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Proceeding Paper

Galactolipids as Potential Biomarkers for Early Diagnosis of Esca Complex Disease in Asymptomatic Grapevine †

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Abstract: Esca complex disease seriously affects vine yield and longevity. Because of the time delay between wood infection by fungi and symptom expression, current disease diagnosis relies on destructive sampling of the wood. The goal of this study was to identify metabolites that could be used as biomarkers for developing a noninvasive biochemical method for early diagnosis of the disease. Results from a lipidomic analysis showed a positive correlation between the level of leaf necrosis and levels of galactolipids, suggesting a role for galactolipids in the etiopathogenesis of the disease. Such information could be used in developing a method for identification of esca-affected vines without the need to rely upon symptomatic descriptors.

Keywords: hydraulic failure hypothesis; lipid profile; elicitor-toxin hypothesis; candidate marker; symptom descriptor; prognostic indicator; galactolipid homeostasis

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1. Introduction

Plants have developed enzymatic and nonenzymatic mechanisms to maintain cellular integrity in response to pathogen attacks [1,2]. During the establishment of immune responses for example, several compounds act as signals to trigger and mediate defense responses [1–4]. These plant responses ultimately impact the processes of infection and the expression of symptoms. A growing number of studies conducted during the last decade indicates that lipid associated molecules play essential roles not only in plant resistance, but also in plant signaling as mediators of signal transduction [5]. Signal transduction is the process in which all cells constantly receive and act in response to new signals from their environment; the phenomenon has been associated with systemic acquired resistance in plants and could explain the latency period observed with some diseases [2,6].

A peculiar characteristic of the pathosystem grapevine/esca complex disease-associated fungi is an undetermined period of latency within the vine (asymptomatic status) [7]. Esca complex is a vascular disease that attacks the perennial organs of vine plants and causes extensive wood necroses via the slow and systemic action of fungi such as *Phaeomoniella chlamydospora*, *Phaeoacremonium minimum*, and *Fomitiporia mediterranea* [8,9]. Leaf symptoms of infected vines are known to appear in a discontinuous manner in time and space in the vineyard [6,7], and because of that, diagnosis is difficult [10,11]. The infection status of asymptomatic vines most often can only be known after cutting and destroying the vines [7]. The complexity of the disease is also related to the lack of efficient and long-term control strategies [10]. A method for an early detection of esca complex could help in preventing its spread in the vineyards and in limiting economic losses it causes [11].

In this study, it was hypothesized that metabolic responses of the host, and in particular compounds involved in signal transduction, could allow the differentiation of healthy and infected vines. Lipid signaling in plants is mediated by an ample range of molecules such as sphingolipids, glycerophospholipids, fatty acids, oxylipins and sterols [2,5]. For example, the galactolipids digalactosyldiacylglycerol (DGDG) and monogalactosyldiacylglycerol (MGDG) have a major role in determining the stability of thylakoid membranes [3,12,13]. Thus, the goal of this study was to identify lipid species differentiating healthy, asymptomatic, and symptomatic vines that can be used as biomarkers for early diagnosis of esca complex. The applicability of leaf candidate markers for predicting the onset of diseases is best achieved using unbiased analyzes [5,14]; therefore, samples were submitted to a lipidomic analysis, which allowed covering a broad range of lipid classes.

2. Materials and Methods

Healthy control (CTL), asymptomatic (ASY) and symptomatic (SY) leaves of the white cultivar Malvasia-fina were collected in the vineyard of Quinta de Nossa Senhora de Lourdes located in the Douro wine-growing region of Portugal [6]. Symptomatic leaves were arbitrarily categorized into two groups based on symptom intensity: chlorotic leaves (SY1), and scorched leaves (SY2). Six continuous years of annual inspections were required to identify all the vines showing external and/or internal signs of esca complex. External symptoms were identified visually, while wood cores were extracted with an increment borer in order to assess internal infections, as fully described in Goufo et al. [6].

A platform was developed for lipidomic analyses using ultra-performance liquid chromatography (UPLC), gas chromatography (GC) and a Q-Exactive Hybrid Quadrupole-Orbitrap high resolution/accurate mass spectrometer. The platform is based on the extraction of lyophilized powdered leaves in two steps, followed by four UPLC injections and one GC injection. Extraction of lipids, chromatographic separations, mass spectrometry analyses, and compound identification were as described by Goufo et al. [15]. Detected lipid species were grouped by lipid classes and the raw data deposited in a Mendeley Data repository. Mean values were calculated for each leaf group and Welch's two-sample *t*-tests were applied to screen potential candidate biomarkers. Whenever necessary, other classification methods were used, e.g., fold changes, volcano plots, hierarchical clustering, principal component analysis, pathway mapping and rich factor analysis.

3. Results

Untargeted analyses in positive and negative modes permitted the detection of 258 molecular lipid species. The lipid species belonged to the biochemical classes fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, prenol lipids, sterol lipids, hormones, carotenoids, chlorophylls, carnitine metabolism, and choline metabolism. The four leaf groups exhibited distinct lipidomic profiles with clear variations in the levels of lipid species among classes. The latency period before symptom appearance was associated with 53 lipid species (25 down + 28 up); symptom emergence was associated with 74 lipids species (41 down + 34 up); and symptom progression was associated with 124 lipid species (43 down +81 up).

Of all the biochemical classes, the galactolipid class (glycerophospholipids) was the only variable that explained differences among experimental groups. Indeed, the levels of 12 galactolipids increased in leaves of asymptomatic vines, and progressively decreased with symptom emergence and progression (Table 1). The asymptomatic state (wood infection, but asymptomatic leaves) was associated with increases in the abundance of all galactolipids, except for MGDG. Among the strongly changed galactolipids were DGDG (0.25-fold change), 1-palmitoyl-2-linolenoyl-digalactosylglycerol (16:0/18:3) (34:3-DGDG, 0.26-fold change), and 1-palmitoyl-2-linolenoyl-galactosylglycerol (16:0/18:3) (34:3-MGDG, 0.26-fold change). The appearance of symptoms in the leaves was found to be negatively associated with all galactolipids (except for a 0.74-fold increase for MGDG). With symptom progression, levels of galactolipids were depleted in diseased leaves (Table 1).

Table 1. Variations in levels of galactolipids in *Vitis vinifera* L. 'Malvasia-fina' affected by Esca complex disease. CTL = control healthy leaves; ASY = asymptomatic leaves; SY1 = symptomatic leaves with chlorosis; SY2 = symptomatic leaves with scorches. Levels of individual lipid species are expressed as scaled imputed data [15,16]. Different colors represent different directions and intensities of change (light orange = positive change; light blue = negative change) at $p \leq 0.10$ (Welch's two-sample *t*-test, $n = 6$).

Galactolipids	Abbreviation	CTL	ASY	SY1	SY2
1-palmitoyl-2-linoleoyl-galactosylglycerol (16:0/18:2)	34:2-MGDG	1.10	1.29	0.88	0.85
1-palmitoyl-2-linolenoyl-galactosylglycerol (16:0/18:3)	34:3-MGDG	1.04	1.25	0.97	0.76
1,2-dilinoleoyl-galactosylglycerol (18:2/18:2)	34:4-MGDG	1.02	1.12	0.94	0.93
1-linoleoyl-2-linolenoyl-galactosylglycerol (18:2/18:3)	36:5-MGDG	1.19	1.23	0.83	0.83
1-linolenoyl-2-hexadecatrienoyl-galactosylglycerol (18:3/16:3)	34:6-MGDG	1.12	1.39	0.94	0.75
1,2-dilinolenoyl-galactosylglycerol (18:3/18:3)	36:6-MGDG	1.03	1.13	0.94	0.85
1-palmitoyl-2-linoleoyl-digalactosylglycerol (16:0/18:2)	34:2-DGDG	1.03	1.22	0.85	0.87
1-palmitoyl-2-linolenoyl-digalactosylglycerol (16:0/18:3)	34:3-DGDG	1.01	1.20	0.98	0.80
1,2-dilinoleoyl-digalactosylglycerol (18:2/18:2)	36:4-DGDG	0.97	1.07	0.95	0.93
1-linoleoyl-2-linolenoyl-digalactosylglycerol (18:2/18:3)	36:5-DGDG	1.10	1.16	0.82	0.83
1,2-dilinolenoyl-digalactosylglycerol (18:3/18:3)	36:6-DGDG	1.02	1.15	0.94	0.82
monogalactosylglycerol	MGDG	0.88	0.87	1.46	1.13
digalactosylglycerol	DGDG	1.10	1.30	0.93	0.73

Comparisons between SY1 and SY2 showed that the greater the damage on the leaves, the larger were decreases in the levels of galactolipids. For example, the level of DGDG fell by 22% (0.25-fold change) with chlorosis apparition in leaves (SY1) and fell further by 108% (0.59-fold change) with the evolution of symptoms from chloroses to scorches, as compared to control leaves (Table 1). For Lyso-galactolipids, a different pattern of change was observed, with unchanged levels in asymptomatic leaves and depleted levels in symptomatic leaves (Table 2).

Table 2. Variations in levels of lyso-galactolipids in *Vitis vinifera* L. 'Malvasia-fina' affected by Esca complex disease. CTL = control healthy leaves; ASY = asymptomatic leaves; SY1 = symptomatic leaves with chlorosis; SY2 = symptomatic leaves with scorches. Levels of individual lipid species are expressed as scaled imputed data [15,16]. Different colors represent different directions and intensities of change (light orange = positive change; light blue = negative change) at $p \leq 0.10$ (Welch's two-sample *t*-test, $n = 6$).

Lyso-galactolipids	Abbreviation	CTL	ASY	SY1	SY2
1-linolenoyl-galactosylglycerol (18:3)	18:3-LMGDG(1)	1.32	1.24	1.02	0.43
1-palmitoyl-digalactosylglycerol (16:0)	16:0-LDGDG(1)	0.45	0.50	0.61	0.67
1-linolenoyl-digalactosylglycerol (18:3)	18:3-LDGDG(1)	1.06	1.09	1.14	0.59
2-linolenoyl-galactosylglycerol (18:3)	18:3-LMGDG(2)	1.23	1.16	1.22	0.42
2-palmitoyl-digalactosylglycerol (16:0)	16:0-LDGDG(2)	0.75	0.60	0.91	0.47
2-linolenoyl-digalactosylglycerol (18:3)	18:3-LDGDG(2)	1.26	1.17	1.44	0.43

4. Discussion

The remodeling of membrane lipids contributes to the tolerance of several plants to biotic and abiotic stresses [3,5,12,13]. Increment in the levels of galactolipids has been observed in several plant as a response to disease pressures and marginal environments [5]. Under heat stress for example, plants exhibit increased levels of 18:2-containing galactolipids and decreased levels of glycerolipids containing ω-3 trienoic fatty acids (18:3 and

16:3), which are the major constituents of thylakoid membranes [12]. In this study, accumulation of galactolipids was observed only in the leaves of asymptomatic vines, except for MGDG. MGDG and DGDG function nonredundantly to regulate the levels of salicylic acid and systemic acquired resistance in plants [3,13]. Observed increases in the level of galactolipids in the leaves of esca-asymptomatic leaves and variations in the MGDG/DGDG ratio with disease progression can be interpreted as supporting involvement of the galactolipid class in the delay of foliar symptoms.

Esca complex is an insidious disease because the first visible foliar symptoms may develop 5 to 10 years after invasion of wood by associated fungi [7]. During the latency period, the vine might lose its vigor and productivity, and predispose other vines to the pathogens [16]. For most farmers, the detection of the disease is performed by visual observation of foliar symptoms. The monitoring of the disease in asymptomatic vine, however, is complicated since it involves a destructive sampling of the woods i.e., sectioning of trunks or harvesting of pieces of woody tissue in order to observe the characteristic wood necroses [10,16]. In this study, a logistic regression model predicted progression of esca complex disease based on changes in galactolipid levels (Data not shown). This implies that levels of galactolipids could be used as a prognostic indicator of esca complex. A simple and fast diagnostic approach may implicate a biochemical method based on the measurement of levels of galactolipids in asymptomatic leaves compared with symptomatic leaves

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