were done at 35 °C) and chloroform, methanol, ddH2O and butylated hydroxytoluene (2 mL; 1:2:0.8 vol., 0.05% BHT) were added to the tubes, and left in 10 °C for 40 min. The remaining five leaf-discs from each well were collected and treated the same way as previously described, but were frozen in liquid nitrogen and thawed at room temperature (RT) for one hour before boiling in 2-propanol. The tubes were then sonicated (approximately 45 min, until the leaf discs were de-pigmented) where after phase separation was induced by adding 500 μL potassium sulphate (0.38 M) and 500 μL chloroform with BHT (0.05% BHT). After separation, the lower phase from each tube were transferred to new tubes, 1 mL chloroform with BHT (0.05% BHT) were added to the old tubes, vortexed, centrifuged (5 min; 2000 rpm) and the lower phases were then collected and pooled with those previously collected. The pooled phases were then dried under nitrogen on heating-block and resuspended in 100 μL chloroform. Silica columns (Discovery® DSC-Si SPE, 52654-U, 500 mg) were primed with 2.5 mL chloroform. The samples were then added to the columns, washed with chloroform (2 x 2.5 mL), moved to new tubes and eluted with a mixture of methanol and acetone (2 x 2.5 mL; 10% methanol in acetone) to collect the lipid fraction. The fractions were then dried out under nitrogen on heating-block, resuspended in 35 μL acetonitrile, vortexed and transferred to vials for the high performance liquid chromatography (HPLC).

Instrumentation and software
The HPLC (HP Series 1050) were used together with an Evaporative Light Scattering Detector (ELSD; Sedex 45, connected by HP interface 35900E). The instruments were controlled by the HP ChemStations for LC software, also used for data analysis. The HPLC were run with gradients of acetonitrile:ddH2O (85:15; solvent A) and 2-propanol (solvent B) in a pre-programmed method (100% solvent A from 0 to 5 min, 20% solvent A from 45 to 50 min, 100% solvent A from 55 to 70 min). The column used for HPLC were Prevail C18 (3 μm, 150 x 2.1 mm with guard column), with a flow at 0.2 mL/min. Detection with ELSD at 40 °C, 2.3 bar pressure and gain 7. Arabidopside were detected by UV absorbance at 220 nm.

Gel electrophoresis
Agarose gels (SeaKem® LE Agarose) and TAE buffer (from 1L 50x stock: 242 tris base, 57.1 mL glacial acetic acid, 100 mL 0.5M EDTA pH 8.0) where used as running buffer for electrophoresis.
Results

Ecotypes

The amounts of arabinosides were measured in five weeks old plants of twelve ecotypes, both before and after freeze-thawing, and were found to be lower than expected. The expression of LOX2 has been shown to increase with age/maturing, and the low values were believed to be connected to the age of the plants. The same plants were re-sampled two weeks later and the amounts of arabinosides were found to be higher (Figure 1). The C24 ecotype had significantly lower amount of arabinosides compared to the other ecotypes at seven weeks of age, making C24 a good candidate to cross with Col-0 for genetic mapping. An age-series experiment were started to see how the levels of arabinosides changed with age in Col-0 and C24.

Age-series

Plants were collected once a week at four to seven weeks of age (discarded after sampling). The amount of arabinosides was higher in sampled plants that were somewhere between six and seven weeks of age compared to younger plants (Figure 2). This gave a reference age at seven weeks for sampling F2 plants, to ensure that the collected material would be easily distinguishable in arabinoside levels (either high like Col-0 or low like C24). The amounts of acyl-MGDG decreased with increased age in both ecotypes (Figure 3). A similar experiment was conducted using the pathogenic bacteria Pseudomonas syringae (DC3000:AvrRpt2). The results were similar to freeze-thawing, but the amounts of arabinosides in Col-0 peaked between four and five weeks instead of six and seven weeks of age (results not shown).

Figure 1: The sum of arabinosides E and G were higher in seven week old plants (7w) compared to the five week old plants (5w). The levels of arabinosides A and B did not affect the results (not shown).
Figure 2: Levels of arabidopsides (A-G means total sum of A, B, E and G) after freeze-thawing increased during maturation and peaked in plants between six and seven weeks of age.

Figure 3: The amounts of acyl-MGDG after freeze-thawing decreased in both ecotypes during maturation.

Time-series
When measuring the amount of arabidopsides one hour after freeze-thawing, there were almost exclusively arabidopside E and G, and very little A and B. To see how the accumulation of arabidopsides changes with time an experiment was done with different thawing-times before extracting lipids. Arabidopsides A and B were high after five minutes of thawing, whereas arabidopsides E and G were low. As the amounts of A and B decreased in the following time-points, E and G increased at the same rate (Figure 4).
Arabidopsides A+B and E+G after freeze-thawing

Figure 4: Levels of arabidopsides after freezing and 0 to 120 minutes thawing in Col-0. Arabidopsides A and B decreased while E and G increased at the same rate.

F2-plants

Sampling of F2-plants was done when they were seven weeks of age. Plants with the highest (most like Col-0) and with the lowest (most like C24) amounts of arabidopsides were selected for DNA mapping (see example of data in Figure 5). A direct connection between lipoxygenase 2 (LOX2) and the levels of arabidopsides was suspected, but when using a LOX2-marker (constructed by Anders Nilsson, unpublished results) with the F2-plants, the results showed that LOX2 is not directly responsible for the accumulation of arabidopsides (data not shown). Numerous markers were tested and the best match was on chromosome four. Further mapping narrowed the area of interest down to between the markers GOT6 and GOT9 (constructed by Anders Nilsson; Figure 6. Also see Appendix I, Table A1). This section contain approximately 648 genes (data from TAIR), whereof 86 were predicted to contain a chloroplast transfer peptide using ChloroP 1.1 [11]. All F2-plants resembled Col-0 more than C24 (in coloration, leaf thickness, hairs etc.) and no connection between visible phenotype and levels of arabidopsides were found.
Figure 5: Levels of arabidopside (A-G means total sum of A, B, E and G) in 44 F2-plants and parentals (Col-0 and C24) after freeze-thawing, sorted on arabidopside levels.

Figure 6: Mapping of genes responsible for arabidopside accumulation on chromosome four. The area of interest were found to be between the GOT6 and GOT7 markers.
Discussion

The genes responsible for arabidopside accumulation were found to be between the markers GOT6 and GOT9 on chromosome four. Previous studies by Staal et al. (2006) have identified areas on chromosome four responsible for resistance against the fungal pathogen *Leptosphaeria maculans* [12]. Arabidopside accumulation have been thought to have connection to the LOX2 gene on chromosome two, supported by the findings of Glauser et al. (2009) [8], but the results from this study show that the LOX2 gene is not responsible for arabidopside accumulation. No previous studies show any connection between genes on chromosome four and arabidopside accumulation, thus we have identified a novel locus crucial for arabidopside accumulation.

The result from the time-series experiment indicates that arabidopside A and B may be converted to E and G within 30-60 minutes after freezing. Enzymatic transfer of OPDA to arabidopside A molecules to form arabidopsode E were suggested by Andersson et al. (2006) [6], and the fact that arabidopside E and G increases at approximately the same rate as A and B decreases supports that theory. These results also show that freezing can be used as a high throughput method for determining accumulation of arabidopside after wounding.

The age-series clearly show that the age of sampled plants must be taken into consideration before conducting experiments or comparing results from different studies. The initial results from testing five weeks old plants from twelve different ecotypes showed little difference in arabidopside levels between the different ecotypes; the difference between Col-0 and C24 were too low to be useful for mapping. All ecotypes except C24 had much higher arabidopside levels two weeks later. This confirms earlier studies that have shown large variations in the levels of arabidopside between the Col-0 and C24 ecotypes.

Only a few F2-plants had lower amounts of arabidopside than C24 controls. This result indicates that that low levels of arabidopside in C24 could be a recessive trait, and that there are probably more than one gene responsible for arabidopside formation. The majority of the plants with low levels of arabidopside used for mapping were slightly higher in arabidopside levels than the C24 control plants, explaining why some of the plants sorted as “low” in arabidopside were found to be heterozygote (Appendix I, Table A1).

The age of the plants in weeks is not the best way of determining the maturation level since small differences in environment can give large changes in growth and maturation over time. In this study all experiments were done on plants grown together with control plants used to get the best results. This way the maturation of the F2-plants would be as similar to the control plants as possible.

Summary

The amounts of arabidopside A and B is high shortly after damage, but decreases quickly as arabidopside E and G increases. The amount of arabidopside is also dependent on the age/maturation of the plants.

The gene/s responsible for the increased levels of arabidopside after wounding are likely positioned on chromosome four, somewhere between the GOT6 and GOT9 markers. Interestingly the lipoxygenase 2 gene earlier reported to be responsible for the accumulation of arabidopside is not present in the interval. Thus we have identified a novel locus crucial for arabidopside accumulation.
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References

Table A1: Genetic mapping with different markers. The area of interest is between the GOT6 and GOT7 markers.